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Research Article

Association of *Bacillus* Species with the Mid-gut of *Anopheles arabiensis* Mosquito Larvae in Sudan

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ABSTRACT

Background: *According to the WHO, over 70% of the Sudanese population living in endemic areas are at risk of malaria. Malaria spreads by the female of the Anopheles species of mosquito. Several studies have characterized various bacteria in the mid-gut of Anopheles mosquitoes. Therefore, this study aimed to isolate bacterial strains, mainly Bacillus species from mid-gut of Anopheles arabiensis mosquito larvae.*

Results: *Fifty-three isolates were examined bacteriologically, showing all isolates were members of Bacillus species. The most prevalent Bacillus spp. Was B.thuringiensis and constituted (33%) of the total number of Bacillus isolates. Moreover, some of Bacillus spp. Which were isolated in this study: (23%) for both B.cereus and B.sphaericus B.lentus (16%) B.brevis (13%), (10%) for both B.popilliae and B.mycoides and (6.7%) B.laterosporus.*

Conclusion: *In this study, we were able to isolate and identify Bacillus species related to the mid-gut of Anopheles arabiensis mosquito larvae.*

Keywords: *Microbial control, Bacillus species, Anopheles arabiensis, mid-gut, mosquito larvae, insectary Rearing.*

INTRODUCTION

Malaria is the most important vector-borne disease affecting humans, with approximately 3.2 billion people exposed globally (WHO, 2015). It remains a significant global health problem, with 92 % of all deaths occurring in Africa (WHO, 2018). Vector-borne diseases are among the leading causes of illness and death in many developing countries. Mosquitoes, the six-legged invertebrates of the class Insecta's family Culicidae, profoundly affect humans.

Mosquito species divided into two sub-families (Anophelinae and Culicinae) (WHO, 2006 & Trari et al., 2017).

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They are responsible for transmitting the most important vector-borne diseases, including malaria, lymphatic filariasis, Japanese encephalitis, and dengue as well as yellow fever and other forms of encephalitis (WHO, 2006).

Malaria is an important vector-borne disease is Plasmodium parasites cause the greatest cause of death in humans. *Plasmodium falciparum, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium vivax* (Service 2001 & Rowe et al ., 2006). These parasites are transmitted by infective bites of Anopheles mosquito vectors, causing malaria in human beings (Cox, 2010).

They cause significant mortality and morbidity, e.g. malaria alone cause fatalities in Africa, around one million every year, the vast majority of them being children under five years (John et al., 2010). Over 90% of malaria cases and deaths are due to *Plasmodium falciparum* and occur in sub-Saharan Africa, [WHO World Malaria Report. (Geneva, 2015), Moss et al., 2012).

The majority of the population is at risk of contracting malaria. About 37 million people were at risk of contracting the disease, and an estimated 4,000 deaths were recorded.

According to the latest data, in 2016 (FMoH, 2017 & WHO, 2017).

In the past, it was believed that the methods of controlling mosquitoes were by using chemical pesticides such as: chlordane, malathion, gammaxane and dichloro diphenyl trichloroethane (DDT). Furthermore, increasing of pesticide-resistant mosquitoes has been managed by developing an alternative.

Recent evidence suggests that biological control is a promising alternative (Poopathi & Tyagi, 2004). The important biological pathogens is B.thuringiensis and B.sphaericus, which belong to the genus Bacillus (Pauchet et al., 2005).

Studies to isolate and identify bacterial species in Anopheles and Aedes mosquitoes collected in the field using microbial breeding techniques have reported a wide range of bacterial taxonomic groups in the mid-gut (Gusmao et al., 2010 & Ngo et al., 2015). Moreover, the intestinal microflora varied depending on the glucose and blood-feeding status of the mosquitoes, with reduced sensitivity of these mosquitoes to parasite development (Pumpuni et al., 1996).

Insects are inescapably associated with an extremely large variety of microorganisms due to their widespread distribution. Bacteria is one of them, and the major species are spore-forming bacilli (Vilmos & Kurucz, 1998). Many different species of bacilli have been isolated from living or dead insects. Bacillus species are generally known as insect pathogens, such as *B. popilliae*, *B. lentimorbus*, *B. larvae*, *B. thuringiensis*, and some strains of *B. sphaericus* (deBarjac, 1981).

