ISOLATION AND SCREENING OF BACILLUS SPECIES AGAINST CULEX LARVAE IN ZARIA AREA

BY

UMARU, NUHU FILIBUS
(MSc/Sci/7674/92-93)

THIS THESIS IS SUBMITTED TO THE POSTGRADUATE SCHOOL OF AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (M.Sc.) DEGREE IN ZOOLOGY IN THE DEPARTMENT OF BIOLOGICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA

NOVEMBER 2000

DECLARATION

I hereby declare that this thesis is a record of my research work except where literatures cited are acknowledged. It has never been presented by me in any previous application for a higher degree.

UMARU, N.F.	29/11/2000	
Name	Date	Sign

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CERTIFICATION

This thesis entitled ISOLATION AND SCREENING OF BACILLUS SPECIES AGAINST CULEX LARVAE IN THE ZARIA AREA by Umaru, Nuhu Filibus meets the regulation governing the award of the degree Master of Science (Zoology) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

01-12-2000

Dr. I. H. Nock
Chairman, Supervisory Committee
Department of Biological Sciences
Ahmadu Bello University
Zaria

Date

15/3/2001

Dr. M. Galadima
Memebr, Supervisory Committee
Department of Microbiology
Ahmadu Bello University
Zaria

Date

14-3-01

Head of Department
Department of Biological Sciences
Ahmadu Bello University
Zaria

Date

14/05/01

Dean Postgradaute School
Ahmadu Bello University
Zaria

LIBRARY

Date

DEDICATION

This thesis is dedicated to my wife Mrs Felicia N. Umaru and children.

ACKNOWLEDGMENTS

I first of all wish to thank the Almighty God for His continuous guidance and protection throughout my academic pursuit. I also wish to thank my Supervisors Dr I. H. Nock and Dr. Musa Galadima for their enormous contributions, encouragement, intelligent criticisms without which the completion of this work would have been very difficult. I equally wish to thank the WHO laboratory centre in Paris, France for confirming my isolates.

My thanks also go to all the Departmental technical staff particularly Mrs E. R. Lasisi for her special assistance and motherly advice. I would want to equally express my appreciation to Dr. Iortsuun for her concern over my work and encouragement.

Finally, I wish to thank Mrs. B. Roberts for taking pains in carefully typing this project.

ABSTRACT

Studies on isolation and identification of Bacillus species against mosquito larvae were carried out in Zaria between the months of February 1995 and May 1997 with a view of establishing potent strains with bio-control potentials against mosquito larvae.

Samples of mud and dead mosquito larvae were collected on filter paper separately and dried in an oven at 30°C for seven (7) days and later heated at 80°C for 20 minutes to destroy non-spore forming bacteria. The samples were screened for spore-forming bacilli by streaking on nutrient agar plates enriched with 0.3% yeast extract; isolates were identified and then tested for their ability to kill Culex mosquito larvae in water.

A total of 78 soil mud samples and 150 dead mosquito larvae were collected: out of the soil samples, 31 (39.28%) produced colony growth on nutrient agar but none showed cidal activity against mosquito larvae. Of the 150 mosquito larvae samples, 27 (18%) produced colony growth on nutrient agar; 23 (85.2%) of this showed activity against Culex mosquito larvae. The identities of the mosquito killing bacteria was revealed as Bacillus cereus(2 lor 91.3%), Bacillus thuringiensis pakistani 11-13, (lor 4.3%, and Bacillus thuringiensis tohokuensis H-17, (lor 4.3%).

Thus, Bacillus cereus emerged as the dominant species isolated from dead mosquito larvae with potent activity against Culex larvae.

The lethal concentration that could kill 50% of the Culex larvae (LC50) were determined to be 0.025 mg/L for Bacillus cereus, 0.012 mg/L for Bacillus thuringiensis Pakistani H-13, and 0.013mg/L Bacillus thuringiensis tohokuensis H-17. A reference standard strain of B. sphaericus (no SPH 88 - 2362) exhibited an LC50 at 0.005mg/L.

There was significant difference ($P<0.05$) in activity between Bacillus cereus, Bacillus thuringiensis and the standard - Bacillus sphaericus and the null hypothesis was rejected. On the other hand, no significant difference($p>0.05$) in activity was observed between both varieties of Bacillus thuringiensis isolates and Bacillus sphaericus (standard), thus the null hypothesis is accepted and Bacillus thuringiensis pakistani and B. thuringiensis tohokuensis H-17 are accepted as potential agents found in the Zaria area.

The study therefore concludes that isolates of B. thuringiensis with bio-control potential against Culex larvae exist in the Zaria environment.

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CHAPTER ONE

1.0 INTRODUCTION

Vector-borne diseases transmitted to man and animals by mosquitoes and other blood sucking insects are among the most prevalent of the communicable diseases in Nigeria and other tropical African Countries (Fontain and Rosen, 1970; WHO, 1975; 1980a).

Such diseases have been of great concern to the World Health Organisation (WHO) and the world at large. The development and spread of vectors and the diseases they transmit are usually aided by man's activities such as the construction of dams, roads, irrigation channels, poor drainages and sanitary conditions which provide conducive breeding sites and atmosphere (WHO, 1980a, 1987, IRRI/PEEM, 1988). Vector borne diseases include malaria, filariasis, leishmaniasis, onchocerciasis, trypanosomiasis and dracunculiasis, yellow fever etc: close to 43% of these are transmitted by mosquitoes.

Mosquitoes are widely distributed but are found mostly in the tropical countries of West and East Africa, South-East Asia, the Caribbean, North and South America, Europe etc (Hopskin 1951, Service, 1963, Katherine *et. al*, 1971, IRRI/PEEM, 1988.

Malaria, yellow fever, filariasis etc are mosquito borne diseases which cause health concern to humans especially in Africa, South America and South-East Asia. Worldwide, malaria alone is responsible

for the death of hundreds of thousands of people particularly infants annually (Hendricks *et.al.*, 1971; Katherine *et.al.*, 1971; Bruce - Chwatt, 1982). It is also estimated that half of the world 's population live in an area where malaria is endemic, though the number of people at risk now has reduced due to more effective control methods adopted by WHO and other related bodies (WHO, 1990).

Despite the efficacy of current control measures, the disease still poses a serious threat to humans. The estimates show that about 500 million Africans made up of 64 million Nigerians especially children of five years and below contract malaria and out of these, about 2 million die every year (WHO, 1985a, 1990).

The control of the vectors of these diseases is a powerful means of controlling the diseases which they transmit. This is achieved most efficiently by attacking these agents at their breeding and resting places.

Since World War II, insecticides have been used in the eradication of vectors of malaria. Despite the efficacy of the method, malaria eradication has failed to materialise in tropical Africa partly because of the emergence of resistant strains to insecticides coupled with increasing cost of production and low specificity on non-target organisms.

It is in view of these that the, unit of vector biology, of the WHO, shifted its emphasis to biological and integrated control rather than the exclusive reliance on insecticides (WHO, 1984b). The bio-control agents are exploited to regulate the vector population growth naturally. Most promising bio-control agents for use against vectors include: bacteria, fungi, protozoa, nematodes, viruses, fishes, algae etc (Clausen, 1956: WHO, 1982, 1984). Biological control therefore, can be augmentative or classical. It is highly dynamic in nature as it continues to operate when incorporated into systems and the effect is permanent once the control is accomplished. It is relatively safe on the environment and on non-target organisms (WHO, 1982, 1984b).

Radiation sterilization has also been used which aims at exposing male insects to high doses of x-ray and when the resulting sterile male mates with the wild female, the latter produces sterile eggs which never hatch. With this the number of insect vectors is reduced. Insect pheromone, repellants, attractants etc have also been used to control insect vectors (Clausen, 1956).

Other methods include the integrated control (which combines the use of two or more methods), environmental management and cultural practices which are aimed at manipulating the environment to discourage or reduce breeding and resting sites of vectors. Mechanical

and physical methods as well as various trapping methods and animal bait have been used to reduce vector population (WHO, 1982).

Based on the literature available, (WHO, 1975; 1979, 1980, 1982, 1985b; 1990), biological control methods seem to offer more promising results because they are cost effective, provide more permanent solutions, have high degree of specificity on non-target organisms and safe on the environment.

It has been known that the control of any vector of disease is best achieved by attacking it at the larval stage in their breeding places because the larval stage lasts longer and thus provides enough time for the larvicide to act on them (WHO, 1984).

Bacteria have been known to be the best candidates for the bio-control of mosquito larvae. Infact two important species viz: Bacillus thuringiensis and B. sphaericus have been screened and formulated for use as wettable powders based on their high cidal activities against the insect(WHO, 1982c, 1984, 1985, 1987).

Although there is adequate knowledge on biological control of mosquitoes by bacteria and other organisms like larvivorous fishes, algae, snails, protozoans, fungi, viruses, nematodes, etc, to our knowledge, apart from Shehu 1986 who obtained powdered Bacillus thuringiensis H-14 from the WHO and carried out a laboratory test on

the effect of Bacillus thuringiensis on culex mosquito larvae, there is no other available literature on Bacillus species that are active against mosquito larvae in the Zaria area where mosquito population is very high. However ,B. sphaericus has been isolated around Ibadan, Nigeria, in 1980 which showed 100% mortality on Culex larvae at 48 hours of exposure(WHO 1984a, 1985b, Shehu, 1986). There is therefore the need to isolate and screen for more candidate strains with good bio-control potentials.

Accordingly, this work was designed with the following objectives:

(a) To isolate and identify Bacillus species in dead mosquito larvae and soil samples in the Zaria area.

(b) To test the toxicity of the identified species against mosquito larvae.

It is expected that the accomplishment of this study will lead to the identification of Bacillus species with the potential of biological control against mosquitoes in the Zaria area. It is hoped that this will lead to the integration of a biological control programme into the overall mosquito control programme.

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CHAPTER TWO

LITERATURE REVIEW

2.1 MOSQUITOES

Mosquito is a delicate slender, fragile two winged troublesome insect measuring about 4-6mm. It belongs to the phylum arthropoda, class insecta order diptera, family culicidae, and the sub-families of anopheline and culicine(Anthony, 1979).

2.1.1 GENERAL DESCRIPTION

The entire body of a mosquito is covered by a flexible exoskeleton and divided into head, thorax and abdomen. The female mosquitoes are blood feeders and suck the blood of vertebrates with well developed piercing stylet like mouth parts adapted for that purpose; the males have poorly developed mouth for this purpose and therefore suck plant juice(Vines and Rees, 1971; Anthony, 1979).

2.1.2 DISTRIBUTION

Mosquitoes are widely distributed and found mostly in the tropical countries of West and East Africa, South-East Asia, the Caribbean, North and South America, Europe, etc (Hopskin, 1951; Service, 1963; Katherine, *et.al*, 1971;WHO, 1981).

2.1.3 LIFE CYCLE OF A MOSQUITO

The Anopheles and Culex mosquitoes undergo complete

metamorphosis from egg - larva - pupa and adult. The eggs of Culex are laid in batches each contains about 50-150 eggs, while Anopheles eggs are laid singly, boat shaped each with a pair of floaters. The eggs of both hatch out to larvae within 2-3 days by cutting the circular slit of the egg shell.

The first instar larvae that emerge are small, pale, inactive and don't have well defined head, thorax and abdomen. Soon after that they moult, become active and resume active feeding. The Culex larvae are bottom feeders while Anopheles larvae are surface feeders. After about 4 casts the larvae transform to pupa in about 3-5 days.

The pupa is a comma shaped creature with a cephalo and abdomen which ends in a paddle like structure. It does not feed. Three days after, air collects in transparent skin, this lift it up to the water surface. It straightens out to break this skin and the young adults emerge. The male and female mosquitoes engage in a swarm dance. The male seizes the female with the aid of its claspers and insemination takes place immediately. After fertilization the males seek for plant juices and female for blood before laying her eggs though sometimes, females too feed on plant nectar in the absence of blood meal (service 1963). This unending life cycle continues in the mosquito's life time.

The female mosquitoes have well developed stylet piercing needle

like mouth parts and before partaking in blood meal give high pitched 'piping' sound, caused by vibrating wings and settle down in the human skin unperceived. The stylet mouth parts of the female mosquitoes which can penetrate thick clothes is retractable and after piercing the skin the saliva is released through a channel into the animal and human blood stream. This causes irritation and swelling of the skin during which various parasites that cause fever and other illness are released.

2.1.4 **IMPORTANCE**

Mosquitoes are regarded as pests to man because they interfere directly or indirectly with his health and socio-economic well being. Mosquitoes are vectors of many human diseases and stand out distinctly among the numerous species of blood sucking arthropods; beyond the transmission of diseases they annoy man and other warm blooded animals by virtue of their bites and the sound they provided (IRRI/PEEM, 1988).

Mosquitoes are the sole vectors of malaria, yellow fever; they also transmit parasites that cause filariasis/elephantiasis and dengue fever among others. Diseases transmitted by mosquitoes have rendered vast areas of tropical countries unfit for living. It has been noted that malaria fever alone is still one of the most important vector diseases

transmitted by mosquitoes where millions of people contract the diseases and at least 2 million die of it every year especially children, (Hendricks *et.al*, 1971; Katherine, *et al* 1971; Bruce-Chwatt, 1982; UNDP/WHO, 1990).

2.2 CONTROL OF MOSQUITOES

Due to the devastating and catastrophic effects of mosquitoes and other insects on humans, man had to look for any available control means that can yield quick result in dealing with the insects and their associated problems.

2.2.1 CHEMICAL CONTROL

The earliest best available means to man that can give instant results not minding its phytotoxicity is no other than chemical pesticides (Isa, 1988). The history of modern pest/vector control dates back to the second half of the 19th century, when Colorado beetles spread rapidly across the United States and seriously threatened food production and national economy (Helmut, 1976). After much argument, it was finally decided to take the unprecedented step of spraying the potato crops with a human poison (arsenic in the form of Paris Green, Helmet, 1976). This then opened the way to the use of biocides (destroyers of life) on crops destined for human consumption.

The first residual contact insecticides were the organo chlorines e.g. the DDT, BHC, heptachlor, aldrin, and dieldrin(Helmet, 1976). These were extensively used as emulsion and wettable powders. They are not very toxic to man, but many are extremely persistent and remain in the environment for a long time. These insecticides are believed to act by inhibiting the enzyme cytochrome oxidase which mediates gas exchange in the process of respiration in all animals which use blood circulation as a gas carrier; the insecticides also de-stabilize the nervous system(WHO, 1985).

