Identification of *Bacillus thuringiensis* and *Bacillus sphaericus* by conventional methods and parasporal crystal formation

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PREFACE

This work has been carried out at the Department of Microbiology, Faculty of Veterinary Science University of Khartoum, under the supervision of Dr. Khalid Mohammed Suliman.

Dedication

To my Father,

Mother,

uncle,

sisters

and other members of my family.

To all those who are on the line, dealing with microbes.

ACKNOWLEDGMENT

Thanks, in the first place, be to Almighty Allah, gave me health and aptitude to complete this work. Next, I wish to express my sincere thanks and gratitude to Dr.kHalid Mohammed Suleiman, wright for his helpful supervision, suggestions and continualed support throughout this work. I am also grateful to the entire staff at the Department of Microbiology, Faculty of Veterinary Sceince, University of Khartoum, for the friendly atmosphere that helped me achieve this work. The help of senior technician, Abdel Aziz, Fawzia, Mona, the media preparation staff, Hashem, Elias, Abdel Azeem and Abdalla is greatly appreciated. I am indebted to Dr. Ahmed Zaki for his generous help in data collection from internet. My thanks are also extended to all colleagues, but my special thanks are due to Amany for her generous assistance. I am also indebted to my family, father, mother, and sisters, and special thanks to my uncles for their continuous assistance, support and encouragement.

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Abstract

In this study 80 samples of mosquitos' larvae comprising several genera were collected with the objective of isolation and identification of the bacillus species capable of producing insecticidal parasporal crystals namely *B. thuringiensis and B. sphaericus.* Larvae were collected from White Nile and Khartoum states. Samples were transported to the laboratory in a thermoflask on ice. In the process of isolation, larvae were treated by heating at 80° C for 5min to kill vegetative cells. They were then crushed using sterile glass beads and the preparation were streaked on nutrient agar and incubated at 37° C for 24 hr. Using conventional biochemical methods, 74 isolates were identified as *Bacillus* spp. The species isolated included *B. thuringiensis, B. sphaericus, B. cereus, B. mycoides, B. alvei, B. megaterium, B. licheniformis, B. subtilis, B. pumilis*, *B. circulans, B. polymyxa, and B. coagulans* 74 isolates were identified as *Bacillus* spp. of which 3 were *B. thuringiensis* and 5 were *B. sphaericus*. Isolated *Bt* and *Bsph* were further confirmed by their ability to produce parasporal crystals demonstrated using Gurr's improved R66 Geimsa stain, which was proved to be avaluable method for special staining of *Bacillus thuringiensis and Bacillus sphaericus* parasporal crystals.

ملخص الدراسة

فى هذه الدراسة جمعت 80 عينة من يرقات الناموس تشمل جنس الكيولكس والانوفلس من ولايتى النيل الأبيض و الخرطوم بغرض عزل و تعريف انواع من جنس الباسلس والتى لها المقدرة على انتاج بلورات جنيب ا لبوغ ذات الاثر القاتل على يرقات عدد من انواع الحشرات كالباعوض والذباب.

نقلت العينات الى المعمل محفوظة فى الثلج ومن ثم عولجت بالحرارة (80 درجة مئوية لمدة 5 دقيقة) وذلك لقتل الخلايا الخضرية المصاحبة لها، ثم سحنت هذه اليرقات بحرازا ت زجاجية معقمة وزرعت فى وسط الاجار المغذى.

فى الدراسة عزلت 74 عصية من جنس الباسلس 3 انواع منها عصيات *thuringiensis* و 5 منها عصيات *sphaericus*. بالاضافة الى هذا فقد عرفت انواع اخرى من جنس الباسلس تشمل عصيات *polymyxa, alvei, mycoides, cereus, coagulans, megaterium, lechenefomis, circulans, subtilis* and *pumilis.*

تم التاكد من عزلات الباسلس *thuringiensis* والباسلس*sphaericus* باستخدام صبغة (غوورز المحسنة) لصبغ البلورات جنيب البوغ المميزة لهذين النوعين.

Chapter One

INTRODUCTION

Bacillus thuringiensis and *Bacillus sphaericus* are ubiquitous Gram-positive, spore forming bacteria that form, parasporal crystals during the stationary phase of their growth cycle. The organisms were initially characterized as an insect pathogens, and their insecticidal activity was attributed largely or completely to parasporal crystals. This observation led to the development of bioinsecticides based on *B .thuringiensis* and *B. sphaericus* products for control of mosquitoes and blackflies in addition to other insect species such as, *Lepidoptera, Coeloptaer*, *Hymenoptera*, *Homoptera*, *Orthoptera*, *Malophaga* and also against *Nematodes*, *Mites* and *Protozoa*. Malaria is a worldwide disease transmitted to human through *Anopheles* spp. In Sudan malaria have serious impact on the people health and economic development. At present, the best control methods for this disease are based on vector control by using synthetic insecticide. Additionally, mosquitoes have developed resistance to the major chemical insecticide groups, for instance, global DDT spraying to control mosquito populations succeeded for only 8 years as mosquito resistance appeared, furthermore chemical insecticides are environmentally unfriendly.

B. thuringiensis and *B. sphaericus* products are now a useful alternative or supplement to synthetic chemical pesticide application in mosquitoes control and commercial agriculture forest management. These biological insecticides are which usually save to environment. The insecticide products of *B. thuringiensis* and *B. sphaericus* were highly specific, harmless to humans, vertebrates, and plants, and are completely biodegradable with no residual toxic products accumulating in the environment.

Chapter Two

LITERATURE REVIEW

2.1 Genus *Bacillus*

Species of the genus *Bacillus* are mainly Gram-positive rods motile (some non motile forms occur) and non acid fast. They produce heat resistant spores under aerobic conditions (Gordon *et al*., 1973). Most bacilli are aerobic, some species are facultatively anaerobic, usually oxidase variable, and catalase positive. Species of the genus differ in the manner in which they attack sugars (Clause *et al*., 1986).

Bacillus species are widely distributed in the environment mainly because of their highly resistant endospores. In soil endospores of *B. anthracis* prototype of the genus, can survive for more than 50 years, able to tolerate extremely adverse conditions such as desiccation and high temperatures (Quinn *et al*., 2002).

2.2 Classification and Identification of the genus *Bacillus*

The genus *Bacillus*, the type genus of the family *Bacillaceae*, included more than 60 species (Sneath, 1986). Classification of the genus *Bacillus* was made on the basis of aerobic growth of spore forming rods (Smith *et al*., 1952).

Generally the *Bacillus* species are identified by methods based

on spore morphology and biochemical reactions. Molecular methods such as DNA homology (Seky *et al*., 1978 and Krych *et al*., 1980), pyrolysis gas chromatography and pyrolysis mass spectrometry (O'Donnell *et al*., 1980) were also used in the identification of *Bacillus* species.

There is considerable evidence that *Bacillus thuringiensis* (*Bt*) and *Bacillus spharicus* (*Bsph*) should be considered a single species (Baumann *et al*., 1984). Many reports reviewed that classical biochemical and morphological methods of classifying bacteria have consistently failed to distinguish *B. thuringiensis* from *B. spharicus* (Kampfer, 1991).

2.3 Habitat

Bacillus species are ubiquitous, inhibiting soil, water, and airborne dust (Schnepf *et al*., 1998). Thermophilic and psychrophilic members of the genus can grow at temperatures ranging between (5 – 58)° C and can flourish at extremes of acidity and alkalinity ranging from pH 2-10 (Gordon *et al*., 1973). Therefore, *Bacillus* species can be recovered from a wide variety of ecologic niches. Some species may be part of the normal intestinal microbiota of humans and other animals (Elmer *et al*., 1997).

Most *Bacillus* species encountered in the laboratory are saprophytic contaminants or members of the normal flora (Smith *et al*., 1952).

2.4 Morphology and cultural characteristics

The *bacillus* cell ranges from 0.5 - 1.2 μm width to 2.5 - 10 μm length in diameter (Merchant *et al*., 1967). The organisms usually grow well on blood agar, producing large, spreading, gray white colonies, with irregular margins (Barrow *et al*., 1993). Many clinical isolates are β-hemolytic, which a helpful characteristic in differentiating various *Bacillus* species from *B. anthracis*, which is non hemolytic (Elmer *et al*., 1997). Catalase is produced by most species and sporulation is not inhibited by most incubation temperatures, positive characteristics that aid in distinguishing genus *Bacillus* from bacteria can grow aerobically on nutrient medium at 37° C, although the optimal temperature for growth of these bacteria, termed mesophiles, is 37° C, they can grow at temperature ranged of (20-45)° C. Those with an optimal incubation temperature of 15° C, are termed psychrophiles and those with an optimal incubation temperature close to 60° C, are termed thermophiles (Quinn *et al*., 2002).