A second group of the residual contact insecticides was produced in the early 1950s. These are the organophosphorus compounds such as parathion, Malathion, TEPP, dimeton etc. They differ from the organochlorine in being less persistent and are much more poisonous to both insects and man (Helmut, 1976). They are also non-specific and are believed to act by inhibiting the hydrolysis of the acetyl-choline by combining with the enzyme acetyl-cholinesterase.

The third group of the residual contact insecticide is the carbamates whose persistence and toxicity lay intermediate between the organochlorines and the organophosphorus. Carbamates have been used in the control of caterpillars and other surface feeders. They also interfere with the functions of the nervous system(Helmut, 1976).

The World Health Organisation in realization of the threat being posed by vectors of diseases particularly mosquitoes to the human race introduced in 1955, the malaria eradication programme whose aim was to eradicate the vectors of malaria by use of chemical biocides(Vines and Rees, 1971, Katherine et al, 1971). Although success was recorded at the inception of the programme, but soon after that, its application became restricted or banned as the case was in other parts of the world because of its serious phytotoxicity and environmental consequences, (Helmute, 1976, 1980). In addition to this, the use of the chemical insecticides witnessed the emergence of insecticide resistant strains which simply multiply without restraint and may cause a far worse vector problem than ever before (Helmut, 1976). Another problem is the ever increasing cost of production and also its low target specificity (WHO, 1980, 1982). A classical example of low target specificity is associated with the death of many birds in the UK, popularly known as 'No Bird Sing' following the application of the chemical pesticides(Helmut, 1976). Thus, the exclusive reliance on chemical insecticides for the control of mosquitoes is no longer the best desired method.

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2.2.2 **BIOLOGICAL CONTROL**

As a result of this, the problems associated with the use of

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chemicals as (stated above), alternative methods of control which will offer more promising results, were sought. This alternative method is 'Biological control. According to Wilson (1972), the term biological control was first used by Smith in 1919 to denote insect-pest control by the use of pests of any kind. More recently the term has been employed to include various biology-based forms of control that have no connection with natural enemies, (Clausen, 1956; Daniel, 1976). This now include the use of predators, parasites, pathogens, plant varieties, insect pheromones, x-ray sterilization (genetic), repellants, attractants, and cultural practices(Helmut, 1976).

The history of biological control dates back to as early as 1762 when predator birds were imported to Mauritius to control the red locusts (Helmut, 1976). The starling success of biological control of Icerya purchasis (Cottony cushion scale) in 1889, provided the real foundation of biological control (Wilson, 1972; Helmut, 1976).

According to WHO (1975), Davidson (1981) and Lacey and Undeen (1986), the use of biological and microbial agents have more advantages over chemical pesticides which include:

(a) Host range: Biological agents are more specific and thus safer on non-target organisms than chemical pesticides.

(b) Safety of the environment: It is ecologically more acceptable to

the environment than chemical pesticides.

(c) It is aimed at controlling a specific species of arthropod vector-borne disease rather than their complete eradication as they may on one hand be important in maintaining balance in the ecosystem.

(d) The biological agents that are mostly used are readily biodegradable and cheaper to produce from local renewable raw materials.

(e) They rarely induce resistance in the target organism.

2.2.3 GENETIC CONTROL

This is the concept of utilizing sexually sterilized males of an insect species to act as agents in the elimination of the species (La Brecque, 1956). Genetic control of vectors has proved operationally successful in the agricultural and veterinary fields, although the method holds less promise against human disease vectors (WHO, 1982).

The successful control of the screw worm (Calitroga sp) of cattle by radiation sterilization is one of the landmarks in the history of pest control of the 19th century. The release of males sterilized by 5000 roentgen unit cabalt bomb on an Island resulted in an eradication of the pest in eight weeks (Knipling, 1950; Helmut, 1976). When these

sterile males are released into the population, they compete for females with the wild males and all the eggs being produced by the females were non-fertile (Helmut, 1976). The limitation of this method is the problem of mass rearing and maintenance of males, as well as timing for release when the native male population is low. Existence of natural barriers and other environmental factors also tend to limit this success hence vectors and insect pest problems still exists.

2.2.4 USE OF REPELLANTS AND ATTRACTANTS

Repellants are chemical substances or sounds, reflecting surfaces which deter or make animals and its food unattractive to pests and vectors of diseases etc. Chemical repellants have been investigated most fully with respect to mammals and birds. Before alternatives of insecticides became available, control of, for example, cabbage root fly relied heavily on tarred discs placed around the bases of plants to deter oviposition by the flies. Other repellants signals include amplified bat calls to derive moths from orchards (Helmut, 1976). It has also been found that the short wave length light (sky) reflected from pieces of aluminium foil laid between the rows in a crop can greatly reduce the number of aphids landing on the plants by inducing descending individuals to fly upwards.

Food sources can also be made unattractive by chemical

repellants, a principle that is much used in protecting man from blood sucking insects like mosquitoes(Risebeth, 1972). It has also been found that even after an insect vector has been attracted to a suitable host it can be deterred from feeding by the use of antifeedants sprayed on the food sources (Munakata, 1970). Examples of some of these chemical repellants include indalone and diethyltoluamide (Chandler and Read, 1960). The disadvantage of this method is that the material and labour costs are high.

2.2.5 **ATTRACTANTS**

Here the feeding or sex behaviour of insects pest may be used to lure the insects into traps or encourage them to consume a lethal dose of toxicant e.g. use of poison baits. Attractants may be combined with other control methods, to achieve better results e.g. attractive baits may be treated with insecticides and distributed within and upon contact or ingestion by insects like mosquitoes poisoning and death result.

2.2.6 **USE OF PATHOGENS**

Ideally, microbial pathogens to be used as biological control agents should according to WHO, (1984a), be able to be cultured in sufficient quantities at reasonable cost, be infective to the host, and

capable of dissemination from host to host and should have residual activity. It should also be able to cause rapid mortality, specific for target organisms and safe for man and domestic animals. The pathogen should be capable of being stored under tropical conditions for at least one year and should have a stable genetic constitution with respect to shift in pathogenicity. In addition, it is desirable that pathogens should be recoverable from an infected specimen so that the cause of its mortality can be properly assessed.

These microbial organisms include nematodes, protozoan, viruses, fungi and bacteria (Price *et al*, 1974; WHO, 1982; Helmut, 1976; Clause, 1956).

2.2.7 **NEMATODES**

Most Nematodes at present being considered for development of biological control agent belong to the family Mermithidae. These nematodes are obligate parasites which must undergo part of their development within a living host. Three species are currently receiving study and evaluation among the mermithid group that seems to be the most promising for mosquito control(WHO, 1982a and b)).

These species are Romanomermis culicivorax, R. Iyengari and Octomyomermis musprattic. Adult of these species lay eggs in mud or detritus of mosquito habitats. After growing within the larvae for about

seven (7) days, the nematodes emerge, killing the larvae by exerting through the cuticle. After emerging, the nematode drops to the bottom of the habitat, moults, mates and the females begin laying eggs. Most of these nematodes have high infectivity against insect larvae generally including mosquitoes (WHO, 1984a).

Octomyomermis musprattic also have great tolerance to alkalinity, salinity and pollutions and offer great potentials for vector control. Another advantage in the use of nematodes is their ability to invade late larval instars and develop into their adult stage providing a natural mechanism for its dissemination (WHO, 1982c).

The problem associated with the use of nematodes as a bio-control agent is how to determine the conditions required to obtain a synchronous hatching of eggs for effective use (WHO, 1982c).

2.2.8 **FUNGI**

Many strains of fungi that have vector control potentials belong to the classes Oomycetes, Zygomycetes, and Deuteromycetes. Most of them are obligate parasites of mostly mosquito and blackfly larvae (Price *et al*, 1974; WHO, 1982 a & b). Two genera of fungi Beauveria and Entomophora have been used effectively against caterpillars, aphids and other small insects respectively. This was done by spraying the surface of water in which mosquitoes or blackflies breed. The fungi

will then penetrate the cuticle of the insects on contact, form conidia in some cases which eventually stick to the larval siphon valves, germinates and kills the larvae apparently by suffocation (WHO, 1982). The popular Colorado beetle was also controlled by the fungi Entomophora (Helmut, 1976).

Fungi are also used to control plant diseases. According to Rishbeth (1972), Philips *et al* (1967) when wounded roots of tomato seedlings were exposed to the fungus Cephalosporium sp, vessels became obstructed and the ability of the wilt fungus Fusarium oxysporum to spread in the tissues after subsequent inoculation was limited. Phiatophora redicicola (fungus) was used to control wheat 'Take All' disease caused by Ophiobolu graminis. Canker formulation which is caused by fungus the Endothia parasitica which causes 'chest nut blight' could be reduced to about 1/5 by inoculating certain fungal saprophytes at the wound sites e.g. Penicillium rubrum which is a good competitor (Rishbeth, 1972).

Some species of fungi particularly those of the deuteromycetes class are good candidates for biological control because they can be produced in deep fermentation, have potential for recycle with good salinity and temperature tolerance (WHO, 1982a; 1982b; Price *et al*, 1974). They can also be grown on artificial media and can maintain

themselves in a habitat without the presence of the host. They are also reported to produce high larval mortality during the spring and early summer.

The main problem with fungi that militates against its effective use and commercialisation is that they need a fairly high humidity of 80% or more before sporulation occurs and the development of epizootics (i.e. disease manifestation) and most of these requirements are dependent on climatic factors (WHO, 1982a; Helmut, 1976). Another problem again is that some of the fungi do not recycle and being obligate parasite requires the presence of both the crustacean and mosquito host for development; most species of coelomyces have proved difficult to study and evaluate (WHO, 1982b).

2.2.9 **VIRUSES**

Viruses offer greatest potentials as microbial control agents because their host range is restricted and is relatively easy to obtain large quantity purified and characterised virus e.g. nuclear polyhydrosis viruses (virus disease of insect larvae characterised by the breakdown of tissues and presence of polyhedral granule) is an excellent example of viruses that have been used in the control of the larvae of blackfly and mosquito (WHO, 1980; WHO 1982b; WHO, 1984).

Irido-viruses have also been used in the control of insect larvae, although it has been found to be lethal to its host. They attack the midgut epithelium of larvae causing an acute disease which results in death in about 48 hours. Virtually all viruses pathogenic to vectors of disease have been isolated from mosquitoes (WHO, 1984).

Virus was also used to control rabbits in Australia in 1950 (Price, *et al*, 1974). The causative virus which is closely related to fowl pox, caused myxomatosis in rabbits producing a kill of over 99% (Murton, 1972).

Although success was recorded, but a few that survived acquired active immunity and genetic resistance to the virus. The shortcomings in the use of virus as a microbial control agent is that they are highly specific obligate pathogens and there is at present no efficient method for their mass production (WHO, 1982b). In addition, there is also the problem of insufficient infectivity or virulence that confront the development of viruses as bio-control agent.

2.2.10 **PROTOZOA**

The protozoans which have vector control potentials belong to the micro sporidia. On the basis of their life cycle, these protozoans can be classified as either monomorphic or dimorphic micro sporidia.

MONOMORPHIC MICRO SPORIDIA: These micro sporidia have

relatively simple life cycles in which only a single spore type is produced (WHO, 1982). These include the Nosema algerae, Vavraia cuticis, and schistosome micro sporidia. These micro sporidia have been shown to have reduced vector populations through attacking their larvae. When they infect adults, are shown to reduce their fecundity and ability to transmit diseases.

Dimorphic micro sporidia on the other hand have two different developmental cycles. In the adult females, these micro sporidia form bacilliform spores which eventually result in the infection of the mosquito eggs. All males hatching from infected eggs develop patent infections and die. In females, larvae which develop from infected eggs, the infection remains benign.

The limitations in the use of protozoans are: Nosema algerae for example has shown little ability to recycle at rates high enough to yield the necessary levels of control in subsequent generations (WHO, 1982).

Another set back also is that recent studies have shown that mortality rates for larvae and adult are low as long as adults are infected.

2.2.11 THE USE OF BACTERIA AS VECTOR CONTROL AGENT

According to WHO (1980) all the bacteria that are considered so far for vector control must be able to form spores and potent stable

toxins (WHO, 1982). They should also be able to be stored for a long period of time even at ambient (high) tropical temperatures and can be formulated as suspensions, wettable powders and dust for easy application with conventional sprayer normally used for chemical pesticides (WHO, 1982; Helmut, 1976). All biological agents selected so far for trial and tested for efficacy have one outstanding merit i.e. they are remarkably safe for man and are virtually harmless to most if not all non-target organisms (WHO, 1982: WHO, 1987). The bacteria tested so far fall into two major groups - Bacillus thuringiensis and B. sphaericus. Both species have been isolated from dead mosquito larvae or larval habitats of mosquitoes (WHO, 1980; WHO, 1982; WHO, 1987; Charnley, 1991). These bacteria do not affect the eggs, pre-pupae, pupae and adult forms of the vector (Davidson, 1981).

2.2.12 CHARACTERISTICS OF BACILLUS THURINGIENSIS H - 14

B. thuringiensis is a Gram positive bacterium which produces crystalline proteinaceous inclusions during sporulation (Chanley, 1991; Shehu, 1986; WHO, 1984). The inclusions contain insecticidal proteins which are proteolytic enzymes (toxins) with a molecular weight of 25,000. The bacterium was discovered in 1977-78 and since then has been used extensively in the control of mosquito and blackfly larvae

(WHO, 1984). Many strains of B. thuringiensis have been isolated and are classified into stereotypes and phage types. The classification of the strains and phage types are based on their insect species activity profile (Chanley, 1991).

2.2.13 MODE OF ACTION OF B. THURINGIENSIS H-14

The toxin crystal of B. thuringiensis is formed along side the spore. Larval enzymes usually digest the crystal, on ingestion, releasing the toxin within seconds of digestion, and the mosquito larvae are killed within hours of ingesting a lethal dose which dissolves their midgut epithelium (WHO, 1982, 1984).

Most commercial preparations of B. thuringiensis for applications are mixture of spores and crystals which upon ingestion of sufficient quantity cause death of the target organism. The insecticidal principles of B. thuringiensis is the delta - endotoxin which is present as a protein in the parasporal crystal (body) and is released in the midgut of the host larva on ingestion (WHO, 1984 a, *de* Barjac, 1979).