2.5 Pathogenicity

Bacterial pathogens of insects has been used to control crop and forest's pest for almost five decades. However, it has been used as an effective agent since 1971's, for its importance to public health, primarily in mosquitoes and blackflies control (Tuason *et al*., 1979). Numerous *Bacillus* spp. has been identified as insect pathogens except *B. anthrarcis* the causative agent of human and animal anthrax (Terranova and Blake, 1978).

Bacillus cereus was found associated with food poisoning and other opportunistic infections in human (Bergdoll, 1981). Other *Bacillus* species which may be viewed as potential opportunistic pathogens, include *B. subtilis*; *B. sphaericus; B. megaterium; B. pumilus; B. circulans; B. licheniforms; B. mycoides; B. macerans; B. coagulans and B. thuringiensis* (Sliman *et al*., 1987).

2.6 Biotechnology

2.6.1 *Bacillus thuringiensis*

2.6.1.1 History

B. thuringiensis is the best known as pathogen of *lepidopetrous* larvae. It was first isolated at the beginning of the last century from diseased worms in Japan and shortly after that from larvae of the Mediterranean flourmoth in Germany (Rogoff, 1966). Commercial production of pesticides based on Bt began in 1958 in the USA (Margalit *et al*., 1985).

Today, Bt products are the most widely used biological agent for the control of *Diptera* (mosquitoes and blackflies)(Goldberg *et al*., 1977).

2.6.1.1.1 *B. thuringiensis* subsp. *israelensis*

In addition to vector control programmes, public demand for control of nuisance mosquitoes and blackflies has resulted in widespread use of Bti in both developing and developed countries

(Guillet *et al*., 1990). *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) proved so effective that within a few years of discovery (1971) it became the principle compound of the Onchocerciasis Control Programme in West Africa, and there after it was used as an alternative for synthetic chemical insecticides in many mosquito control programmes (Kurtak *et al*.,1989).

2.6.1.2 Phenotypic and genotypic characterizations

There have been many attempts to categorize strains of *B. thuringiensis* based on phenotypic and molecular characteristics including carbohydrate fermentation and nitrate reduction (Elmer *et al*., 1997), ultrastructure of parasporal crystals (Itoua *et al*., 1995), immunoblotting (Michael *et al*., 1995), insecticidal activity against *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles albimanus* larvae, scanning electron microscopy, sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE), and plasmid profile as well as PCR analysis using novel general and specific primers for cry (toxic) and cyt (non toxic) genes encoding proteins active against mosquitoes (Jorge *et al*., 2003).

2.6.1.3 Life cycle of *B. thuringiensis*

The life cycle of Bt has been divided into two major phases, vegetative growth and sporulation. During the first stage, growth is exponential the bacteria are in a nutrient rich environment. When nutrients become scarce, the bacteria sporulate, forming spores that

can remain viable in the environment for long periods until condition favorable to vegetative growth previal (Itoua *et al*., 1995).

2.6.1.4 *B. thuringiensis* serotypes

Bacillus thuringiensis was classified into 39 H-serotypes based on flagellar antigen. Some serotypes can be further divided into sub serotypes by H-antigen subfactors; H3ac, H3abc, H3ade, H4ab, H4ac, H5ab, H5ac, H8ab, H8ac, H8bd, H11ab, H11ac, H20ab, H20ac (De Brjact *et al*., 1990).

2.6.1.5 *Bacillus thuringiensis* pathotypes

Bacillus thuringiensis was classified into pathotypes depending on its pathogenicity to insects. Pathotype A was found to be specific to *Lepidoptera* (Caterpillars), pathotypes B specific to *Diptera* (mosquitoes and blackflies) and pathotype C specific to *Coleoptera* (beetles) (Krieg, 1987).

2.6.1.6 *B. thuringiensis* toxins

Various strains of the *Bt* produce toxins of different types, but the chemical nature and mode of action of the various toxins are not fully understood. Three main toxins have been well documented, the parasporal protein crystals or endotoxin, the thermostable water soluble exotoxin active against the house fly, and the water soluble exotoxin active against saw fly larvae. Other enzymes such as lecithenase and hyaluronidase are also produced (Heimpel, 1967).

2.6.1.6.1 Parasporal crystals

ITOUA *et al.* (1995) reviewed that parasporal crystals morphology was of three types, bipyramidal, pyrmidal, and spherical. Jorge *et al*. (2003) reported that strains of Bt showed small ovoid crystal inclusion was similar to strain of *Bti*.

Parasporal crystal toxin is produced by all types of *Bt*. The toxin vary from strain to strain in shape, size and antigenic composition (Angus, 1956). Parasporal crystal formation in the absence of spore production could occur when cultures are grown at low temperatures (Smirnoff, 1963). Electron micrographs of the surface of the crystal show regular structure (Labaw, 1964). The protein of crystal is synthesized from amino acid resulting from break down of intracellular materials in the cell during the early phase of sporulation (Estes, 1966). The chemical structure of the crystal toxin is complex and solution of the protein contain several different components as judged by physical separation or by antigenic composition (Cooksey, 1968; Pendleton 1966, 1967).

2.6.1.6.2 Mode of action of parasporal crystals

The larvae of mosquitoes and blackflies feed on small particulate matter in their breeding grounds (Knowles *et al*., 1987). Upon ingestion by larvae, the crystal protein dissolves at high pH of insect gut, proteolytic action releases toxic fragments, and the processed toxin binds to specific receptor on the plasma membranes of susceptible cells in the mid-gut epithelium (Vanrie *et al*., 1990). Initial

binding is followed by the creation of small pores in the membrane leading to colloid-osmotic lysis in which equilibration of ions across the pores leads to net influx of ions, an accompanying influx of water, cell swelling and lysis (Knowles *et al*.,1989). Disruption of the epithelial lining kills the larvae rabidly. This model of toxin action was based on studies with the processed cyt protein but the same mechanism of action could account for the toxicity of other Bt toxins, (Chilott *et al*., 1990).

2.6.2 *Bacillus sphaericus*

B. sphaericus, is the second entomopathogenic member of the genus *Bacillus* and although it's target spectrum is restricted to certain types of mosquito larvae, it's high efficacy in polluted water has made it particularly useful against the ubiquitous *Culex* vector of filariasis and certain viral encephalitis. Isolation of mosquito pathogenic strains of *B. sphaericus* pre-dated the isolation of *B. thuringiensis* subsp. *isrealensis* (Kellen *et al*., 1965), but the early strains showed low toxicity (WHO, 1990). Later more highly toxic strains were recovered and these, together with several recently isolated strains, have considerable potential as biological control against (Singer, 1988). In contrast to *Bti* and *B. sphaericus* strains are non toxic towards *simulium* larvae.

Treatment of cesspits and latrines in Tanzania with a dry powder preparation of *B. sphaericus,* strain at 10 g/m^2 provided

control of mosquito larvae for 6-10 weeks. In contrast, *B. thuringeinsis* is unable to grow saprophytically in the environment and it spores and crystals do not persist long in polluted water (WHO, 1990).

2.6.2.1 Mode of action of parasporal crystals

RAMOSKA *et al*. (1977) the *Bsph* toxin has a high activity towards larvae of *Culex* and *Psorophora* and variable toxicity to *Anopheles*. Pathological changes in the larvae following ingestion of toxin largely involve the midgut cells. Large vacuoles or cytolysosomes appear in the gut, eventually these cells separate from one another and slough from the basement membrane (Charles, 1987). In cultured cells treated with parasporal crystals of Bsph, there was very rapid swelling of mitochondrial christae and endoplasmic reticulum within 5 min of treatment (Davidson and Titus, 1987).

At the molecular level the larvicidal toxin of *B. sphaericus* acts by ADP- ribosylating numerous proteins, a mechanism similar to the diphtheria toxin and psuedomonas exotoxin (Schirmer, 2002).

2.7 Applications of *B. thuringiensis* and *B. sphaericus* toxin in controlling mosquitoes larvae in the filed

In the case of *Bacillus* insecticides, the formulation of spore toxin material and carrier is devised to present a suitable amount of the crystal toxin to larvae in an acceptable form for ingestion. Other considerations are of handling, stability both during storage and in the field, and cost (Lacey, 1984). The different habitats and feeding habits

of mosquito and blackfly larvae have resulted in the development of various formulations of Bti and *Bsph*. Wettable powders and liquid flowable concentrates are generally used in conventional aerial and ground sprays to unobstructed breeding sites (Lacey *et al*., 1988). Sustained release formulations such as floating briquettes or semi submersible pellets are designed to provide long-lasting larvicidal activity in containers or small ponds (Lacey, 1984).

2.8 Economic importance of *Bt* and *Bsph* products

Bt and *Bsph* are now the most widely used biologically produced pest control agent. In 1995, worldwide sales of Bt were projected at $90 million representing about 2% of the total global insecticide market (Lambert and Peferoen, 1992). The annual world wide distribution of Bt a mounts to 2.3×10^6 kg. As of early 1998, there were nearly 200 registered Bt products in the USA (Rowe *et al*., 1987).

2.9 Mosquito borne diseases

2.9.1 Malaria

For centuries, malaria outranked warfare as a cause of death to human beings and sapped the strength of human suffering. It continued to be a heavy drag on man's efforts to advance his agriculture and industry (Kennedy, 1962).

It estimated that during the 1980's the annual incidence of the disease

was of the order of 250 million cases with 2.5 million people dying of malaria every year.

In the Sudan, malaria is one of the most prevalent of the indigenous disease (WHO, 1990).

Malaria is endemic all over the country with the exception of some dry areas in the White Nile State and the Red sea province. The disease has a great impact on public health which negatively affects the economic and social life of population. In Khartoum, malaria accounts for 14.3% of all outpatients attending health facilities. Malaria remains the first cause of hospital admission (30.9%) and the commonest cause of death in hospitals (20.3%). In the Sudan, the four types of human malaria have been recorded. However, *plasmodium falciparum* represents approximately 90% of all malaria infections in most parts of the country (Ministry of Health, 1989).

2.9.2 Yellow Fever

Yellow fever is one of the most important human viral disease, transmitted by *Aedes agypti*. Africa is the original home (Carter, 1930).

2.9.3 Dengue

Is a noncontiguous infectious disease of low mortality. The causative agent is unknown, but it was identified as filter passing

organism transmitted by mosquitoes (Simmons,1931).

2.9.4 Filariasis

The disease is caused by *Wuchereria* (filaria) *bancrofti cobbold* or *W. malayi* which are round worms found in the adult stage in man (Brug, 1930).

2.9.5 Encephalitides

A number of viral diseases have been grouped under this title. Mosquitoes were definitely proved as vector of equine encephalomyelitis, a highly fatal disease in horses (Bishopp, 1939).

2.9.6 Rift Valley Fever

A disease affecting sheep, goats, and man, transmitted by mosquito species (Smithburn *et al*., 1948).

2.10 Potential for resistance to *Bt* and *Bsph* bioinsecticides

Bacillus thuringiensis subsp.*israelensis* products have been used in control programmes for mosquitoes and blackflies for more than 15 years. In the contrary, resistance to Bsph has already developed in some populations in India, Brazil and France was reported, and this might be explained by the fact the active ingredient is a single toxin as opposed to the products of Bt products which is

composed of two ingredients, so to date resistance to the products was not reported (Georghiou, 1994).

2.11 Control of mosquito

2.11.1 Chemical control

DDT was the first organochlorine insecticide used as an effective pest control agent. It provided an excellent, wide ranging control at low cost, but persistence in the environment together with accumulation in animal feed led to it being banned. Organophosphate, parathion and malathion are widely used in mosquitoes and blackflies control programmes and have proved effectiveness against midges. Carbamates are particularly useful against adult mosquitoes while the synthetic pyrethroids are effective against both larvae and adult mosquitoes (Aitken, 1946).

2.11.2 Biological control

Microbial control agent might be immune but this is now known to be incorrect and resistance to *Bt* crystal toxin has been observed in several *Diptera,* (Goldman *et al*., 1986). Moreover, cell line of *Culex quinquefasciatus* quickly becomes resistant to add toxin

from *Bsph*, (Schroeder *et al*., 1989). Both Gram-positive, aerobic, endospores forming bacteria of the genus *Bacillus* B.t.i and *Bsph* have been used successfully in many countries for controlling mosquitoes (WHO, 1990).

Chapter Three

MATERIALS AND METHODS

3.1 Collection of samples

A total of 80 samples of mosquito larvae were collected during the period from Janury to May 2004 from four different locations in the Sudan.

* Geizera Aba open drain that containing water of autumn season.
* Geizera Aba large pools as mosquitoes and mosquitoes larvae source.
* University of Khartoum, Faculity of Sceince pools.
* Khartoum North, (Hellt Kuku).

Samples were live mosquito larvae that were collected in clean universal glass containers ful of water.

3.2 Sterilization and asepsis

Sterilization of the glassware equipments including petri-dishes, tubes, pipettes, flasks were boiled, soaked for three days in Losan disinfectant or done in the hot air oven set at 160^{o} C and finally sterilized by autoclaving for 15 min at 121^{o} C.

All media; with the exception of sugars which were sterilized at 110^{o} C for 5 minutes, screw-capped bottles, solutions, and cotton stoppered tubes were sterilized in autoclave at 121^{o} C for 15 minutes.

3.3 Bacteriological investigation media

3.3.1 Peptone water

	(g/L)
Peptone	10
Sodium chloride	5
PH	7.2(approx)

50 grams were added for one liter of distilled water mixed well and distributed into sterile test tubes and sterilized by autoclaving at 121˚ C for 15 minutes.

3.3.1.6 Nutrient broth

	(g/L)
Lab-lemco powder	1
Yeast extract	2
Peptone	5
Sodium chloride	5
PH	7.4(approx)

An amount of 13 grams was added to one liter of distilled water, mixed well and distributed in final containers, sterilized by autoclaving at 121˚ C for 15 min. For nutrient agar 1.5% agar (w/v) were added.

3.3.3 Nutrient agar

Nutrient agar was used as simple media for growth of bacteria and for determination of pigments production of some *Bacillus* spp; like *B. lichienformis* this was done by culturing bacteria and incubated at 37˚ C for 24 hrs and then looking for different types of pigments.

3.3.4 Blood agar base No.2

	(g/L)
Protease peptone	15
Liver digest	2.5
Yeast extract	5
Sodium chloride	5
Agar No.3	12
PH	7.4 (Approx)

40 grams of blood agar base No.2 were suspended in one liter of distilled water, dissolved by boiling and sterilized by autoclaving at 121° C for 15 minutes. The medium was cooled to (45–50) ° C and 7% defibrinated ovine blood was added under sterile conditions, mixed gently and dispensed in sterile Petri-dishes in 15 ml volume each.

This medium used as enriched non-inhibitory media for primary isolation of bacteria and for determination of typical hemolytic activity which was important diagnostic feature for *Bacillus cereus* and *Bacillus anthracis* and other organisms.

3.3.5 MacConkey agar

	(g/L)
Peptone	20
Lactose	10
Bile salts	5
Neutral red	0.075
Agar No.3	15
PH	7.4 (Approx)

47 grams were dissolved in one liter of distilled water by boiling, sterilized by autoclaving at 121° C for 15 minutes and dispensed in sterile Petri-dishes in 15 ml volume each.

3.3.6 Starch agar

	(g/L)
Potato starch	10
Distilled water	50 ml
Nutrient agar	1000 ml

50 grams of starch were triturated with water to smooth cream, and then added to the molten nutrient agar. The mixture was sterilized at 115° C for 15 minutes and distributed into sterilized Petri-dishes.

The medium was used to study the hydrolysis of the starch. It was used to differentiate between different species of *bacillus*.

3.3.7 Lecthovitellin (LV) agar

Lecithovitellin solution (egg yolk saline)

Hen eggs	4
NaCl (0.85%) solution	1000 ml

Egg yolk was separated from egg white and beated in saline to form homogeneous mixture. 25 grams kieselguhr (diatomite) was added, mixed and clarified by filtration through paper and sterilized by filtration.

Lecthovitellin Agar	(ml)
Lecthovitellin solution	1000
Nutrient Agar	900

Nutrient agar was melted and cooled to about 55° C; lecthovitellin solution was added aseptically, mixed and poured into plates.

3.3.8 Casein agar (milk agar)

Milk skim	500 ml
Nutrient Agar (double-strength)	500 ml

Whole fresh milk was stored overnight in refrigerator. The creamy layered was removed, and the milk was steamed for one hour and cooled in refrigerator. Sufficient litmus solution was added to give a bluish-purple colour and the medium was sterilized at 115° C for 10 minutes. It was then cooled to about 50° C and added to double strength nutrient agar which was melted and cooled to 50-55° C, mixed and distributed in Petri-dishes or tubes.

3.3.9 Dorset egg medium

Egg yolk and white	800 ml
NaCl (0.9% sterile)	200 ml

5 ml of homogenous mixture were placed into sterile 30 ml volume screw- capped (1 oz Universal or McCartney) bottles, containers were sloped in an inspissator and heated slowly to 75° C for 1hr. Heat processing was repeated of the following 2 days.