The delta endotoxins released during sporulation are being activated by proteolysis due to insect midgut or crystal associated proteases. WHO (1984) reported that the toxin binds to the midgut wall (membrane proteins) and disrupts cellular regulation of ion balance

thus causing cells to swell and lyse. The infected larva stops feeding, becomes sluggish, with a rapid swelling, distortion and bursting of the midgut cells and consequently death occurs within 15 to 30 minutes (WHO, 1984; *de* Barjac, 1978; Davidson, 1981; Charnley, 1991).

HOST RANGE: Of all the various strains of B. thuringiensis, it is the B. thuringiensis var Israelensis that has been well researched into and has been shown to be effective both in the laboratory and in the field against all the major mosquito and blackfly vector species (Goettel, *et al,* 1982; WHO, 1982, 1984). The bacteria do not persist longer than a few days and regular treatments are necessary during the vector breeding season. According to available evidence, there is no cross-resistance with chemical insecticides, nor is there any indication of the development of resistance to B. thuringiensis. H - 14.

On the other hand, B. thuringiensis has been known to be very specific as it kills only a limited range of insect vectors and pests, while having no effects on birds, mammals and fish. This selectivity is of environmental advantage, though creates problems in an agricultural establishment where a complex of pests have to be controlled. B. thuringiensis H-14 has a low speed of action and does not recycle in the environment particularly polluted water at levels high enough to provide any significant residual activity (WHO 1982, 1984, 1987).

2.2.14 CHARACTERISTIC OF BACILLUS SPHAERICUS

B. Sphaericus was discovered in 1974 and since then it has been undergoing test trials on certain species of mosquito larvae (WHO 1980, 1984). B. sphaericus is a gram positive and spore forming bacteria which have been found to be highly pathogenic for certain insect larvae. It is cosmopolitan in distribution. The pathogenic strains of B. sphaericus have been isolated from dead larvae or larval habitats of mosquitoes in about 11 countries which include California, USA, India, Jakarta, Indonesia in 1974, Nigeria in 1980, Israel, Sri Lanka, Phillippines, El Salvador, Thailand and Romania (WHO, 1984).

The most virulent strains of B. sphaericus that have been extensively studies through Tropical Diseases Research (TDR) support are: Serotype H - 5a, 5b: B. sphaericus 1593, isolated near Jakarta, Indonesia in 1974; strain 2362, isolated in Nigeria in 1980 and serotype H- 25. B. sphaericus also known as MR4, isolated in Sri Lanka in 1979 (WHO, 1984; Maurice and Pearce 1987). The pathogenic strains are genetically different from the non-insecticidal strains. The most active strains of B. sphaericus are known to synthesise parasporal bodies or crystal during sporulation (WHO, 1984).

The toxins of this species which have been proved to be protein in nature are located in the spore coat. The toxins produced by B.

sphaericus is associated with the pre-sporulating cell or spore and it is not secreted into medium. The cell wall is the most toxic structure in the sporulating cell with the spore itself developing later.

Bacillus sphaericus produces a parasporal crystal that is toxic to mostly mosquito larvae, and have more limited host range than Bacillus thuringiensis (WHO, 1980, 1982, 1984). B. sphaericus has been known to be most insecticidal to larvae of Culex spp and to less extent to Anopheles species, while no activity has been recorded with Aedes mosquito and blackfly larvae (WHO, 1982, 1984).

2.2.15 MODE OF ACTION

The molecular mode of action of B. sphaericus is at present not well established, but recent studies of the histopathology caused by B. sphaericus in mosquito larvae has revealed that the midgut epithelium appears to be the primary site of action at least initially (WHO, 1985). Upon ingestion of the sporulated insecticidal strains by mosquito larvae, the crystal dissolves in the gut of the host by proteolytic enzymes within few minutes, toxins are produced which interact with the midgut epithelial cells causing death of the larvae within hours (WHO, 1982, 1984, 1985). The epithelium gets distended and the gut is paralysed. This is followed by epithelial cell lysis and larval death.

The sequence of pathogenesis observed in larvae to which spores of the insecticidal strains of B. sphaericus have been fed is similar to that observed in larvae fed by B. thuringiensis H - 14 (WHO, 1984). Some of these strains produce parasporal bodies and all are more toxic to larvae than strains 1321 isolated earlier in India.

2.2.16 COMPARISON OF BACILLUS THURINGIENSIS WITH B SPHAERICUS

1. Bacillus sphaericus kills its target host by means of a cell-bound toxin that is released by digestion, while B. thuringiensis kills its host by toxins present within the spores on ingestion.

2. No crystalline parasporal body has been identified as the location of the toxin in B. sphaericus, while parasporal body is known to be the location of the toxins in B. thuringiensis.

3. In B. sphaericus, vegetative and sporulating cells as well as spores are relatively toxic with differing activity from strain to strain, while in B. thuringiensis the toxins are known only to be formed along side of the spores.

4. The insecticidal activity of B. sphaericus has been reported to last for 80 - 90 days in sewage and 30 - 50 days in fresh water, while the insecticidal activity of B. thuringiensis last for 24 - 48 hours or less

when in polluted waters (Maurice and Pearce, 1987; WHO, 1984, 1985).

5. B. sphaericus is also known to increase its activity saprophytically in polluted waters, while B. thuringiensis is known to be effective only in clear waters.

6. B. sphaericus can also kill mosquito larvae even in settling tanks which is usually accompanied by turbulent aeration during sewage treatment installations while B. thuringiensis has little or no effect at all as it sinks to the bottom in water.

7. The B. sphaericus may be killed in the chlorination tanks, but its toxins still remain unharmed and can affect any larvae occurring down stream of these tanks. The toxins of B. thuringiensis are killed in chlorination tank and thus will have no effect on the larvae of mosquitoes.

8. Most importantly, B. sphaericus has been known to effectively recycle itself, which means it can provide a continuous control over a long period of time, while B. thuringiensis can not as its effects last only between 24 - 48 hours.

2.2.17 CHARACTERISTICS OF BACILLUS CEREUS

According to Yusuf (1990) Bacillus cereus was first described in 1887, fifteen years after the genus Bacillus was described.

They are Gram-positive spore-forming, rod-shaped organisms commonly found in soil (Stanley, 1978, Shin - Ichiro, *el al.* 1997;). The cell measures 1.0 to 1.2 μ by 3.0 to 5.0 μU and sporulates readily on Nutrient Agar. The spores are ellipsoidal and centrally or para-centrally placed without swelling of the sporangium (Gordon, *et al.*, 1973).

The organism is capable of growing both aerobically and anaerobically at temperature between $10^{o\,c}$ to $45^{o\,c}$ with optimum at $35^{o\,c}$ and several reports show that certain strains possess unsual resistance to heat and radiation, (Parry and Gilbert *et al.*, 1981a; Larry *et al*, 1997; Anderson *et al*, 1998). B. cereus can also be isolated from vegetation, sediments, dust, natural waters, certain dry foods, meat poultry, milk and dairy products (Nygren,s 1962; Mossel, *et al*, 1967; Shin-Ichiro, *et al.* 1997; King-Thom Chungm *et al*, 1986; Yusuf, 1990; Stanley, 1978; Larry, *et al*, 1997). Gordon, *et al.* (1973) reported that certain strains of B. cereus are pathogenic to insects and found them to form crystalline inclusions in such diseased insects.

2.2.18 RELATIONSHIP OF B. CEREUS TO B. THURINGIENSIS

It was reported that B. thuringiensis strains contain an enterotoxin gene very similar to that of B. cereus and that there is a

common spore forming presipitinogen present in all the strains of crystal forming insect pathogens and of B. cereus (Norris, *et al,* 1961). It was also observed that five strains of B. cereus and five other crystal forming strains exhibited the same pattern of fatty acids and that the distribution patterns of fatty acids were the same for two strains of B. cereus, one rhizoid strain, and two strains of B. thuringiensis. (Gordon, et al., 1973)

Krieg (1969), also observed that the antigens of B. cereus and B. thuringiensis overlapped. B. thuringiensis could be distinguished from B. cereus for its production of crystalline inclusion bodies made of insecticidal proteins, although several strains of B. thuringiensis appear to produce enterotoxins similar to that of B. cereus (Carlson and Kolsto, 1993).

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Cloning and sequencing of enterotoxin genes from B. cereus and B. thuringiensis was carried out by shin-Ichiro *et al,* (1997) and the sequence determination revealed an Open Reading Frame (ORF) encoding a 45 -KDa protein with 422 amino acids residues in B. cereus, while B. thuringiensis showed a continuous ORF encoding a 45 - KDa protein of 430 amino acids residues. From the above it could be deduced that since the enterotoxin genes of B. cereus and B. thuringiensis are very much similar, B. cereus could thus exhibit a

similar pathogenicity in insects. This gives meaning to the work of Gordon, *et al.,* (1973) who isolated 15 strains of B. cereus in diseased insets. Shin-Ichro, *et al.,* (1997) and Gordon, *et al.,* (1973) were in agreement that B. thuringiensis might be a variant of B. cereus because of the similarity in their enterotoxin genes, as revealed during cloning. Gordon, et al., (1973) further suggested that the minor but distinct differences between the two strains might be responsible for difference in activities.

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2.2.19 MODE OF ACTION OF B. CEREUS

B. cereus just like other species of bacilli, produce proteolytic enzymes which digest casein which in milk causes abnormal odour, while some strains are known to produce extracellular enterotoxin which cause diarrhoea and emetic food poisoning in higher animals (Gordon, *et al.,* 1973, Douglas, *et al.,* 1994;; Shin-Ichiro, *et al.,* 1997; Vesa, *et al.,* (1998).

They also cause *non-gastro intestinal infection (gastro enteritis)* in both humans and animals (Dryden and Kramer, 1987, Elmer, 1994,). According to Dryden and Kramer (1987), B. cereus can be associated with wound infections, septicaemia, pneumonia, osteomyelitis, meningitis etc.

Jocelyn, *et al.*, (1996) reported that certain strains of B. cereus can be used for control of plant disease. This is due to their ability to control/suppress damping off disease of alfa-alfa caused by the Oomycete pathogen Phytophthora medicaginis . They further added that B. cereus is also associated with production of antibiotics designated Zwittermicin 'A' and antibiotics 'B' (Kanosamine) which are found in extracellular fraction of fully sporulated cultures.

2.2.20 USE OF PREDATORS

Other methods of control include the use of predators e.g Larvivorous fish. Indigenous fishes are usually preferred for this purpose. This is because they are better adapted to local conditions and are also easily and economically established (Price, *et al.*, 1974). Phytophagous fish in their young stages may also feed on mosquito larvae and as adults will clear vegetation, thereby exposing mosquito larvae to attack by larvivorous fish and other predators.

The fish species that are selected for this purpose must have marked preference for mosquito larvae, small adult size which makes for easy colonization and access to shallow depths, rapid maturation, high fecundity, a high degree of tolerance to salinity and pollution, and harmlessness to non-target fauna in the aquatic ecosystem. Oryzias

latipes for example possesses most of these attributes and can withstand a wide range of climatic and ecological conditions (WHO, 1982b,). Other promising fishes belong to the genera Cyprinodontide e.g Aphanius sp. and Gambusia affinis which is one of the well known larvivorous fish.

2.2.21 ALGAE AND AZOLLA

Algae and Azolla have also been found as potential vector control agents. Azolla is a free floating aquatic fern that live in symbiotic association with algae. It fixes atmospheric oxygen in sufficient qualities. Li and Wu (1934) in IRRI/PEEM (1988) reported that the water surface coverage by Azolla anabaena has been found to be detrimental to mosquito especially Culex species. This was further confirmed by later studies supported by WHO in 1986(WHO, 1987).

It was discovered that a complete coverage of the water surface by Azolla will definitely inhabit oviposition of Culex species. Coverage of water surface by Azolla has also been found to be detrimental to the development of 4th - instar larvae and the emergence of adult mosquito

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2.3 CULTURAL METHODS

The concept of cultural control in the altering of the habitat to

make it less favourable to pest reproduction and survival is termed cultural control. Before the advent of the modern insecticides, man's chief weapon against insects was to try to disrupt their life cycles by periodically denying them their food plant and to achieve the maximum control that a manipulation of ordinary agricultural practices would allow (Helmut, 1976; WHO, 1980b).

Cultural method is one of the oldest methods of control and not until recently that its underlying biological principles were known. The biological principles involved include manipulation of the environment to make it less favourable for the pest and more favourable for the natural enemies, other cultural practices include use of planting and harvesting times that will be less favourable for the establishment of pest and also vectors of diseases, cultivation and habitat diversification may also expose the larvae or even adults of pests and vectors of diseases to natural enemies which could be either parasites or harsh climatic conditions

2.4 ENVIRONMENTAL MANAGEMENT

Environmental management may be defined as the planning organization, carrying out and monitoring of activities for the modification and or manipulation of environmental factors or their interaction with man with a view to preventing or minimizing vector

propagation and reducing man-vector-pathogen contact (WHO 1980b). Environmental modification on the other hand is a form of environmental management consisting of any physical transformation that is permanent or long-lasting of land, water & vegetation, aimed at preventing, eliminating or reducing the habitat of vectors without causing unduly adverse effects on the quality of the human environment. Environmental modification includes drainage, filling, land levelling and transformation of impoundment margins.

Environmental manipulation is a form of environmental management consisting in any planned recurrent activity aimed at producing temporary conditions unfavourable to the breeding of vectors in their habitats. This include water salinity changes, stream flushing, regulation of the water level in reservoirs, vegetation removal, shading and exposure to sunlight are examples of environmental manipulation activities, (WHO, 1980; WHO, 1982a).

The short comings of this method is that the method is aimed at reducing the pest or vector of disease below economic threshold level rather than eliminating them. For the method to succeed, one has to understand the life cycle of the pest and vectors of diseases as well as understand the ecological and economic consequences and accurate application of it that will be to the best interest of mankind (Helmut,

1976).

CHAPTER THREE

MATERIALS AND METHODS

3.1. COLLECTION AND REARING OF GRAVID FEMALES AND LARVAE OF MOSQUITOES

(a) A total of 57 gravid female mosquitoes were collected with sweep nets from Suleiman Hall, Ahmadu Bello University, Zaria and Chindit Barracks, Sabon Gari, Zaria.