3.4 Isolation of bacteria

Each sample, containing about 8-10 intact larvae, was selectively heated at 80° C for 5 min to eliminate all vegetative cells and non spore forming bacteria which might be associated with the samples (Itoua *et al*., 1995).

Larvae in each sample were crushed using sterile glass beads to create a homogenous suspension which was used to inoculate nutrient agar plates by streaking method. Plates were incubated at 37^{o} C for 24- 48hrs.

Discreet single colonies in primary cultures were picked and subcultured on nutrient agar plates to obtain pure cultures.

3.5 Preservation of purified cultures

Purified cultures were preserved by subculturing on Dorset egg medium and storage at 4^{o} C until comprehensive bacteriological investigation was carried out. Before investigation isolates were streaked on nutrient agar plates and fresh culture was used in biochemical methods.

3.6 Identification of isolates

Isolates were identified using conventional biochemical methods according to the procedure described by Elmer *et al*. (1997).

3.6.1 Primary methods

3.6.1.1 Colony morphology

Colonies of *B. thuringiensis* species were β- hemolytic, tended to be large frosted-glass appearance initially, but may become opaque and their color vary.

Species of B. sphaericus had round, raised, flat colonies with entire to undulate or rhizoid edges. Colonies of other species were often dry, spreading, Colonies of B. cereus, after overnight growth on blood agar plates, were frequently surrounded by a large zone of B-hemolysis. *B. cereus* colonies were variable in size, depending on growth conditions, raised irregular, with grayish to greenish frosted glass appearance, and undulate margins. Colonies of *B. mycoides* were large, mucoid and had hairy, rhizoid, rootlike outgrowths from the colony margin that spreaded over the surface of the agar which is non motile species.

3.6.1.2 Gram's staining method

Smears were prepeared from purified colonies of cultures. These were dried in air, fixed by heating, stained with the Gram method and examined microscopically.

Colonies with morphology suggestive for bacilli, cellular morphology, arrangement, and spore formation. Suspected single colonies were subcultured on nutrient agar plates to obtain pure cultures.

3.6.1.3 Motility medium

	(g/L)
Peptone	10
Meats extract	3
Sodium Chloride	5
Gelatin	80
Agar	4
Distilled water	1000 ml

Gelatin was soaked in the water for 30 minutes; other ingredients were added, boiled to dissolve and finally the preparation was sterilized at 115° C for 20 minutes.

3.6.1.4 Motility test

Motility medium was inoculated with test culture, and incubated at 37° C for 24 hours.

3.6.1.5 Hugh and Leifson's (O/F) medium

	(g/L)
Peptone	2
Sodium chloride	5
K_2HPO_4	0.3
Agar	3
Distilled water	1000 ml
Bromo-thymol blue	15 ml

The solids were dissolved by heating in distilled water. The pH was adjusted to 7.1 through filtered, then the indicator was added followed by sterilization at 115° C for 20 minutes. 7 ml of sterile glucose solution was then added aseptically in (10 ml volumes into sterile cotton capped test tubes).

3.6.1.6 The oxidation – fermentation (O/F) test

Test culture was inoculated in duplicate into two tubes of Hugh and Leifson's medium (1953). A layer of sterile melted soft paraffin was used to covered one tube to the depth of 1cm and tubes were incubated for 5-7 days and examined.

3.6.1.7 Oxidase test

Drops of oxidase reagent were poured over strips of filter paper in Petri-dish moistened with distilled water and sterilized in a hot oven. Using sterile forceps, strips were laid on a clean slide. Colony on blood agar was picked with sterile glass rod and rubbed on the filter paper. A dark purple colour that developed within five to ten seconds was considered positive reaction.

3.6.1.8 Catalase test

A drop of 3% aqueous solution of hydrogen peroxide was placed on a clean slide. A colony of tested culture on nutrient agar was put onto the hydrogen peroxide drop. Evolution of gas of bubbles indicated a positive test.

3.6.2 Secondary methods

3.6.2.1 Peptone water sugar

	(ml)
Peptone water	900
Andrade's indicator	10

The pH was adjusted to 7.1-7.3 and the Andrade's indicator was added bringing pH to 7.5.

Sugar	10 g
Distilled water	90 ml

The sugar was added to the mixture of peptone and the indicator, mixed thoroughly then distributed in 2 ml volume in sterile test tubes with an inverted inner Durham's tube. They were then sterilized by autoclaving at 115˚ C for 10 minutes.

3.6.2.2 Nitrate broth

	(g/L)
K NO_3	1
Nutrient broth	1000 ml

KNO_3 was dissolved in the broth, distributed in sterile test tubes, and then sterilized at 115˚ C for 20 min.

3.6.2.3 VP. MR medium

	(g/L)
Peptone powder	5
Dextrose	5
Phosphate buffer	5
PH	7.5 (approx)

50 grams were added to liter of distilled water, mixed well and then distributed in test tubes and sterilized by autoclaving at 121˚ C for 15 minutes.

3.6.2.4 Nutrient gelatin

	(g/L)
Lab lemco powder	3
Peptone	5
Gelatin	120
PH	6.8 (approx)

128 grams was suspended in one liter of distilled water, boiled to dissolve completely, mixed well and then poured in sterile Bijou bottles in 2 ml volume, followed by autoclaving at 121° C for 15 minutes.

3.6.2.5 Simmon's citrate agar

	(g/L)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate tribasic	2
Sodium chloride	5
Bromo-thymol blue	0.08
Agar No.3	5

23 grams were suspended in one liter of distilled water, boiled to dissolve completely, sterilized by autoclaving at 121° C for 15 minutes, poured in sterile McCartney bottle aseptically, and allowed to set in slope position.

3.6.2.6 Urea agar base

	(g/L)
Peptone	1
Dextrose	1
Sodium chloride	5
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar No.3	15
PH	6.8 (approx)

2- 4 grams were suspended in 95 ml of distilled water, boiled to dissolve completely, and sterilized by autoclaving at 115° C for 20 minutes. The preparation was cooled to 50° C and 5 ml sterile 40% urea solution was added aseptically, mixed well, then distributed 10 ml volumes into sterile McCartney bottles and allowed to set in the slope position.

3.6.2.7 Sugar fermentation test

The ability of isolate to ferment sugar was tested using peptone water containing 1% of desired sugar. The tubes of medium were inoculated with one to three colonies and then incubated Appearance of reddish colour with gas production was indicated by development of an empty space in Durham's tubes.

3.6.2.8 Indole test

Peptone water was inoculated with test culture and incubated at 37° C for 48 hours. One ml of xylol was added to the culture, shaken well and then allowed to standard until the xylol collected on the surface. 0.5ml of kovac's reagent (which contains p-dimethyl-aminobenzaldehyde) was poured down the side of the tube. A pink ring which appeared on the xylol layer within a minute indicated a positive reaction.

3.6.2.9 Voges-Proskauer (VP) test

The test culture was inoculated in glucose phosphate peptone water and incubated at 37° C for 48 hrs. 0.6 ml of 5% alcoholic solution of a-naphthol and 0.2 ml of 40% KOH was added to one ml of the test culture. A positive reaction was indicated by development of bright pink colour within 30 minutes.

3.6.2.10 Nitrate reduction

Test culture was inoculated in nitrate broth and incubated at 37° C for two days. One ml of solution A (sulphanilic acid) was added to the test culture followed by one ml of solution B (a-naphthylamine). A positive reaction was indicated by development of red colour. If the result was negative Zinc dust was added and the red colour indicated the presence of nitrate (Zobell, 1932).

3.6.2.11 Gelatin hydrolysis

Nutrient gelatin was inoculated with test culture and incubated at 37° C for 2-3 days. Tubes were placed in a refrigerator to test the stability of the gelling property of gelatin.

3.6.2.12 Citrate utilization

This test was applied to test the ability of organism to utilize citrate as sole source of carbon. A light suspension of organism in sterile saline was inoculated in citrate medium with wire loop and incubated at 37° C. A positive test was indicated by change of colour from green to blue.

3.6.2.13 Urease activity

The activity of urease was shown by alkali production (ammonia) from urea solutions. Test culture was streaked on urea agar slope and incubated at 37° C for two days. A positive reaction was indicated by change of colour to pink.

3.6.2.14 Starch hydrolysis

Starch agar plate was inoculated with test culture and incubated at 37° C for 24 hrs. Plate flooded with lugol's iodine solution. Hydrolysis was indicated by clear colour less zones. Starch which had not been hydrolyzed turned blue.

3.6.3 Special stains

3.6.3.1 Spore stain

This special stain was similar to Ziehl-Neelsen's method but ethanol was used for decolrization.