(b) The collected females were taken to the laboratory and released into cages for identification using the methods described by Chandler and Read (1960), Anthony (1979) and Singh (1983). Identification was based on presence or absence of lobbed scutellum, resting position and scales on the wings among others; this separated the mosquitoes into culicine and anopheline. The anopheline were destroyed and not used as earlier attempts to breed towards production of large number of larvae were not successful.

(c) Identified female Culex were separated and put inside wire meshed cages measuring 30 x 30 x 30 cm (Service, 1963; WHO, 1975).

(d) A petri dish containing damp cotton wool soaked in 4% sucrose

solution was placed in each cage to facilitate oviposition. An artificial light source of 60 watts bulb was also provided (Service, 1963; WHO, 1975; Singh, 1983).

(e) A breeding pan (resembling a glass jar) or open clay pot jar half filled with de-chlorinated tap water was provided in the cages (Plate I) for oviposition. Grass (Cyanodon dactylon) infusion was placed in the water jar as source of food upon decay for the emerging larvae (Umaru, 1986).

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Platel: Breeding/Rearing cage made of wire mesh containing (a) breeding pans, (b) a pigeon with skinned chest hung over it as source of blood meal for the mosquitoes (c) petri dish, (d) open clay pot.

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(f) A pigeon with skinned chest was hung over the cage for 24 hours to provide source of blood meal for the females, while daily observations were made for possible oviposition.

(g) Following oviposition, the eggs were removed by a micro pipette, from the dish to a slide containing a few drops of water for identification under light microscope (at x 40) using the method described by Anthony (1979) before transferring them to the breeding pans.

(h) The physico-chemical conditions namely, (temperature, pH and relative humidity and of the breeding water habitat of the gravid females/larvae were determined periodically with the aid of thermometer, pH metre and hydrometer respectively. The corresponding conditions of the breeding laboratory were checked twice weekly to ensure that the conditions were optimal (Sing, 1983).

(I) Newly laid eggs were examined and monitored using a dissecting microscope during transformation in order to ascertain the age of the test larvae and possibly account for any other cause of mortality of the larvae e.g. food scarcity and available dissolved oxygen in the breeding media.

3.2 ISOLATION OF BACILLUS SPECIES AND PREPARATION OF DRY POWDER SAMPLES

The method used for the isolation and identification of Bacillus

species is that described by WHO (1987).

(a) The most probable sources of Bacillus spp, according to WHO (1987), consists of moribund or dead mosquito larvae and mud-samples. These samples were collected along River Kubani which separates Chindit Barracks with Gyellesu, Zaria, and a small stream at Jaji and in gutters on the main campus of the Ahmadu Bello University, Samaru, Zaria, between February, 1995 and May, 1997.

(b) Dead Culex mosquito larvae were collected into small plastic tubes and mud in polythene bags and taken to the laboratory and most of them were identified to be Culex mosquito larvae as recommended by WHO (1987) using the method described by Read and Chandler (1960) and Anthony (1979). Each dead mosquito larva or mud sample (20g) was put on filter paper and held at room temperature or placed in an oven at 30°C, and left to dry for 7 days.

(c) Each dead larva or 1gm out of mud sample was transferred into a 10ml of sterile water in a glass tube and macerated/mixed.

(d) The tubes containing the samples were heated in an oven at 80°C for 20 minutes to kill non-spore forming organisms.

(e) One loopful of the tube content was streaked on sterile nutrient agar plates containing 0.3% yeast extract. The plates were incubated at room temperature.

(f) The plates were examined after 24 and 48 hours for bacterial colonies. When there were no colonies, the plates were re-incubated for additional 48-72 hours and then re-examined.

(g) The colonies were examined for spore formers using the Gram staining technique (Drury and Wallington, 1960). Each colony containing spore formers was then streaked on a fresh plate of nutrient agar (enriched with yeast). The plates were incubated again for between 3-7 days at room temperature for colony growth.

(i) The (g) above were subcultured on a fresh medium enriched with 0.3% yeast extract. A small portion of each colony was placed onto a slide and smear made, dried, fixed and Gram stained bacteria were identified on the basis of the presence or absence of crystalline inclusions, spore shapes, rod-shaped sporangium/swelling and colour among others as reported by Gordon, *et al.*, (1973), Carlson and Kolsto, (1993) and Yon-Biao, *et al.* (1998). The remaining part of the growth was harvested onto a sterile filter paper, dried and ground in a sterile mortar; portions of each were sent to the Institute de Pasteur, Paris France for confirmation of identity.

3.3 PREPARATION OF BACTERIAL SUSPENSION AND CIDAL TESTS ON MOSQUITO LARVAE

The method described by WHO(1987) was adopted for this work.

(a) About 50mg of the dried Bacillus powder prepared from agar plates (3.2) was dissolved in 10ml chlorine free water in a test tube and homogenized thoroughly for five minutes.

(b) One loopful of the suspension was placed on a slide and after Gram staining, the slide was observed under a light microscope (x 1000): the structures/shape and colour of the bacteria including the status of being either positive or negative bacteria was noted (Drury & Wallington, 1960).

(c) The initial Bacillus suspension above was agitated thoroughly and six serial dilutions using dilution factors of 0.80, 0.6, 0.4, 0.2, 0.16 and 0.1 in constant volumes of water (50 ml and 200ml) were made to give the following concentrations of the Bacillus suspensions: 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/l (Appendix 1) in order to get a good distribution of mortality rates (WHO, 1987).

(d) Twenty five (25) of the test Culex larvae in replica of three test tubes per concentration were used. The test was repeated three times to ensure that at least two mortality rates on either side of the LC50 (LC50 = lethal concentrations that will kill 50% of the organisms)

within the range of 10-90 or 95% mortality were achieved. At the end of the trial test above, the whole preparation process of Bacillus suspension was repeated and the test proper was carried out as below.

(e) A stock solution was prepared by putting 50mg of dried Bacillus powder obtained from the culture into 10ml of sterile water in a test tube and mixed thoroughly for 5 minutes to give a solution of 50mg/L.

(f) From this stock, another solution was made by adding 0.1ml of the stock homogenate to 999.9ml of de-ionized water (1:1000) mixed thoroughly to give a concentration of 0.5mg/L.

3.4 TOXICITY TEST AGAINST MOSQUITO LARVAE

(a) 50ml of de-ionized water was dispensed into a triplica of of six 100ml volume beakers and 25 living 2nd instar larvae of Culex were introduced.

(b) The required volumes of Bacillus suspension (40, 30, 20, 10, 8 and 5 micro litres) to give concentration of 0.04, 0.03, 0.02, 0.01, 0.008, 0.005 were calculated. These were added by means of micro pipette to each of the set of beakers containing Bacillus suspension less the volume of Bacillus to be added; the experiment was carried out three times.

(c) Similarly, 200ml of de-ionized water was put into another

triplica of six sets of 250ml volume beakers and 25 living 2nd instar larvae of Culex were introduced into each of them (WHO, 1987).

(d) By using the same method as above (Appendix 1), the calculated volumes of Bacillus suspension required were 160, 120, 80, 40, 32 and 20 micro litres, these were added into the 200ml of water beakers less the volumes of Bacillus to be added to give the same corresponding concentrations of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/L. The reason for using 50ml and 200ml of de-ionized water was to test the efficacy of the Bacillus species on Culex larvae in varying volumes of water since it has been known that Bacillus show difference in activities in varying volumes of water(De Barjac and Thamson, 1971, Shehu; 1986).

(e) After an exposure for 24 to 48 hours, the number of living larvae in the test tube were counted (number of live larvae were counted instead of dead larvae) because dead larvae get decomposed/lysed easily and may thus not give accurate reading.

(f) The number of living larvae in the control were also counted and where mortality rate was higher than 10%, the experiment was repeated as recommended by WHO (1987). The total number of living larvae of Culex mosquito used in this work (both trial and the test proper 9,450.

(g) The percentage mortality rate was calculated and the LC50 was obtained by plotting a graph of mortality rate against concentrations.

(h) The relative toxicity of the test preparation was determined graphically and with respect to the standard given by the formula:

$$P(IU) = \frac{1000 \times LC50 \text{ of standard}}{LC50 \text{ of test preparation}}$$

Where P = Potency

IU = International Unit of toxicity

(Source: WHO, 1987).

(i) Similarly 50mg of the 100mg of lypohised powder of a reference strains of Bacillus sphaericus (SPH 88 - 2362) obtained from the Institute de Pasteur, Paris, France was also prepared and tested against 2nd instar larvae of Culex mosquitoes as above. The results of the former above were compared with those of the standard (B. sphaericus) with a view of establishing the toxicity level of the various isolates.

CHAPTER FOUR

RESULTS

4.1 MOSQUITO BREEDING

A large number of egg rafts were laid on top of water containing grass infusion in the breeding pan, while a few were laid on the petri dish containing cotton wool soaked in 4% sucrose solution. The eggs were observed under low light magnification for shape and morphological changes and identified to be that of eggs of Culex mosquitoes (Plate II). The eggs became darkened and enlarged progressively as they imbibed water and changes started from the poles (anterior and posterior) which was in line with the observation of Chandler and Read (1960).

Eggs started hatching out to larvae after 24 hours and by 48 hours almost all had hatched out to the first larval instar which was small, weak and not observed feeding; head bears egg breakers (hatching spines). The larvae moulted and transformed into 2nd instar larvae after 24 hours of hatching. After ecdysis, the 2nd instar larvae become active, breathing apparatus become visible and body was not distinctly divided into head thorax and abdomen. Following another ecdysis after 48 hours, the 3rd instar larvae emerge with body segments very distinct, sluggish, and head globular.



Plate II: Eggs of Culex mosquitoes

(@ x 140)

Here they stayed for short period and transformed to 4th instar larvae which have more distinct body division into head, thorax and abdomen. They are also sluggish in action and are bigger in size. Shortly after this transformation from 1st to 4th stage larvae which took about 5 days the larvae transformed to the pupal stage. The larvae started transforming to pupae after 5 days and by the 9th day almost all had transformed to young adults. The average time observed for eggs to transform to young adults in Culex mosquito was 15 days (9 - 16 days). There was however little delay in the transformation of some of the larvae to pupae. Some of the emerging larvae were allowed to transform to adult to provide continuous material (larvae) for the project. There was increased frequency of maturity and egg rafts viability from F_1- F_2 generations.

The optimum temperature of the breeding media of Culex mosquitoes recorded throughout the period of the investigation was 28.50°C (26.4°C - 30°C) and compared favourably well with 30°C reported by Omokaro (1985). The optimum pH of the breeding media recorded was 6.9 (6.5 - 7.4) The average relative humidity recorded in this work was 71.6% (48% - 90%).

4.2 ISOLATION AND IDENTIFICATION OF BACILLUS ISOLATES

Based on microscopic examination of Gram stained isolates, two groups of Bacillus were identified as follows:

Group one: colonial morphology appeared whitish creamy on the agar plates; Gram's stain showed blue-black structure with ellipsoidal and intercallary located spores within the cells (Plate III). These were identified as Bacillus cereus following the description of Smith, *et al* (1952); Gordon *et al* (1973); Ahmed, *et al.,* (1983); Shin-Ichiro *et al* (1997). The identity of such isolates were later confirmed as B. cereus by the WHO laboratory in Paris, France (Appendix 2). This group is the most dominant species isolated from dead mosquito larvae within the Zaria area based on its prevalence of about 88%.

Group two: This group has features closely related to the first group. Its morphology also reveals opaque whitish creamy colonies on agar plates; Gram stain revealed spherical spores containing crystalline inclusions. Its cells/spores also appear blue-black with Gram's stain under light power magnification (Plate IV and V). This group is not very common in this area based on the result of this work which represents just 12%. This group follows the description of Bacillus thuringiensis (WHO 1984, 1985, and 1987). The two varieties of Bacillus thuringiensis were confirmed in the Paris laboratory as Bacillus thuringiensis Pakistani H-13 and B. thuringiensis tohokuensis H-17 (Appendix 2.

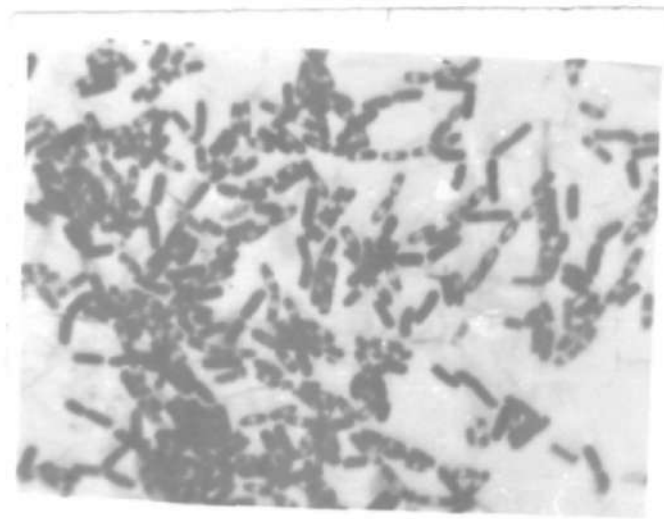


Plate III: Structures/colonial morphology of Bacillus cereus with ellipsoidal/intercallary located spores.

(Gram stain: x 1000)



Plate IV: Creamy whitish colonial morphology of B. thuringiensis on nutrient Agar plate.

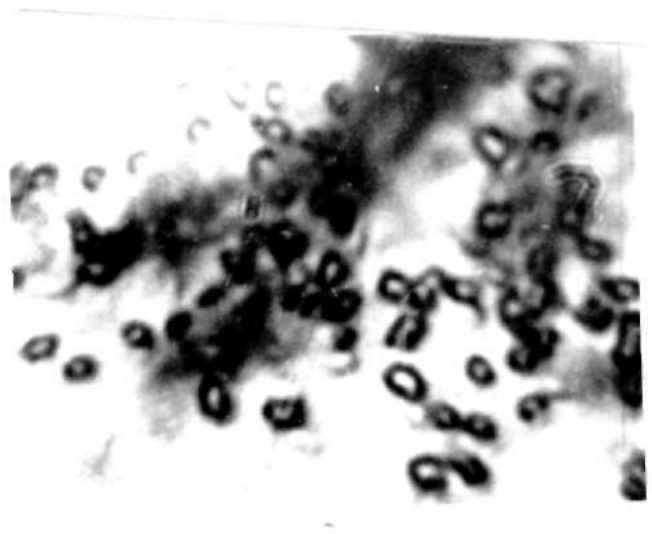


Plate V: Bacillus thuringiensis with spherical spores.

(Gram stain: x 1000)

Bacillus sphaericus (standard) was also grown on the same nutrient agar with the test isolates in order to compare their morphology. Colony appearance was opaque, white and creamy. Gram stain revealed rod shaped sporangia that showed no crystalline inclusion (Plate VI) as described by WHO (1984, 1985) and Charnley (1991). Both mosquito larvae isolates and the soil isolates that yielded growth revealed similar morphological features by Gram's stain i.e. some showed presence or absence of crystalline inclusions, spherical or rod shaped and deep blue-black colour.



Plate VI: Rod shaped sporangia of Bacillus sphaericus.

(Gram stain: x 1000)

4.3 TOXICITY TESTS OF BACILLUS ISOLATES ON CULEX LARVAE

Out of the 78 soil and mud samples tested, 39.7% produced bacterial growth on nutrient agar plates and when tested for cidal activities, all showed no activity on Culex mosquito larvae. Of the 150 dead mosquito larval samples collected, 27 (18%) yielded growth on incubation at 30°C after 24-48 hours, while the remaining 123 (82.0%) samples did not grow even on further incubation for up to 7 days. Out of the 27 isolates from dead mosquito larvae that yielded growth, 23 (85.5%) showed high cidal activity against the mosquito larvae.

About 50% and 72.8% of the Culex larvae tested upon died within 48 hours on exposure to 0.02mg/L Bacillus cereus suspension in 50ml and 200ml volume of water respectively; 5% and 8% died after24 and 48 hours of exposure in the control experiment that had no Bacillus cereus suspension. The difference in mortality rates between treated and non-treated was significant ($p<0.05$).

The mortality rates were also observed to increase with increase in the volume of water used i.e. mortality rates were higher in 200ml volume of water than in 50ml of water (Appendix 4-7). Bacillus thuringiensis pakistani H-13 and B. thuringiensis tohokuensis H-17 which constituted 4.3% each of the total isolates were observed to have higher cidal activity on Culex larvae than B. cereus (Table 1).

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The major differences observed was that of killing time where both varieties of B. thuringiensis killed faster (within 12-24hrs) while B. cereus killed within 24-48hrs. The difference in the mortality rates was significant ($p<0.05$) at all concentrations tested(Table 1).

A known standard Isolate - Bacillus sphaericus suspension displayed a very high activity against second larval instar of Culex mosquito in 200ml volume of water where 93 - 96% mortality was recorded within 24 hours and 98% in 48 hours at concentrations of between 0.02 - 0.04 mg/L (Appendix 3 and 4, Fig 6). The B. sphaericus suspension was observed to have effect on Culex mosquito larvae even beyond 48 hours which confirms its ability to re-cycle itself as earlier reported by DeBarjac (1979), WHO (1984, 1985, 1987) and Charnley (1991). Smilarly, cidal activity was also observed with B. cereus against second larval instars of Culex mosquito larvae after 48 hours, thus suggesting its ability to also have a re-cycling or stay longer action up to 7 days. This was not observed with B. thuringiensis Pakistani H - 13 and Bacillus thuringiensis tohokuensis H - 17.

Another observation made on the larvae killed by B. cereus was the softening and gradual disintegration of the dead larval tissues hours after death. These were not observed in the larvae killed by either of the two varieties of B. thuringiensis or B. sphaericus

TABLE 1: Toxicity level (LD50 and LD90) of Bacillus sphaericus 2362 and Zaria Bacillus isolates in 200ml of water after 24hrs of exposure

S/NO	ISOLATE	LD50	Mean no of dead larvae per test	LD90	Mean no of dead larvae per test	Toxicity acceptance level (at 0.005 - 0.04 mg/L) 15-22 dead per conc.	Level of significance Test Vs Standard
1	Bacillus SPH88 2362 sphaericus	0.005 mg/L	18	0.02 mg/L	22	18 - 22 (within)	WHO Standard
2	Bacillus cereus	0.025 mg/L	5	0.032 mg/L	11	5 - 11 (below)	Significant ($p<0.05$)
3	B. thuringiensis pakistani H-13	0.012 mg/L	15	0.028 mg/L	18	15 - 18 (within)	Insignificant ($p>0.05$)
4	B. thuringiensis tohokuensis H-17	0.013 mg/L	14	0.025 mg/L	19	14 - 19 (within)	Insignificant ($p>0.05$)

(standard).

At low concentrations the activities of the various isolates were low and with increase in concentration, exposure time and volume of water giving almost a straight line graph at concentrations of between 0.005mg/L - 0.02mg/L beyond which there was a decline in mortality rate. Thus giving a dom-shaped/Sigmoid curve(Fig. 1-6) which is in line with the observation earlier reported by De Barjac and Larget, (1979) and Shehu, (1986). The mortality rate of the various isolates and the standard were generally low in 50ml volume of water than in 200ml volume, but all exhibited the same pattern of activity curve in relation to exposure time and concentrations from 0.005 - 0.02mg/L (Fig. 3 - 8). Bacillus cereus however has its activity peak from 0.02mg/L - 0.03mg/L before a decline in activity sets in.

Generally, an increase in concentration and exposure time were proportional to the mortality rate up to certain level and thereafter became a curve and changed from being arithmetical to exponential. At this stage an increase in exposure time and concentration became inversely proportional to the mortality rate.

The lethal concentrations that will kill 50% (LD50) of the test organisms were determined for each isolate graphically using graph sheets and by the formula earlier given by WHO (1987).

Thus the toxicity of Bacillus cereus (which gave 23% to 75% mortality after 24 hours exposure in 50ml and 200 ml of water was found to be 0.032 mg/L on the graph and LD50 was 0.025 mg/L (Fig 1, 2). The toxicity of Bacillus thuringiensis was 32.5% - 78% at 24 hrs exposure in 50ml and 200 ml volume of water which fall on 0.02mg/L on the graph and its LD50 was 0.013mg/L (Fig 3 and Table 1). The toxicity level of the standard (B. sphaericus) was from 72% - 96.2% after 24 hours exposure and 80-98% in 48 hours in 50ml and 200 ml volume of water respectively and its LD50 was 0.005mg/L (Fig 7 and 8). The test of significant level results show that 18-22 larvae out of 25 died per each treatment with the standard (B. sphaericus) within 24 and 48 hours exposure in 200ml volume of water.

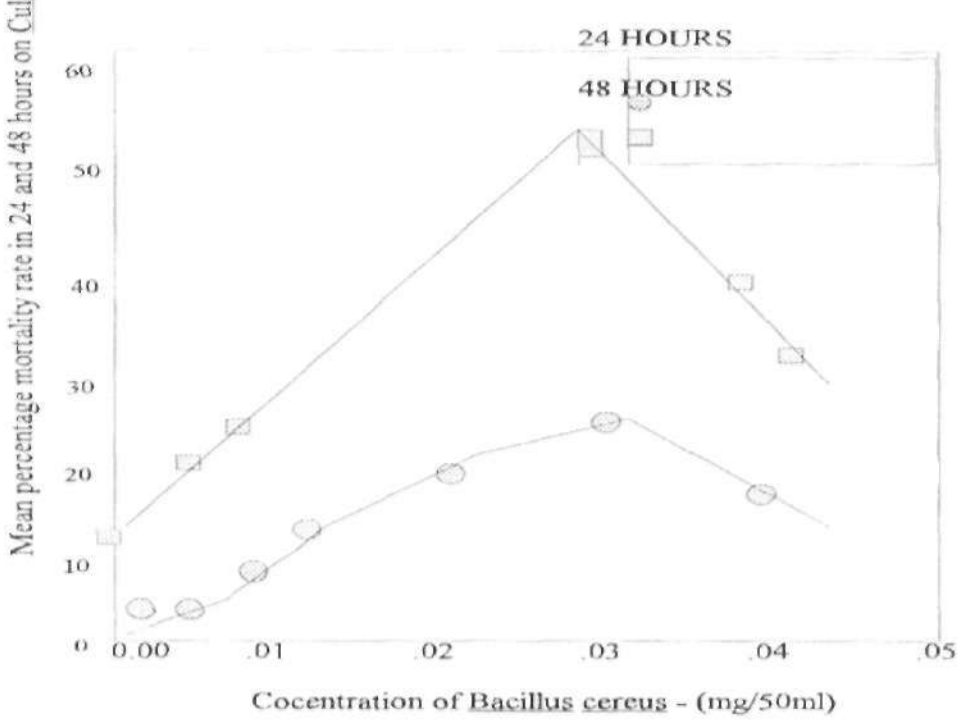


Fig. 1 Toxicity of B. cereus to second instar larvae of Culex mosquito after exposure for 24 hours in 50ml of water

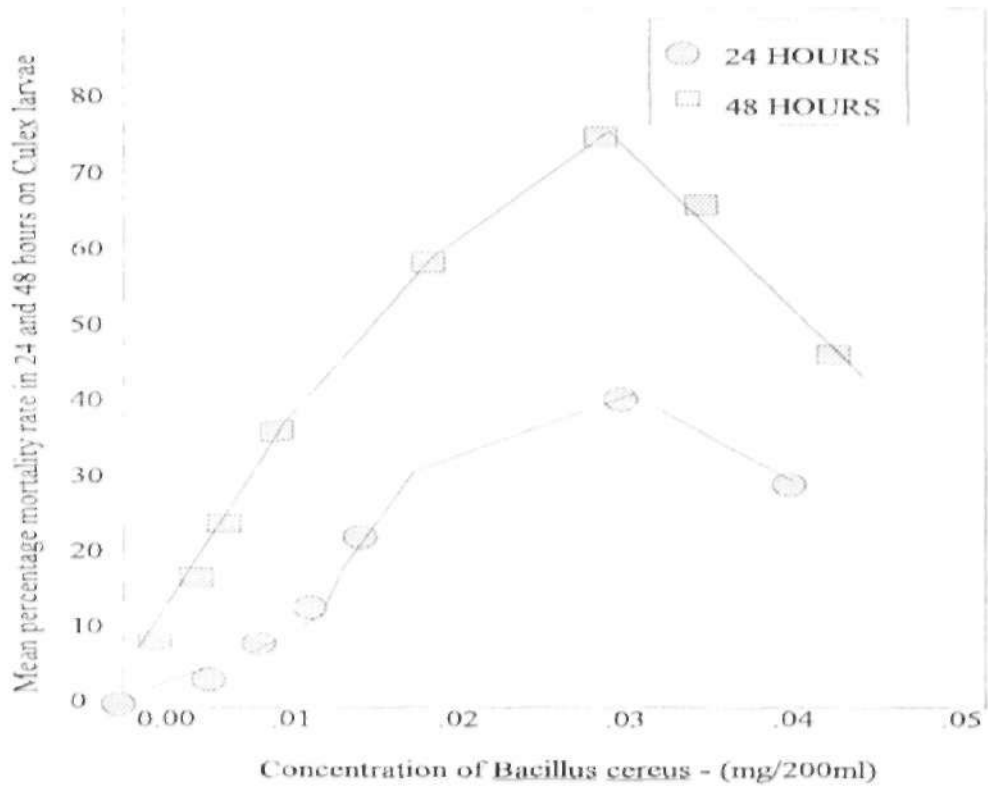


Fig. 2 Toxicity of B. Cereus to second instar larvae of culex mosquito after exposure for 24 and 48 hours.

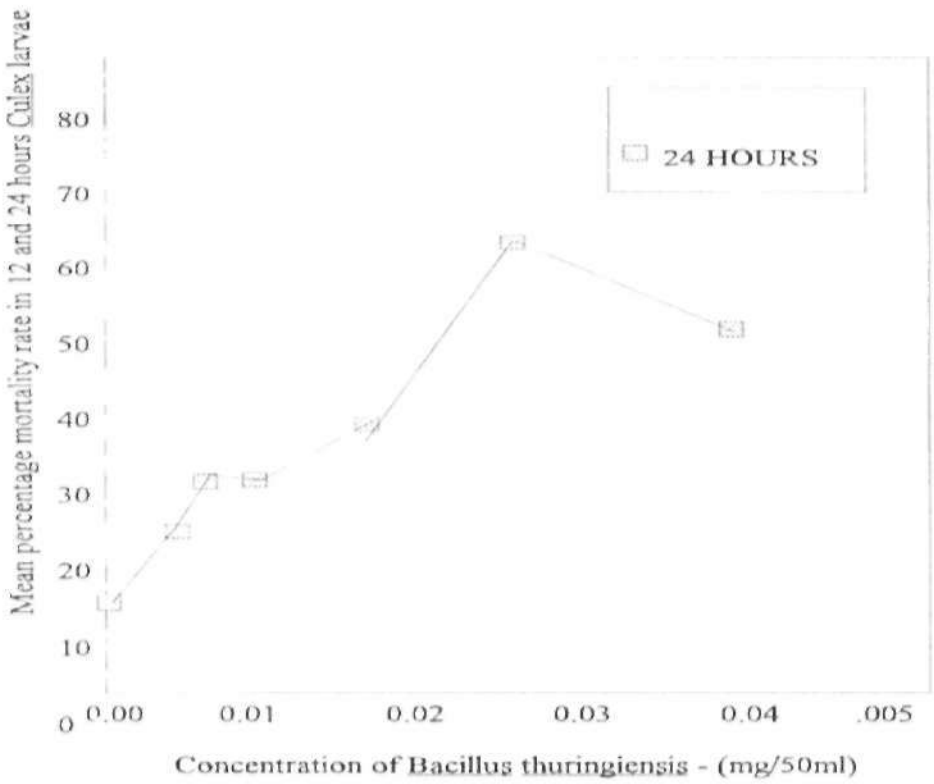


Fig. 3 Toxicity of B. thuringiensis pakistani H-13 to second instar larvae of Culex mosquito after exposure for 24 hours.