Air dried and heat fixed the smear by passing the slide with carbol fuchsin (for preparation see above), the slide was heated until steam rinsed. Allow for 3-5 minutes to stained. Then washed with tap water. Then decolorized by pouring acid- alcohol (3ml concentrate hydrochloric acid, 97 ml methylated spirit or 70% ethanol) over the slide until the smear is a faint pink color. Then washed with tap water, and stained with counter stain 0.5% methylene blue.

3.6.3.2 Gurr's improved 66R Giemsa stain

Chilcott, (1988) in this method the bacterial cells and crystals stained black and spores were differentially stained pale to light blue with a dark blue margin.

Giemsa Stain-(Gurr's)	(g/L)
AzurII eosin	3.0g
AzurII	0.8g
Glycerol	250 ml
Pure methanol	250 ml

Or 3.8 g of compound giemsa stain power may be use instead of Azur II place all in flask, plugneck loosely with cotton wool and worm on a waste both to 1hr, cool and filter.

Thin films of aqueous suspensions of *B. thuringiensis* grown on Nutrient Agar plates, were placed on microscope slide, air dried, and incubated at 100° C for 10 min .

The hot slide was placed into naphthalene black 12B solution (1.5 naphthalene black 12B in 35% v/v glacial acetic acid) for 2 min.

Washed with tap water.

Immersed in Gurr's improved R66 Giemsa stain (BDH) for 1min.

The slides were washed and dried.

Chapter Four

RESULTS

4.1 Isolation of *Bacillus* species

The selection heating of larvae samples at 80° C for 5 min, offered a good opportunity for recovery of spore forming bacilli, where 74 *Bacillus* species were isolated from a total of 80 mosquito larvae samples collected from White Nile and Khartoum states and treated by selective heating prior to inoculation onto growth media. The bacilli isolated comprized 3 strains of *B. thuringiensis*, 2 of which were obtained from samples collected from Khartoum North and the third strain was isolated from Geizera Aba pools specimens. Samples from Geizira Aba open drains and Univrsity of Khartoum pools were negative for *B. thuringiensis*.

The study documented the isolation of 5 *B. sphaericus* strains, 3 isolates were obtained from samples of Geizera Aba open drians and the other two isolates were recovered from U of K (Faculty of Science pools).

The different *Bacillus* species isolated from mosquito larvae are shown in Table (1). The most frequent *Bacillus* species isolated from the different samples was *B. cereus* (20.28%) followed by *B. mycoides* (17.57 %) (Fig. 1).

Table(1): The sampling areas and the *bacillus* spp isolated

Batch No.	Area	*Bacillus* type	Total No. of isolates(%)	Total No. Batch isolates (%)
One	Geizera Aba (open drains)	*B. megaterium* *B. licheniformi* *B. subtilis* *B. sphaericus* *B. pumilis* *B. circulans* *B. polymyxa* *B. coagulant*	7 (9.46) 2 (2.7) 1 (1.35) 3 (4.05) 1 (1.35) 4 (5.41) 1 (1.35) 1 (1.35)	20 (27.03)
Two	Geizera Aba (pools)	*B. thuringiensi* *B. cereus* *B. mycoides* *B. megaterium* *B. pumilis*	1 (1.35) 11 (14.86) 6 (8.11) 1 (1.35) 1 (1.35)	20 (27.03)
Three	University of khartoum (Faculty of Science pools)	*B. cereus* *B. mycoides* *B. licheniformi* *B. sphaericus* *B. subtilis*	3 (4.05) 3 (4.05) 5 (6.06) 2 (2.7) 7 (9.46)	20 (27.03)
Four	Khartoum North(Helt kuku pools)	*B. thuringiensi* *B. cereus* *B. mycoides* *B. alvei* *B. subtilis* *B. pumilis* *B. circulans* *B. polymyxa* *B. coagulant*	2 (2.7) 1 (1.35) 4 (5.4) 1 (1.35) 1 (1.35) 1 (1.35) 2 (2.7) 1 (1.35) 1 (1.35)	14 (18.92)

4.2 Frequency of *Bacillus* species isolated from mosquito larvae

A total of *bacillus* species were isolated from the mosquito sample collected from Geizera Aba open drains. *B. megaterium* was the most prevalent isolate of this batch (35%) (Fig.2).

B. cereus was the principal isolate in batch two 20 samples (55%) (Fig.3). In batch three (20 samples), collected from U of K (Faculty of Science pools) *B. subtilis* was the predominent *Bacillus* species (35%) (Fig.4). In samples of the 14 mosquito larvae collected from Helt kuku area *B. mycoides* was the most frequently species isolate of this batch (28.57 %) (Fig.5).

4.3 Biochemicals methods

Identification of the spore of the genus *bacillus* were performed using the chart developed by Elmer *et al*.,(1997).

Lecithinase production differentiated the genus *bacillus* into two groups, lecithinase and non lecithinase producers. Lecithinase producers included *B. cereus*, *B. thuringiensis*, and *B. alvei*. All *bacillus* species studied were motile except *B. mycoides*.

For nitrate reduction test *B. cereus*, *B. sphaericus*, and *B. pumilis* were negative, the rest of tested species with regard to indole test

4.4 Parasporal inclusion morphology and biological activity

The new staining procedure described, stained parasporal of *B. thuringiensis* and *B. sphaericus*. The bacterial cells and crystals stained black, and the spores were differentilly stained pale to light blue with adark blue margin when examined under oil immersion lens. Spore of *B. cereus* when stained with the same methods appeared pale without the blue margin associated with spores of crystal producing species (Fig.6,7,8)

Table (2): The biochemical properties of the *Bacillus* isolates.

***Baciullus.* spp.**	**Lr**	**Mo**	**Nit**	**Ind**	**VP**	**Cit**	**Gel**	**Glu**	**Sta**	**Ma**
B. cereus	+	+	-	-	+	-		+	+	
B. thuringiensis	+	+	+	-	+	+		+	+	
B. mycoides	+	-	+	-	+	-		+	+	
B. alvei	+	+	+	+	+	-		+	+	
B. megaterium	-	+	+	-	-	+		+	+	
B. licheniformis	-	+	+	-	+	+		+	+	+
B. subtilis	-	d	+	-	+	+		+	+	-
B. sphaericus	-	+	-	-	-	-		-	-	
B. pumilis	-	+	-	-	d	-		d	d	
B. circulans	-	+	d	-	-	-		+	d	
B. polymyxa	-	+	+	-	+	-	+	+	d	
B. coagulans	-	d	+	-	+	-	-	-	-	

Cat: catalase, Lr: Lecithenase reaction, Mot: Motility, Nit: Nitrate, Ind: Indol test, VP: voges proskuer, Cit: Citrate test, Gel: Gelatin hydrolysis, Glu: Glucosetest, Sta: Starch hydrolysis, Ma: Maltose test, d: different reactions.

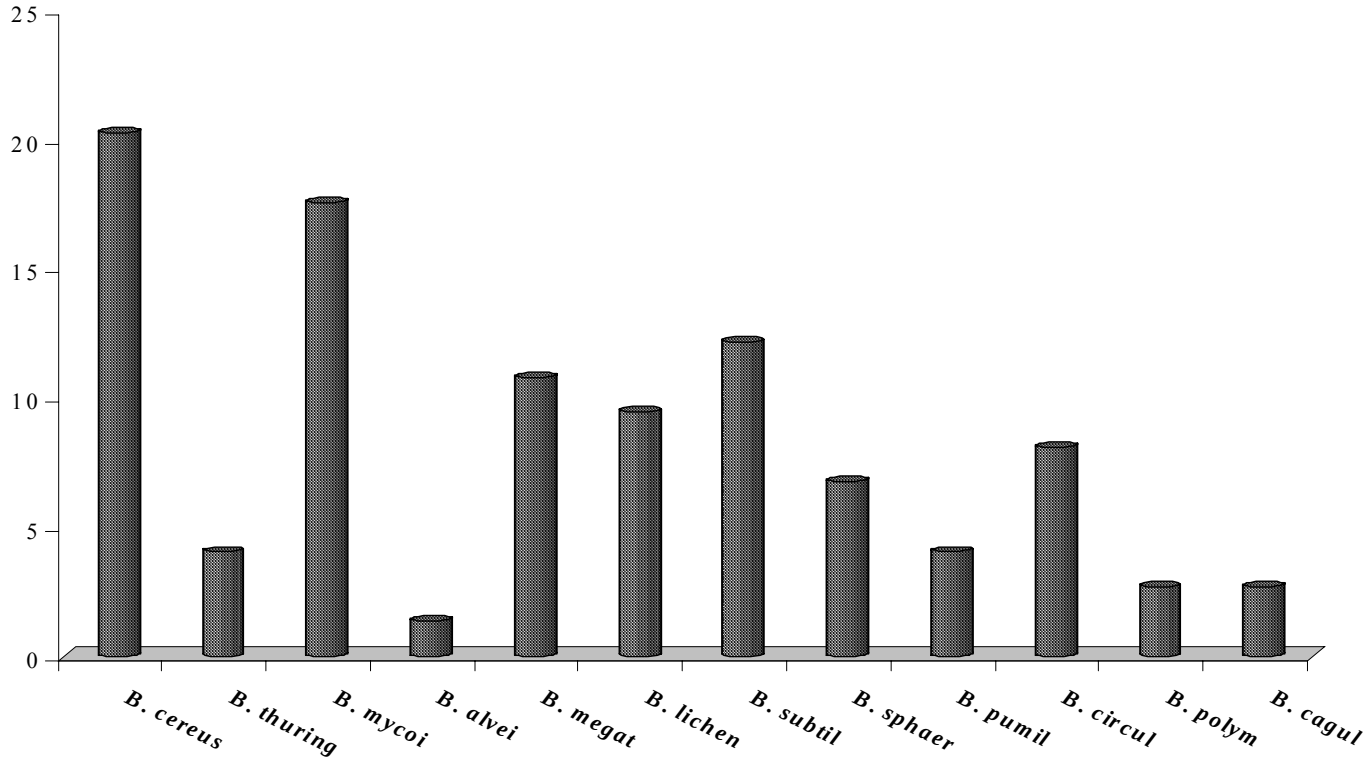

Fig. (1): Frequency of *Bacillus* species isolated from White Nile and Khartoum States

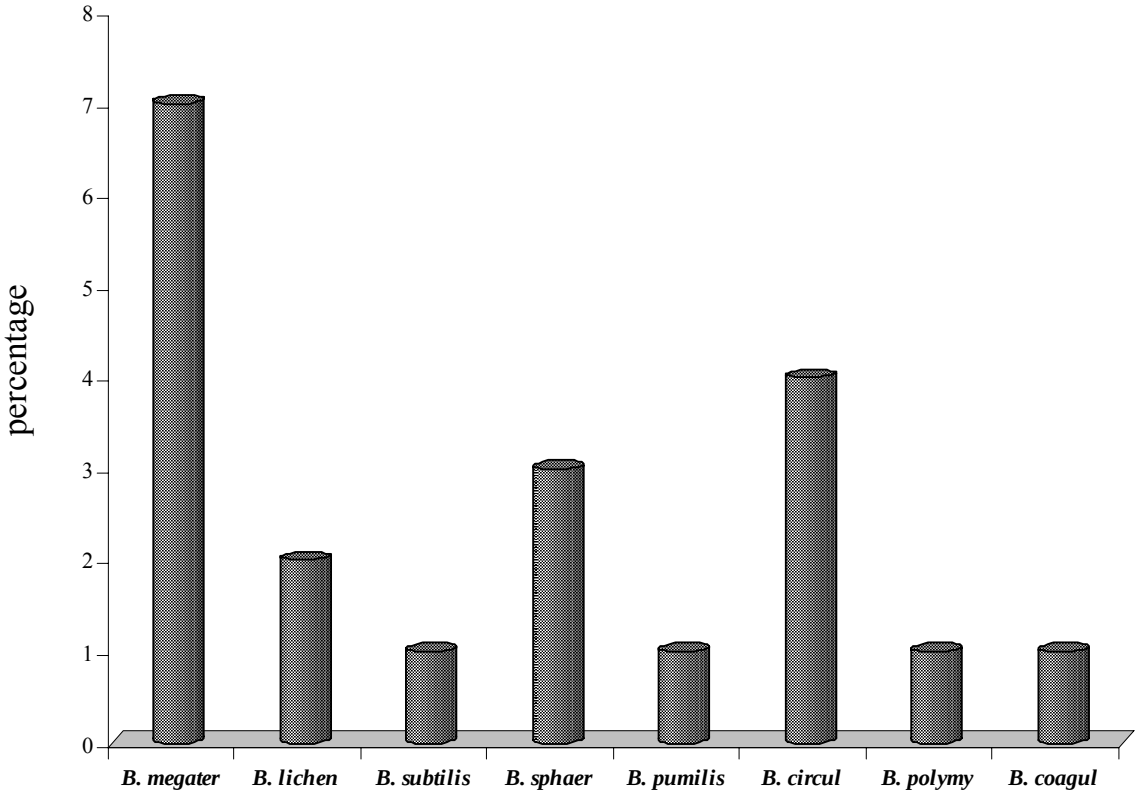

Fig. (2): Frequency of *Bacillus* species isolated from mosquito larvae from Giezera Aba open drains

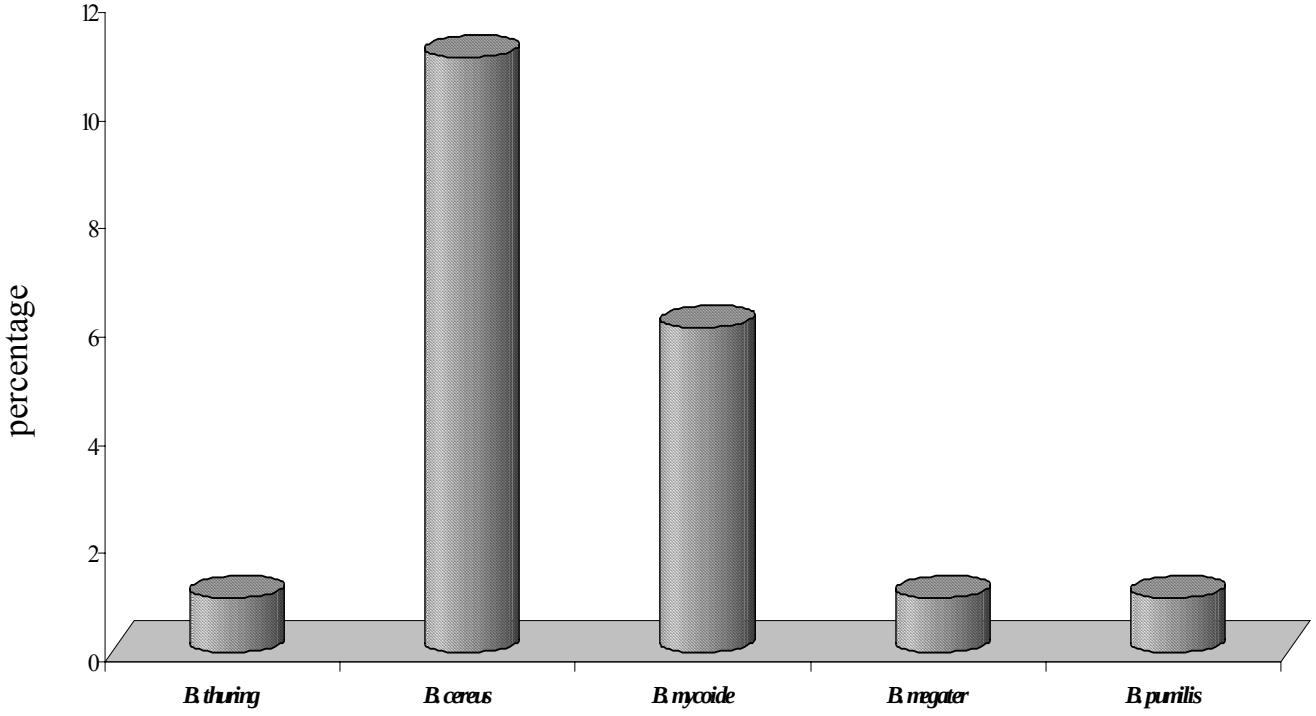

Fig. (3): Frequency of *Bacillus* isolated from mosquito larvae from Giezera Aba pools