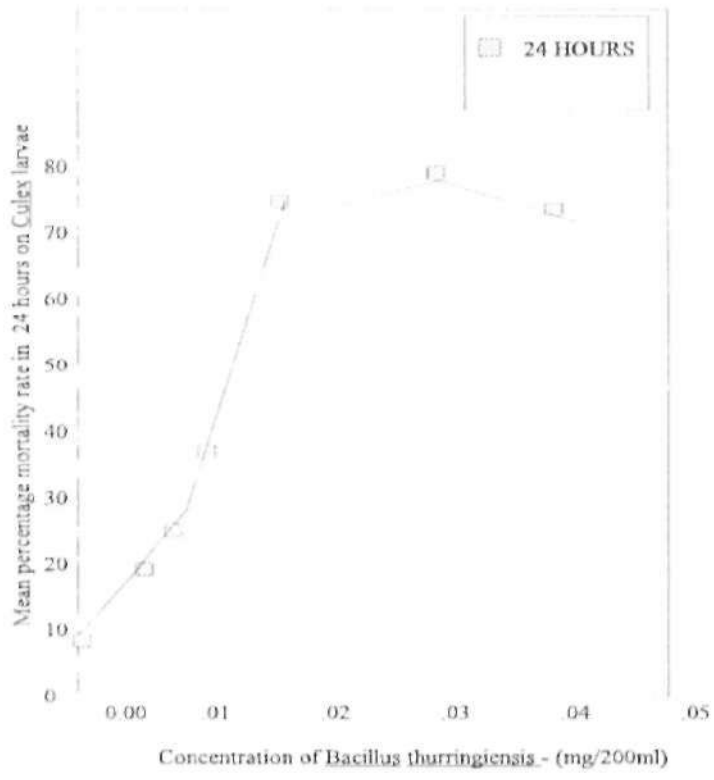


Fig. 4 Toxicity of B. thurringiensis pakistani H-13 to second instar larvae of Culex mosquito after exposure for 24 hours.

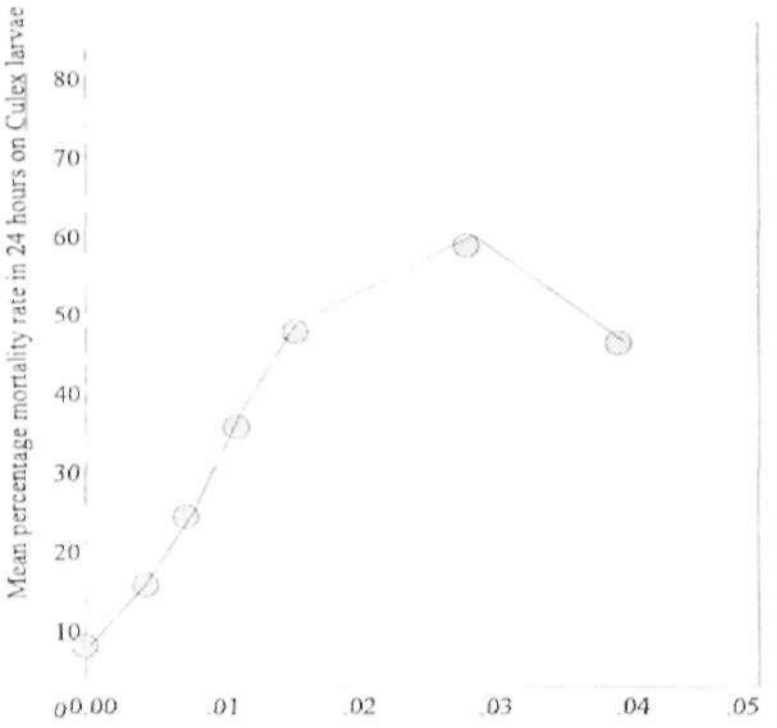


Fig. 5 Toxicity of B. thuringiensis to 2nd instar larvae of Culex mosquitoes after exposure for 24 hours.

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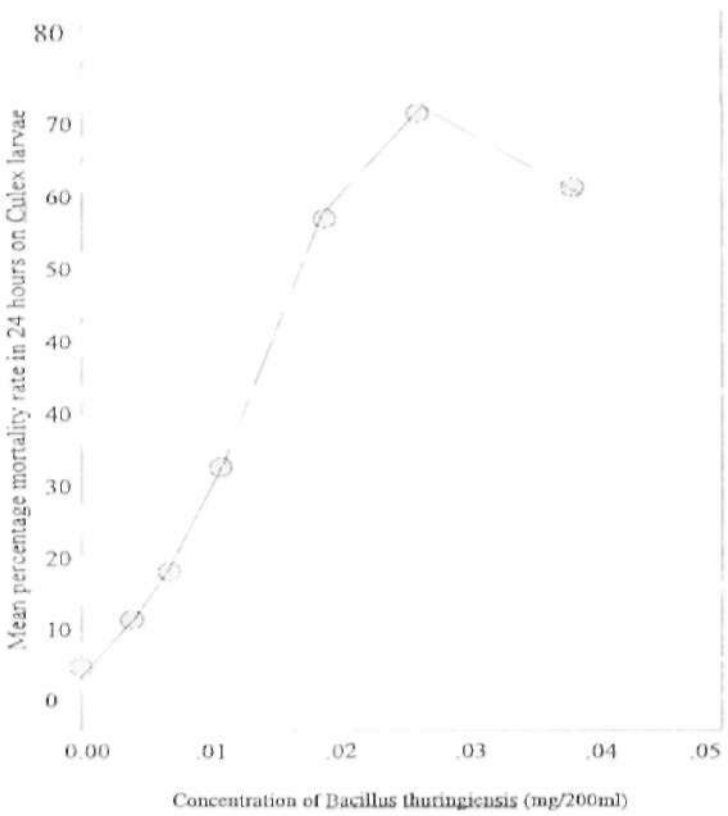


Fig. 6 Toxicity of B. thuringiensis tohokuensis H-17 to 2nd instar larvae of Culex mosquitoes after exposure for 24 hours.

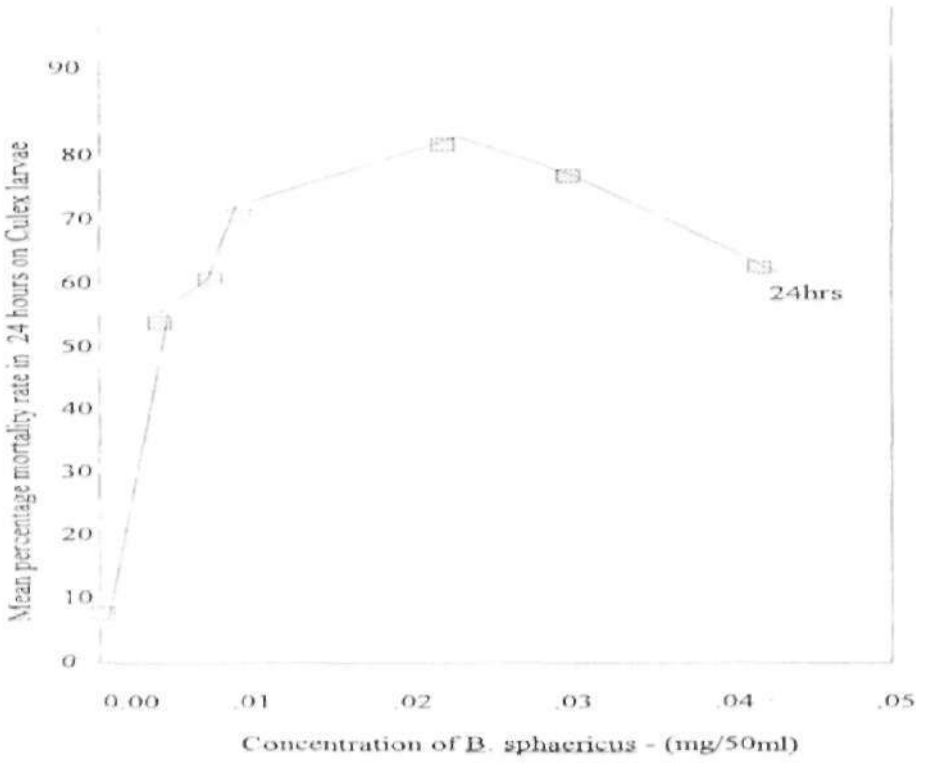


Fig. 7 Toxicity of B. sphaericus to second instar larvae of Culex mosquito after exposure for 24 hours.

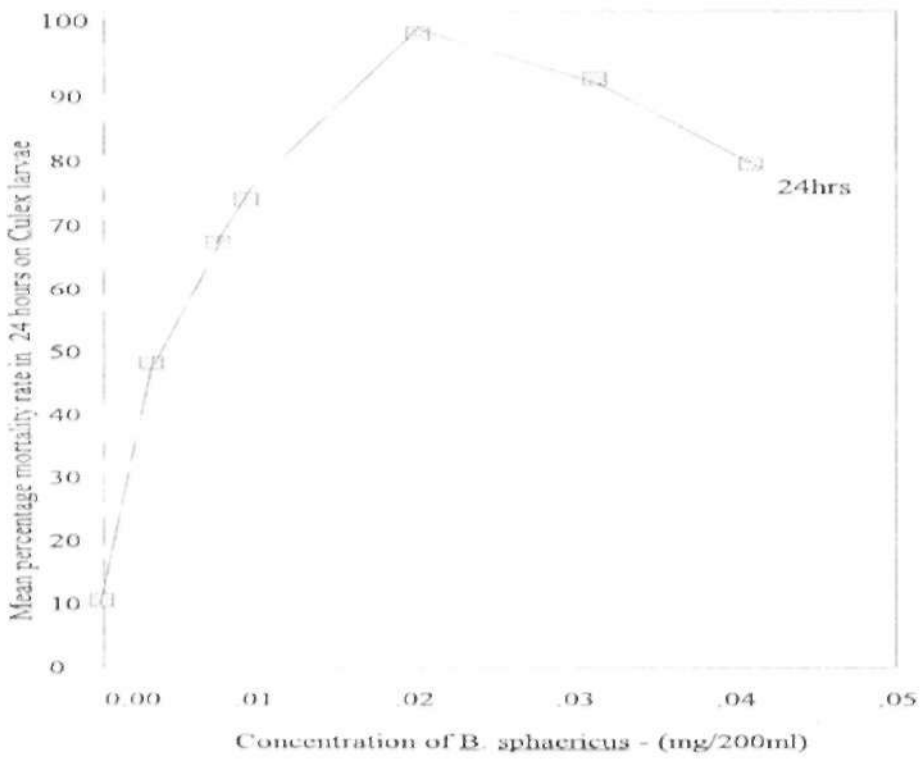


Fig. 8 Toxicity of B. sphaericus to second instar larvae of Culex mosquito after exposure for 24 hours.

CHAPTER FIVE

5.1 DISCUSSION

Part of this work focused on colonization of mosquitoes to provide available test material for the isolated Bacillus species. The successful colonization of Culex mosquitoes under laboratory conditions of temperature, PH and relative humidity was achieved in line with report by Jupper and Brown (1967); WHO (1975) and Umaru (1986). The period of transformation from one stage to another compared favourably with those described by Chandler and Read (1960).

In this work, gravid female Culex mosquitoes adapted well to the laboratory conditions and were successfully reared up to four generations (F4) with each generation showing increased acclimatization. The optimum temperature (28.5°c) of the breeding media of the Culex mosquitoes compared favourably with the 30°c earlier reported by Omakoro (1985).

Differences in larval stages also played a very important role in this work just as it has been reported by earlier workers. The first stage larvae were not found suitable because, they were weak, small, inactive and hardly feed (Hopkins 1967, Umaru 1986).

The second instar larvae were more suitable because they are very active, fed well and rate of metabolism was high (WHO 1975, 1982, 1984, 1985, Shehu, 1986).

After successful isolation of the Bacillus species that showed activity against Culex mosquito larvae based on colony characteristics and Gram staining technique, two broad groups emerged as Bacillus cereus and B. thuringiensis. Based on colony morphology on agar, there is no much distinctive features that differentiate Bacillus cereus from both the two varieties of Bacillus thuringiensis and also Bacillus sphaericus - WHO Standard. This is because all of them show the same pattern of colony growth on nutrient agar plates: opaque, creamy - whitish in colour and mucoid.

However, microscopic examination revealed ellipsoidal/intercallary located spores in Bacillus cereus (Plate III), while B. thuringiensis have spherical spores (Plate V) and are said to contain crystalline inclusions (Charnley, 1991). The microscopic examination of B. sphaericus revealed a rod shaped sporangia (Plate VI) that are said to lack crystalline inclusions (Chanley, 1991). These two groups showed significant difference in activity which could be due to some factors particularly the constituent nucleotide sequence(Shin-Ichiro, et al., 1997). The difference in activity between

B. cereus and B. thuringiensis was significant ($p<0.05$) while there was no significant ($p>0.05$) difference in activity between B. sphaericus (WHO standard) and B. thuringiensis. This suggests that B. sphaericus and B. thuringiensis might have similar constituent nucleotide sequence.

Shinagawa *et al* (1991) observed that there were variations in the constituent nucleotide sequence of the extracellular enterotoxin genes of Bacillus thuringiensis and B. cereus. Shin-Ichiro *et al* (1997) also reported that the nucleotide sequence determination of Bacillus cereus revealed an open reading frame (ORF) encoding for a 45-KDa protein with 422 amino acid residues, while that of B. thuringiensis showed a continuous ORF encoding a 45-KDa protein of 430 amino acid residues.

From these findings, one can then simply link the significant differences in toxicity (Appendix 5) of B. thuringiensis over B. cereus to the differences in the constituent of their extracecullar enterotoxin genes where B. thuringiensis showed higher activity in relation to its higher nucleotide sequence (430) than B. cereus that has less (422). These differences exhibited above by the two broad groups isolated (B. cereus and B. thuringiensis) are reflected in the results of their LC50 and level of significance where B. cereus fall short of the level of

significance compared to the B. sphaericus (standard) and B. thuringiensis (Appendix 7-9 and Table 1).

The exact mode of action of B. thuringiensis and B. cereus has not yet been well established, but earlier workers (De Barjac, 1979; Davidson, 1981; WHO, 1984, 1985, Charny, 1991) have suggested that upon ingestion, the midgut epithelium appears to be the primary site of action at least initially. According to them, upon ingestion of insecticidal bacterial strains, the crystals is dissolved in the gut of the host insect by proteolytic enzymes: toxins are produced and cause death of the larvae within few hours followed by epithelial cell lysis.

The duration of killing time in the standard - B. sphaericus and B. thuringiensis (isolate) were shorter and actions were faster and were completed within 24 hours, unlike in the B. cereus where there was a delayed action which was also slower and prolonged up till 48 hours or even longer. This could be due to differences in their constituent enterotoxins produced where B. sphaericus and B. thuringiensis are more toxic and consequently active on mosquito larvae having protein with 435 and 430 amino acids residues respectively than B. cereus whose enterotoxins have protein with 422 amino acids residues as observed by (WHO, 1984, 1985, Shin-Ichiro et al, 1997).

The mortality rate increased arithmetically with increase in exposure time from 24-48 hours (see Fig 1 - 8). The volume of suspending fluid were also found to be directly proportional to rate of killing to a certain level and beyond which there was decline in the death rate as seen in the graphs (Fig 1-6). These observations are in line with earlier work reported by DeBarjac and Larget (1979) who stated that the second instar larvae of mosquitoes are 7 folds sensitive to Bacillus thuringiensis at high temperature, in deeper containers with more exposure time up till 24 hours. These according to them make the medium slightly warmer thus results in higher metabolism and feeding of bacteria by the larvae; ultimately there is high number of bacteria in the gut of the larvae which consequently leads to more infectivity and death of the larvae.

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These explains the reason why more larvae died in 200ml volume of water containing suspension of Bacillus than in 50ml at the same exposure time, temperature and concentrations (Fig 1-8). Thus the mortality rate observed in the larvae between 24- 48 hours exposure period can purely be attributed to the bacterial activities because in this work, laboratory factors were not allowed to set in though the work was extended beyond 48 hours record of such results were not used due to the fact that addition of synthetic dietary

substances into the medium could have been necessary as recommended by WHO (1975) and Jupper and Brown (1967) after 48 hours and this was not done.

The bioassay tests carried out to determine how much concentrations of Bacillus species were required to cause death in the population of the target insect - (the mosquito) were variable. Thus the lethal concentration that killed 50% of the insect population (LD50) by the various isolates showed that 0.025 mg/L concentration of Bacillus cereus was required to reduce the larval population of Culex mosquitolarvae by 50%, while 0.012 mg/L and 0.013mg/L of Bacillus thuringiensis pakistani H-13 and Bacillus thuringiensis tohokuensis H-17 respectively were required to cause the same mortality within 24 hours; for the standard Bacillus sphaericus, 0.005 mg/L concentration was needed to reduce the population by 50% at the same exposure time. This shows that Bacillus cereus requires more killing time and higher concentration than Bacillus thuringiensis and B. sphaericus.

Bacillus cereus, though found very common in this area is not considered a good candidate because of its low toxicity (Table 1) but more importantly, due to its pathogenicity as a disease causing organism in humans it is therefore not ideal to use as a biological

agent(Dryden, *et al, 1987*).

Its high prevalence in mosquito larvae as shown in this study, however suggest that it could be biologically active (though low) in regulating mosquito populations in the natural habitat. The isolation of Bacillus cereus from dead mosquito larvae in addition to exhibiting some bio-control potential against Culex larvae is being reported to our knowledge, for the first time.

From the results obtained, it was observed that Bacillus cereus exhibited low cidal activity of between 23 to 73.5% mortality per each treatment, in 50ml and 200ml volume of water respectively, while Bacillus thuringiensis pakistani H-13, and Bacillus thuringiensis B. tohokuensis H-17 exhibited high level of cidal activities of 32-78% mortality was recorded per each treatment, similar to the B. sphaericus where 78-98% mortality per each treatment were recorded in 50 and 200ml volumes of water at same exposure time(24-48 hours) and thus fall within the level of acceptance as a biological control agent (Table 1). These could be regarded as strains found within the Zaria area that can be used in the control of mosquito larvae.

The application of B. cereus as a bio-control agent however is not favoured because of its low toxicity against mosquito larvae as

seen in this work and did not compare favourably with Bacillus sphaericus - WHO standard (Table 1). In addition, B. cereus is also known to be pathogenic to animals including humans and thus cannot be recommended for application as a bio-control agent. The two varieties of Bacillus thuringiensis (H-13 and H-17), though were not found to be common in this work, their activity however show high potency against Culex mosquito larvae. The literature of the two varieties of bacilli - Bacillus thuringiensis pakistani H-13 and tohokuensis H-17 isolated in dead mosquito larvae in this work were not available which suggest that they may not be very common Bacillus species. The study has thus revealed that there exist Bacillus species within Zaria area that have biocontrol potentials. It also shows that Bacillus cereus exist in some insect larvae and not only in food, thus suggesting that it's also pathogenic not only to man and other animals but also insects.

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*IRRI/PEEM = International Rice Research Institute/Panel Expert Committee on Environmental Management.

APPENDIX 1: DETERMINATION OF CONCENTRATIONS OF BACILLUS SUSPENSION IN VARYING VOLUMES OF WATER

S/No	Volume of water (ML) used (a)	Dilution factor used (b)	Vol (ul) of Bacillus suspension to be added to each beaker c = a x b	Corresponding concentrations of Bacillus suspension (mg/L) in vol. of water used (d)
1		x0.8	50x0.8 = 40	$\frac{40}{50} \times \frac{50}{1000} = 0.04$
2		x0.6	50x0.6 = 30	$\frac{30}{50} \times \frac{50}{1000} = 0.03$
3	50	x0.4	50x0.4 = 20	$\frac{20}{50} \times \frac{50}{1000} = 0.02$
4		x0.2	50x0.2 = 10	$\frac{10}{50} \times \frac{50}{1000} = 0.01$
5		x0.16	50x0.16 = 8	$\frac{8}{50} \times \frac{50}{1000} = 0.008$
6		x0.10	50x0.10 = 5	$\frac{5}{50} \times \frac{50}{1000} = 0.005$
7		x0.8	200x0.8 = 160	$\frac{160}{200} \times \frac{50}{1000} = 0.04$
8		x0.6	200x0.6 = 120	$\frac{120}{200} \times \frac{50}{1000} = 0.03$
9	200	x0.4	200x0.4 = 80	$\frac{80}{200} \times \frac{50}{1000} = 0.02$
10		x0.2	200x0.2 = 40	$\frac{40}{200} \times \frac{50}{1000} = 0.01$
11		x0.16	200x0.16 = 32	$\frac{32}{200} \times \frac{50}{1000} = 0.008$
12		x0.10	200x0.10 = 20	$\frac{20}{200} \times \frac{50}{1000} = 0.005$

1 Concentration of stock solution = 0.5mg/L

2 Dilution factor of x0.8 - 0.10 (WHO, 1987)

3 Volume of water - 50ml No. (1-6) 200ml (No. 7-12)

4 Corresponding concentrations of Bacillus suspension in varying volumes of water.

$$= \frac{\text{Vol of Bacillus added}}{\text{Vol of water}} \times \frac{50 \text{ (mg of Bacillus stock)}}{1000 \text{ (litre)}}$$

$$\text{i.e.} = \frac{\text{Vol of Bacillus}}{\text{Vol of water}} \times \frac{50 \text{ (mg/L)}}{1000}$$

KASUM ... LIBRARY

APPENDIX 2: RESULTS OF THE CONFIRMATION AND CHARACTERIZATION OF THE BACILLUS ISOLATES BY WHO COLLABORATIVE LABORATORY CENTRE IN PARIS, FRANCE.

Institut Pasteur
, rue du Dr ROUX, 75724 PARIS CEDEX 15

Bactéries Entomopathogènes

33-1-45 68 82 27
33-1-40 61 30 44

W.H.O. Collaborating Centre
for Entomopathogenic Bacillus

Paris, le 13 Janvier 99

Dr Nock
Department of Biological Sciences
Faculty of Science
Ahmadu Bello university
Zaria
Nigeria

have been identified in the following way :

Nig1 -> Bacillus cereus
Nig2 -> Bacillus thuringiensis pakistani (H13)
Nig3 -> Bacillus cereus
Nig4 -> Bacillus cereus
Nig5 -> Bacillus cereus
Nig6 -> Bacillus cereus

he strains : NIG 7, NIG 8, NIG 9, NIG 10, NIG 11, NIG 12, NIG 13, NIG 14, NIG 15, NIG 6, NIG 17,

at you sent in 13/07/98

have been identified in the following way :

KASHIM IBRAHIM LIBRARY

NIG 7 -> Bacillus cereus
NIG 8 -> Bacillus cereus
NIG 9 -> Bacillus cereus
NIG 10 -> Bacillus thuringiensis tohokuensis (H17)
NIG 11 -> Bacillus cereus
NIG 12 -> Bacillus cereus
NIG 13 -> Bacillus cereus
NIG 14 -> Bacillus cereus
NIG 15 -> Bacillus cereus
NIG 16 -> Bacillus cereus
NIG 17 -> Bacillus cereus

Sincerely yours,

M -M. Lecadet

by order,
Bernard PAPIEROK, Head
Entomopathogenic Bacteria and
Fungi Laboratory

16/2/99

APPENDIX 3: Toxicity of Bacillus isolates at varying concentrations on second instar larvae of Culex mosquitoes in 50ml of water after 24 hr exposure

S/NO	ISOLATE	MEAN % MORTALITY RATE AT 0.04 (mg/L)		MEAN % MORTALITY RATE AT 0.03 (mg/L)		MEAN % MORTALITY RATE AT 0.02 (mg/L)		MEAN % MORTALITY RATE AT 0.01 (mg/L)		MEAN % MORTALITY RATE AT 0.008 (mg/L)		MEAN % MORTALITY RATE AT 0.005 (mg/L)	
		50ml:	200ml	50ml:	200ml	50ml:	200ml	50ml:	200ml	50ml:	200ml	50ml:	200ml
1	NIG. 1: B. cereus	18.50:	25.00	22.20:	38.78	16.85:	32.00	8.25:	15.20	6.00:	7.25	4.20:	5.88
2	NIG. 2: B. thuringiensis pakistani H-13	58.00:	73.89	38.33:	66.78	52.20:	75.33	30.60:	32.00	26.00:	32.00	23.02:	26.00
3	NIG. 3: B. cereus	20.65:	25.14	22.70:	38.78	16.80:	32.01	8.88:	15.20	6.02:	7.22	4.01:	5.85
4	NIG. 4: B. cereus	18.25:	25.20	22.22:	38.88	16.50:	32.05	8.20:	15.10	6.00:	7.25	4.02:	5.50
5	NIG. 5: B. cereus	18.50:	25.22	22.65:	38.25	16.70:	32.88	8.15:	15.05	6.04:	7.35	4.04:	5.60
6	NIG. 6: B. cereus	18.28:	25.15	22.30:	38.92	15.90:	32.11	8.00:	15.05	6.06:	7.00	4.40:	5.45
7	NIG. 7: B. cereus	18.60:	25.00	22.30:	38.35	16.25:	32.90	8.50:	15.25	6.06:	7.73	4.20:	5.25
8	NIG. 8: B. cereus	18.10:	25.15	22.05:	38.21	16.40:	28.02	8.40:	15.20	6.00:	7.00	4.10:	5.40
9	NIG. 9: B. cereus	18.70:	25.25	22.25:	38.65	15.50:	53.84	8.00:	15.28	6.08:	7.00	4.08:	5.60
10	NIG. 10: B. thuringiensis tohokuensis H-17	58.50:	73.00	66.25:	72.82	52.60:	75.84	30.10:	32.00	27.10:	30.00	26.10:	5.45
11	NIG. 11: B. cereus	18.22:	25.00	22.55:	38.00	16.25:	27.55	8.90:	15.60	6.10:	7.00	4.25:	5.28
12	NIG. 12: B. cereus	18.25:	25.55	22.40:	38.45	16.45:	28.02	8.30:	15.50	6.04:	7.20	4.21:	5.40
13	NIG. 13: B. cereus	18.29:	25.02	22.45:	38.01	16.20:	28.25	8.70:	15.15	6.02:	7.25	3.88:	5.20
14	NIG. 14: B. cereus	18.15:	25.60	23.01:	38.22	16.70:	28.18	8.65:	15.80	6.04:	7.30	3.66:	5.50
15	NIG. 15: B. cereus	18.75:	25.50	22.80:	38.25	16.55:	28.20	8.30:	15.90	6.06:	7.50	4.01:	5.20
16	NIG. 16: B. cereus	18.28:	25.00	22.25:	37.05	16.50:	28.48	8.35:	15.00	5.88:	7.35	4.02:	5.10
17	NIG. 17: B. cereus	18.75:	25.45	22.20:	38.68	16.28:	28.24	8.40:	15.05	5.90:	7.00	4.20:	5.20
18	NIG. 18: (B. cereus)	18.65:	25.00	23.00:	37.50	17.15:	29.80	8.00:	15.30	6.00:	7.20	4.40:	5.00
19	NIG. 19: (B. thuringiensis)	57.75:	69.50	56.50:	72.85	51.30:	74.30	30.00:	31.00	27.80:	29.80	29.80:	27.00
20	NIG. 20: (B. cereus)	18.50:	25.20	22.50:	36.20	16.25:	27.50	8.00:	15.00	6.08:	7.25	4.40:	5.00
21	NIG. 21: (B. cereus)	18.20:	18.10	22.80:	38.25	15.85:	28.00	8.01:	15.00	5.80:	7.00	4.40:	5.05
22	NIG. 22: (B. cereus)	18.30:	25.00	22.05:	37.30	16.05:	26.50	8.02:	15.00	5.88:	7.00	4.20:	5.10
23	NIG. 23: (B. cereus)	18.05:	25.05	22.25:	25.37	16.05:	27.40	8.08:	15.03	6.01:	7.20	4.40:	5.15
24	B. sphaericus(standard)	65.05:	81.33	80.25:	81.33	80.05:	96.00	70.00:	72.00	61.00:	69.00	45.02:	49.00

NIG = NIGERIA - tagged by Institue Pasteur
* 18 - 23 not confirmed by Institue Pasteur

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APPENDIX 4:Toxicity of various concentration of Bacillus Isolates on Second Instar Larvae of Culex Mosquitoes in 200ML of Water after 24hr Exposure