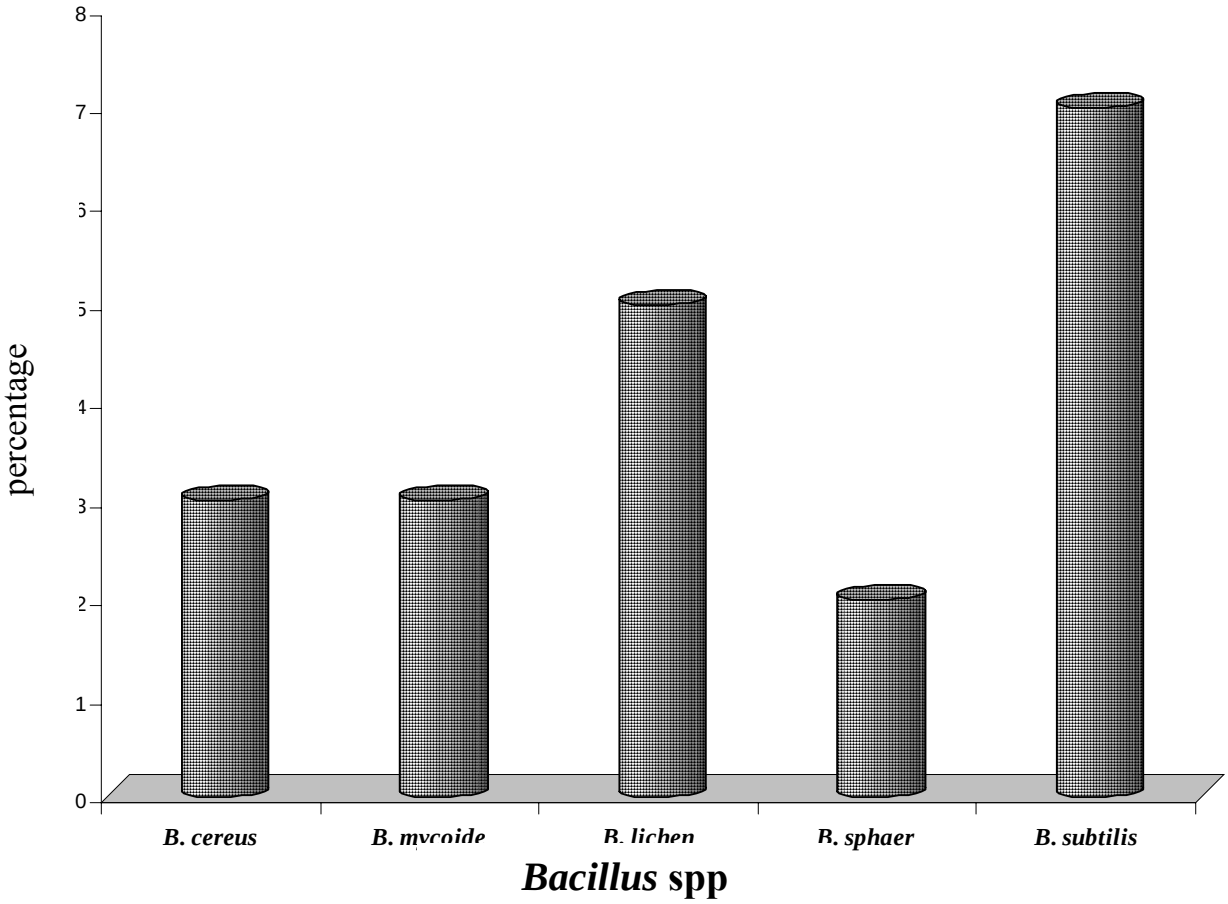

Fig. (4): *Bacillus* species isolated from mosquito larvae from University of Khartoum (faculty of science pools)

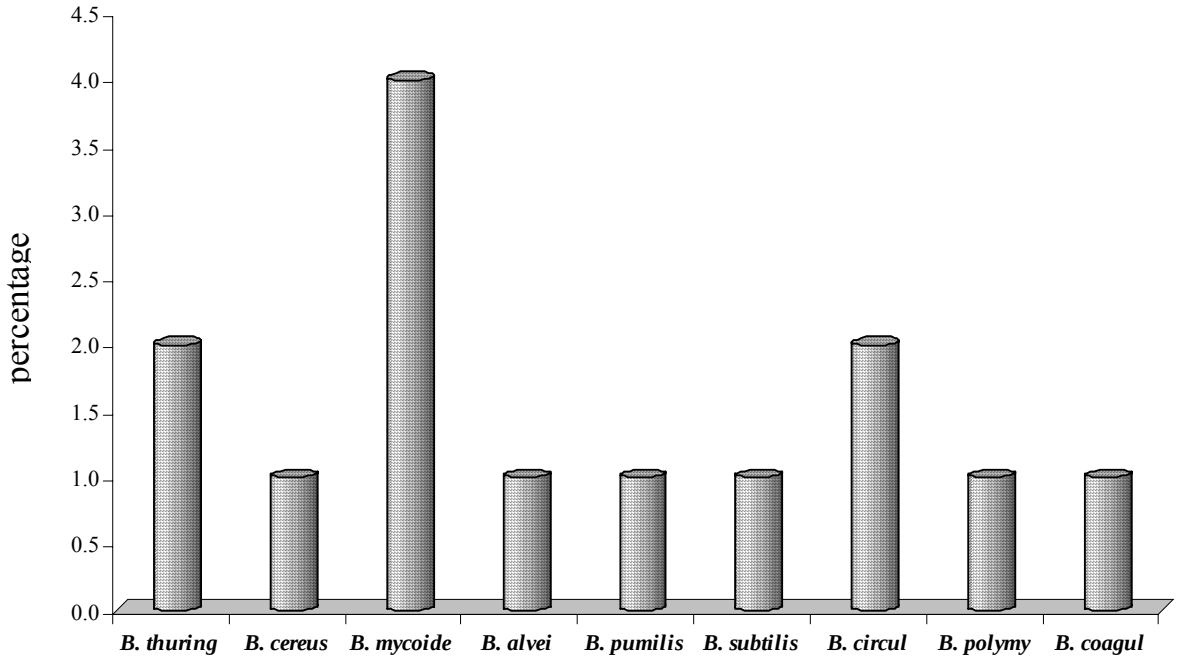

Fig. (5): *Bacillus* species isolated from mosquito larvae from Khartoum North (Helt kuku pools)

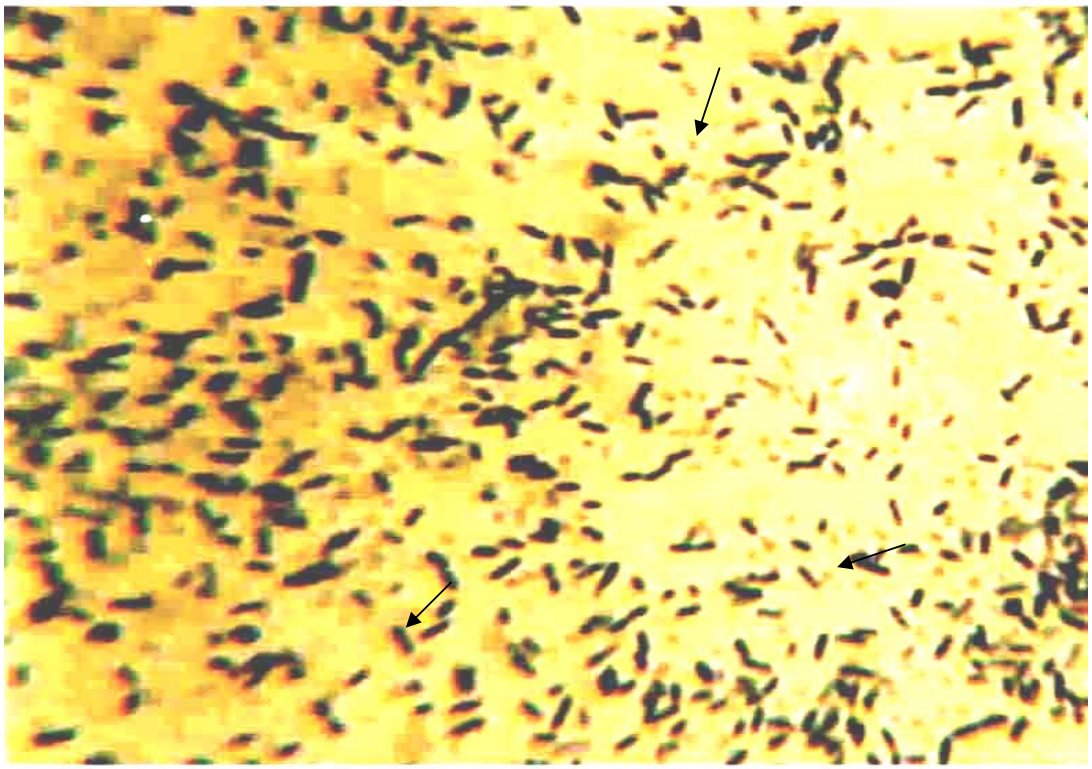

Fig. (6): *Bacillus thuringiensis* parasporal crystals (Gurr's staining methods)×100

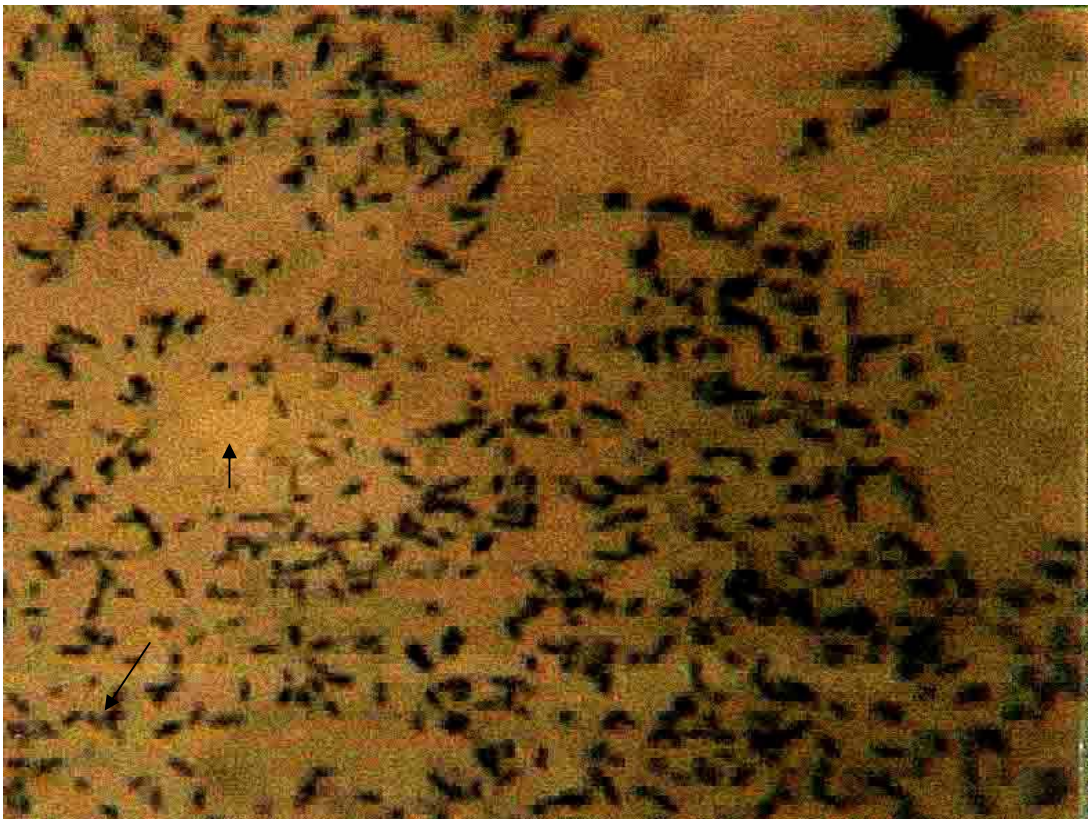

Fig. (7): *Bacillus sphaericus* parasporal crystals (Gurr's staining methods)×100

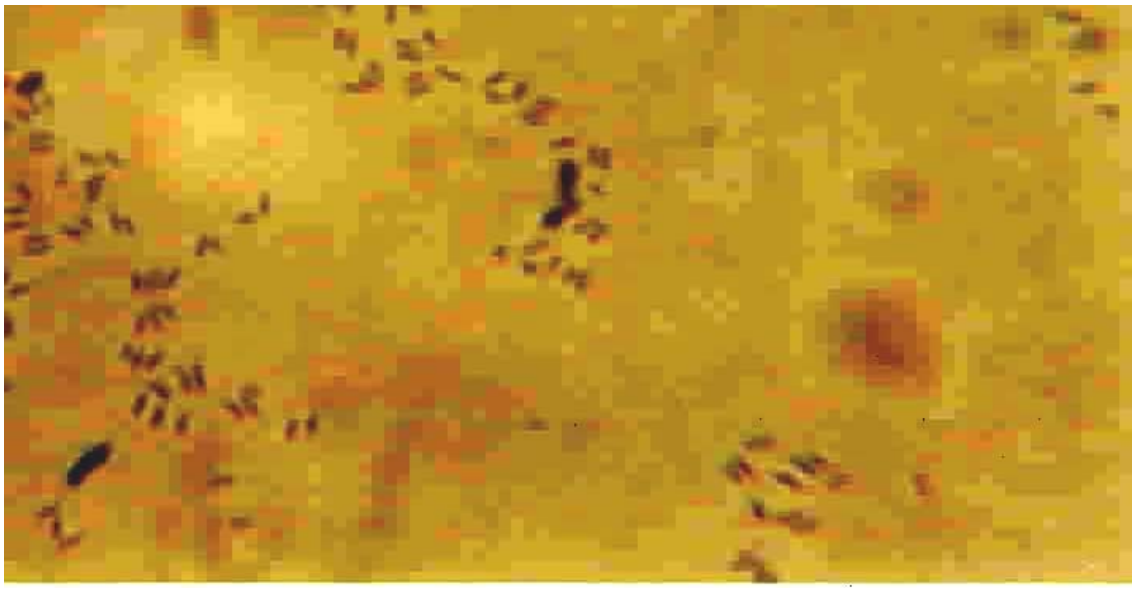

Fig.(8): *Bacillus cereus* spores (Gurr's staining methods)×100

Chapter Five

Discussion

The discussion covered the results of this investigation which included biochemical properties of *Bacillus* isolates as well as the special staining method for demonstrating of parasporal crystals of *Bt* and *Bsph*.

The primary objective of the study was to isolate local Sudanese isolates of *Bt* and *Bsph* which would have potential insecticidal activity against mosquitoes and blackflies in addition to agricultural pest. The study is the initial phase of a project aimed to control mosquitoes and blackflies using bioinsecicidals.

The Sudan, with its different climatic regions, and diversity of insects, provide the opportunity of isolating novel entomopathogenic bacteria. In this study, we isolate three strains of *Bt* and five strains of *Bsph*. Initial isolation of *Bacillus* species was based on selective

heating of mosquitoes samples at 80° C for 5 min to get rid of vegetative bacterial cells. The method proved to be very efficient in the primary isolation of *Bacillus* species. Similar results were obtained by Itoua *et al*. (1995) who used the method to selectively isolate *Bacillus thuringiensis* from dead and diseased larvae obtained from a laboratory colony of the European sunflower moth, *Homoeosoma nebulella.*

According to the identification chart lecithinase test was one of the key reactions which differentiated *B. cereus, B. thuringiensis, B. anthracis, B. mycoide*, from other *Bacillus* spp. In our study to differentiate positive lecithinase reaction of *B. cereus* group, our results substantiated the techincal note of an improved method for differential staining of *Bt* and *Bsph* crystals. The present finding also disagreed with Gordon *et al*. (1973) and Elmer *et al* . (1997) who stated that only *B. cereus* group were have lecithinase activity, but *B. alvei* also have the same activity, this finding attributed to be mutated strain.

Parasporal crystals demonstrated by method have been used for detecting Bt colonies in surveys, by phase contrast microscopy which is time consuming compared to the other new staining method (Gurr`s improved 66R Giemsa stain) which is easer and reliable described by Chilcott *et al*. (1988) which was originally used to stain bacterial smears.

The unique character that distinguished Bt and Bsph from other *Bacillus* species is parasporal crystals formation, which it was first described by Logan *et al*. (1985) to differentiate between these spp. Its diffecult to differentiate *Bt* from *B. cereus* for similarty on the colony morphology and almost all biochemical reactions this idea agreed with Kampfer, (1991). This result is consistant the fact that even analytic methods such as DNA homology, pyrolysis gas chromatography and pyrolysis mass spectrometry, failed to differentiate between these species. Differentiation between *B. subtilis* and *B. licheniformis* is normally achieved by virtue of the ability of the later to ferment carbohydrates, this in agreement with Gordon *et al*. (1973). Numerous isolates in this study hydrolysis starch and this test distinguished Bsph from *B. megaterium* and *B. circulans* where thelater two species hydrolysis starch, while the fermer species did not. Results of indol test in this study agreed with that obtained by Smith *et al* .,(1952) that all *Bacillus* species were negative except *B. alvei*.

From results obtained variable reactions of nitrate reduction was shown by 12 isolates, this was in contrast to the results reported in the tables of the *Bacillus* species characters described by Smith *et al*., (1952) which showed defined reactions.

The previous studies reported that non toxic Bt strains were isolated at higher frequency than these which slow insecticidal

activity, for this reson the potential insecticidal activity of *Bt* and *Bsph* isolates obtained in this study against effective mosquito species remain to be determined.

The study documented the isolation and identification of local sudanese isolates of *B. thuringiensis* and *B. sphaericus* which was confirmed by demonstration of parasporal crystals characteristic for both species.

Recommendations

Future studies using *B. thuringiensis* and *B. sphaericus* of this study which could deal with the following**:**

- Analysis of the parasporal crystals by SDS-PAGE, determination of the molecular weight of its proteins and comparing them to that of a reference strains by i
- Molecular characterization of the isolates and sequencing of genes encoding the larvicidal crystals.
- Demonstration of larvicidal effect of the local Sudan isolates.

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