S/NO	ISOLATE	MEAN % MORTALITY RATE AT 0.04 (mg/L)	MEAN % MORTALITY RATE AT 0.03 (mg/L)	MEAN % MORTALITY RATE AT 0.02 (mg/L)	MEAN % MORTALITY RATE AT 0.01 (mg/L)	MEAN % MORTALITY RATE AT 0.008 (mg/L)	MEAN % MORTALITY RATE AT 0.005 (mg/L)
1	NIG. 1: B. cereus	25.00	38.78	32.00	15.20	7.00	5.00
2	NIG. 2: B. thuringiensis pakistani H-13	75.89	38.33	32.33	32.00	28.00	23.85
3	NIG. 3: B. cereus	25.11	38.78	32.01	15.20	7.22	4.85
4	NIG. 4: B. cereus	25.20	38.88	32.05	15.10	7.25	4.00
5	NIG. 5: B. cereus	25.22	38.25	32.88	15.05	7.35	4.00
6	NIG. 6: B. cereus	25.15	38.92	32..11	15.05	7.00	4.06
7	NIG. 7: B. cereus	25.00	38.55	32..90	15.25	7.73	4.06
8	NIG. 8: B. cereus	25.15	38.21	28.02	15.20	7.00	7.00
9	NIG. 9: B. cereus	25.25	38.65	56.84	15.28	7.00	4.27
10	NIG. 10: B. thuringiensis tohokuensis H-17	73.00	72.82	73.84	32.00	27.00	23.00
11	NIG. 11: B. cereus	25.00	38.00	27.55	15.60	7.00	5.00
12	NIG. 12: B. cereus	25.55	38.45	28.02	15.50	7.00	5.00
13	NIG. 13: B. cereus	25.02	38.01	28.25	15.15	7.00	5.00
14	NIG. 14: B. cereus	25.60	38.22	28.18	15.80	7.00	5.00
15	NIG. 15: B. cereus	25.50	38.25	28.20	15.90	7.00	5.00
16	NIG. 16: B. cereus	25.00	37.05	28.48	15.00	7.00	5.00
17	NIG. 17: B. cereus	25.35	38.68	28.24	15.05	7.00	5.00
18	NIG. 18: (B. cereus)	25.00	37.50	26.80	15.30	7.00	5.00
19	NIG. 19: (B. thuringiensis)	69.50	72.85	72.30	31.00	26.00	22.00
20	NIG. 20: (B. cereus)	25.20	36.20	27.50	15.00	7.00	5.00
21	NIG. 21: (B. cereus)	25.10	38.25	28.00	15.00	7.00	5.00
22	NIG. 22: (B. cereus)	25.00	37.30	26.50	15.00	7.00	5.00
23	NIG. 23: (B. cereus)	25.05	37.80	27.40	15.00	7.00	5.00
24	B. sphaericus(standard)	81.33	93.33	96.00	72.00	69.00	49.00

NIG = NIGERIA - tagged by Institue Pasteur
* 18 - 23 not confirmed by Institue Pasteur

APPENDIX 5 Toxicity of various concentration of Bacillus isolates on Culex mosquitoes in 50ml of water after 48 hrs exposure

S/NO	ISOLATE	MEAN % MORTALITY RATE AT 0.04 mg/L in 50ml:	200ml	MEAN % MORTALITY RATE AT 0.03 mg/L in 50ml:	200ml	MEAN % MORTALITY RATE AT 0.02 mg/L in 50ml:	2000ml	MEAN % MORTALITY RATE AT 0.01 mg/L in 50ml:	200ml	MEAN % MORTALITY RATE AT 0.008 mg/L in 5oml:	200ml	MEAN % MORTALITY RATE AT 0.005 mg/L in 50ml:	200ml
1	NIG. 1: B. cereus	27.56:	46.22	47.56:	72.89	36.09:	63.11	24.20:	36.05	21.22:	22.50	16.00:	17.00
2	NIG. 2: B. thuringiensis sakstaffi H-43	61.50:	75.25	65.30:	76.50	56.00:	78.10	43.25:	63.50	38.50:	59.35	36.00:	48.00
3	NIG. 3: B. cereus	27.56:	46.22	47.56:	72.80	36.02:	63.11	24.25:	36.00	21.20:	22.00	16.40:	18.00
4	NIG. 4: B. cereus	27.66:	46.12	47.65:	72.78	36.11:	63.80	24.05:	36.20	21.20:	23.00	16.45:	17.50
5	NIG. 5: B. cereus	26.98:	46.08	47.58:	72.88	36.17:	62.98	24. 25:	36.20	21.00:	23.00	16.40:	17.20
6	NIG. 6: B. cereus	27.70:	46.05	47.60:	72.58	36.25:	63.15	24.00:	36.28	20.80:	24.00	16.30:	17.30
7	NIG. 7: B. cereus	28.00:	46.25	47.74:	72.95	36.22:	63.45	24.40:	36.45	20.22:	23.45	16.40:	17.25
8	NIG. 8: B. cereus	27.53:	46.30	46.91:	72.62	36.55:	63.15	24.35:	36.60	21.45:	23.45	16.00:	17.00
9	NIG. 9: B. cereus	28.10:	46.15	48.06:	72.55	36.78:	63.20	24.30:	36.40	20.80:	24.00	15.90:	17.80
10	NIG. 10: B. thuringiensis kenkuenshis H-17	61.30:	74.00	62.00:	75.00	66.35:	78.40	44.30:	65.00	39.80:	49.24	38.20:	49.25
11	NIG. 11: B. cereus	28.08	46.10	47.44:	72.80	36.08:	63.18	24.28:	36.00	20.20:	23.00	16.20:	17.20
12	NIG. 12: B. cereus	27.55:	45.90	47.52:	72.77	36.00:	63.3	24.27:	36.40	20.40:	22.20	16.05:	17.13
13	NIG. 13: B. cereus	27.69:	46.05	47.81:	72.25	35.88:	63.42	24.20:	36.60	20.20:	22.50	16.80:	17.00
14	NIG. 14: B. cereus	26.95:	46.12	46.75:	71.95	35.89:	63.44	24.15:	36.45	20.20:	22.00	16.40:	17.00
15	NIG. 15: B. cereus	27.77:	45.88	47.25:	72.00	36.15:	63.22	24.45:	36.35	21.40:	23.00	15.85:	17.40
16	NIG. 16: B. cereus	27.58:	46.25	47.55:	72.45	36.28:	63.02	24.20:	36.20	21.00:	23.00	16.40:	17.05
17	NIG. 17: B. cereus	27.65:	46.05	47.48:	72.66	36.35:	63.02	24.25:	36.70	21.20:	23.50	16.20:	17.0
18	NIG. 18 (B. cereus)	26.80:	45.45	46.50:	71.55	35.60:	63.25	24.00:	36.80	20.80:	22.50	16.40:	16.90
19	NIG. 19: (B. thuringiensis)	60.70:	75.80	61.10:	71.55	63.40:	76.88	42.50:	68.50	38.45:	47.00	35.15:	47.00
20	NIG. 20: (B. cereus)	27.00:	46.00	47.00:	70.50	36.00:	62.20	24.60:	36.90	21.48:	22.20	16.60:	16.95
21	NIG. 21: (B. cereus)	26.45:	46.00	46.55:	71.80	36.05:	63.00	24.00:	35.35	21.48:	21.60	16.40:	17.20
	NIG. 22: (B. cereus)	27.10:	44.95	47.10:	72.60	36.05:	61.90	23.88:	36.20	21.28:	23.00	16.20:	17.10
	NIG. 23: (B. cereus)	27.30:	45.75	46.85:	71.20	36.10:	62.20	24.05:	36.20	21.20:	22.50	16.70:	17.50
24	B. sphaericus(standard)	80.50:	96.50	82.25:	98.25	85.25:	99.50	78.60:	84.70	75.70:	77.50	55.65:	71.75

NIG = NIGERIA - tagged by Institue Pasteur
* 18 - 23 not confirmed by Institue Pasteur

APPENDIX 6 :Toxicity of various conc of Bacillus on second instar larvae of culex mosquitoes in 200ml of water after 48 hrs exposure

S/NO	ISOLATE	MEAN % MORTALITY RATE AT 0.04 (mg/L)	MEAN % MORTALITY RATE AT 0.03 (mg/L)	MEAN % MORTALITY RATE AT 0.02 (mg/L)	MEAN % MORTALITY RATE AT 0.01 (mg/L)	MEAN % MORTALITY RATE AT 0.008 (mg/L)	MEAN % MORTALITY RATE AT 0.005 (mg/L)
1	NIG. 1: B. cereus	46.22	63.11	72.89	36.05	22.00	15.00
2	NIG. 2: B. thuringiensis pakistaffi H-15	Activities completed	Activities completed	Activities completed	Activities completed	Activities completed	Activities completed
3	NIG. 3: B. cereus	46.22	63.11	72.89	36.00	22.00	15.00
4	NIG. 4: B. cereus	46.12	63.08	72.78	36.20	22.00	15.00
5	NIG. 5: B. cereus	46.08	63.10	72.88	36.20	22.00	15.00
6	NIG. 6: B. cereus	46.05	62.98	72.58	36.28	22.00	15.00
7	NIG. 7: B. cereus	46.25	63.25	72.95	36.45	22.00	15.00
8	NIG. 8: B. cereus	46.30	63.45	72.62	36.60	22.00	15.00
9	NIG. 9: B. cereus	46.15	63.20	72.55	36.40	22.00	15.00
10	NIG. 10: B. thuringiensis tolworthi H-17	Activities completed	Activities completed	Activities completed	Activities completed	Activities completed	Activities completed
11	NIG. 11: B. cereus	46.10	63.18	72.80	36.00	22.00	15.00
12	NIG. 12: B. cereus	45.90	63.33	72.77	36.40	22.00	15.20
13	NIG. 13: B. cereus	46.05	63.42	72.25	36.60	21.48	14.80
14	NIG. 14: B. cereus	46.12	63.44	71.95	36.45	22.00	16.40
15	NIG. 15: B. cereus	45.88	63.22	72.90	36.35	22.40	15.20
16	NIG. 16: B. cereus	46.25	63.11	72.45	36.20	22.50	15.20
17	NIG. 17: B. cereus	46.05	63.02	72.66	36.70	22.20	15.40
18	NIG. 18: (B. cereus)	45.45	62.25	71.55	36.80	21.28	14.80
19	NIG. 19: (B. thuringiensis)	Activities completed	Activities completed	Activities completed	Activities completed	Activities completed	Activities completed
20	NIG. 20: (B. cereus)	45.55	62.30	70.50	36.00	22.00	15.00
21	NIG. 21: (B. cereus)	46.00	63.00	71.80	36.35	21.60	15.00
22	NIG. 22: (B. cereus)	44.95	61.90	72.60	36.20	20.70	15.00
23	NIG. 23: (B. cereus)	45.75	62.20	71.20	36.20	22.00	15.00
24	B. sphaericus(standard)	96.5	98.25	88.5	81.7	76.5	71.75

NIG = NIGERIA - tagged by Institue Pasteur
* 18 - 23 not confirmed by Institue Pasteur

APPENDIX 7: Determination of 95% confidence limits to the potency level of standard (b. Sphaericus)

Bacillus sphaericus (Standard)

N = 18 (sample size i.e number of treatment)

$\sum X$ = 351: (sum of dead larvae in a treatment in 24 hrs)

X = 19.5 (Mean of dead larvae in a treatment in 24 hrs)

$\sum X^2$ = 7121 (sum of squares of dead larvae in a treatment

$(EX)^2$ = 123201 (Square of sum of dead larvae)

to .05 = 2.110 = T-test at 5% level with /7 degrees of freedom

(Source: Parker (1974); Moses (1990).

Standard Error (SE) of the mean = $\sqrt{\frac{EX^2 - \frac{(EX)^2}{N}}{N(N-1)}}$

S.E = $\sqrt{\frac{7121 - \frac{123201}{18}}{18\,(18-1)}} = \sqrt{\frac{7121 - 6844.5}{306}}$

S.E = ±0.9506

95% confidence limits = X ± to .05 x S.E
= 19.5 ± 2.11 x 0.9506

= 19.5 ± 2.006
= 18 - 22 (dead larvae per treatment)

APPENDIX 8 : Determination of 95% confidence limits/significance level of the potency of bacillus thuringiensis

Bacillus thuringiensis pakistani H-13 and B thuringiensis var tokhokuensis

H- 17

N = 18 (Sample size i.e. number of treatment)

$\sum X$ = 302 (Sum of dead larvae in the treatment)

X = 16.78 (Sample mean of dead larvae)

$\sum X^2$ = 5166 (Sum of square of dead larvae)

$(\sum X)^2$ = 91204 (Square of sum of dead larvae)

to. 05 = 2.11 (from table - T-test at 5% level with df - 1 = 17

Standard Error of the mean = $\sqrt{\frac{\sum X^2 - \frac{(\sum X)^2}{N}}{N(N-1)}}$

$= \sqrt{\frac{5166 - \frac{91204}{18}}{18(18-1)}}$

$= \pm 0.569$

955 = X ± S. E x 2.11

16.78 ± 0.569 x 2.11

95% confidence limits = 16.78 ± 1.2006

= 15 to 19.00

APPENDIX 9: DETERMINATION OF 95% CONFIDENCE LIMITS/LEVEL OF SIGNIFICANCE OF THE POTENCY OF B. CEREUS

Bacillus cereus at 24 hours in 24 hours

$\sum X$ = (Sum of dead larvae in a treatment) = 188

X = (Mean of dead larvae in treatment) = 10.44

$\sum X^2$ = (Sum of squares of dead larvae) = 35344

to. 05 (T - test at 5% level with 17 degrees of freedom) = 2.11

N = Number of treatments = 18

S. E = (Standard Error of the mean) = ?

95% confidence limits = ?

$$\text{S. E} \quad \sqrt{\frac{\sum X^2 - \frac{(\sum X)^2}{N}}{N(N-1)}} = \sqrt{\frac{2030 - \frac{35344}{18}}{18(18-1)}} = \sqrt{\frac{66.445}{306}}$$

S.E = ± 0.466

95% confidence limits = X + to.05 x SE

= 10.44 ± 0.466 x 2.11

= 10.44 ± 0.983

95% confidence limits of B. cereus at 24 hours = 9 to 11 (dead larvae per treatment)

95% confidence limits of Bacillus cereus at 48 hours

N = 18
X = 16.33 S.E = $\sqrt{4832 - \frac{86436}{18}} = \sqrt{\frac{30}{306}}$
EX = 294
$(EX)^2$ = 86436 $\sqrt{\frac{}{18\,(18\text{-}1)}}$
EX^2 = 4832 $\sqrt{0.098}$
S.E ± 0.313

95% confidence limits = x ± to.05 x S.E

= 16.33 ± 0.313 x 2.11
= 16.33 ± 0.661

95% confidence limits

of B. cereus at 48 hours = 15 to 17 (dead larvae per treatment within

48 hours).