Microorganisms infecting mosquitoes and other insects as entomo -pathogens (Seigal & Novak, 1996). Many species of bacteria were isolated from a number of mosquitoes species (Chao & Wistreich, 1959; & Fulton et al., 1974), but a few of them are considered to be true pathogens that cause host death. Ninety–one pathogenic bacterial strains were isolated from infected mosquito larvae; 40 were identified as *B. alvei* and *B. circulans* and 51 belonged to *B. sphaericus* group (Singer, 1973).

This study aimed to investigate the bacterial micro-flora from the mid-gut of Anopheles mosquitoes, mainly entomo-pathogenic *Bacillus*. *spp*.

MATERIALS AND METHODS

Rearing of *Anopheles arabiensis* mosquito:

Mosquito rearing in the insectary required four months instead to establish a good colony of mosquito. The insectary rooms were maintained at 28°C and 80%humidity, with 12hrs. Day/night cycle. 3-5 day old female mosquitoes were fed blood to lay eggs. The females laid eggs for two days, and eggs were allowed to hatch to larvae during the next days. Growing larvae were fed everyday with fish food. The pupae were allowed out to the adults for the next 2-3 days, and food was given daily to the larvae/pupae by carefully removing the net to avoid escape of the adults.

Collection of samples:

Alive and dead larvae were collected from the insectary and were removed from the water and washed several times with tap water followed by sterile distilled water then sterile normal saline. Finally they were dipped in 70% alcohol for a few seconds and then washed and transferred to a sterile slide, for dissection. The whole body was decapitated using sterile blades and tweezers, and the intestines of mosquito larvae were then excised.

Cultivation of samples:

Thirty mid-guts of Anopheles mosquito larvae were inoculated on both Blood Agar and Mac Conkey Agar culture media. Culture were performed under aseptic condition then plates were incubated at 37ºC under aerobic condition for 24-48 hrs. Subsequently, the sects with large growth were selected for further isolation.

Isolation of Bacteria from the mosquito larvae:

Isolation was conducted by comparing the morphology of the grown bacterial colonies based on size, color, margins, elevation and spreading. Morphologically different colonies were considered as different colonies. Depending on Gram stain to determine gram positive and gram negative bacteria, and to identify the bacterial cells` morphology (cocci, bacilli, comma , spiral) for each different colony.

The isolated bacteria were identified according to Barrow and Feltham (1993).

Gram staining:

Gram staining is done according to the method described by Cruickshank et al. (1975). Bacteria colored in red were classified as Gram-negative organisms, and the violet color was classified as Gram-positive organisms.

Spore staining:

Spore staining was done according to the method described by Schaeffer-Fulton method, Murray and Robinow, (1981) was used to stain the spores, the endospores appear, as green-blue, while they are vegetative cells are brownish red.

Biochemical characteristics:

All the biochemical tests were performed according to Sneath, (1986) and Barrow and Feltham (1993). These tests were catalase production, motility, hydrogen sulfide production, anaerobic growth, production of indole, citrate utilization, starch hydrolysis, gelatin hydrolysis, ability to grow at pH 5.7, decomposition of casein, growth in sodium chloride, maximum and minimum growth temperature, production of gas and acid from glucose, Voges- Proskauer test, aesculin hydrolysis and urease production.

RESULTS

A total number of 30 mid-guts of intestinal contents of *Anopheles* mosquito larvae collected from insectery and subjected to bacteriological examination. Fifty-three of the isolates produced positive growth for *Bacillus* species. Microscopic examination of isolates revealed rod-shaped, round or squared at the ends, More than one bacillus species could be isolated from the same sample. On blood agar, colonies of *Bacillus* species produced complete haemolysis. In both media a surface scum maybe formed, with or without turbidity, or a heavy flocculent or membranous deposit.

In this study, the percentage of *Bacillus spp*. was 60% .The highest isolate was *Bacillus thuringiensis* 30%, then both *B.cereus* and *B. sphaericus* were 23% only one *B.lentus* was 16%, the same with *B. brevis* was 13%. Three isolates record 10% they were *B.popilliae* and *B.mycoides*, *B.laterosporus, B.pantithenticus* and *B.alvei*. Eight species of Bacillus record the lowest percentage 3.3% they were *B.alvei, B.larvae, B.polymyxa, B.firmus ,B.firmus , B.badius ,B.megaterium B.circulans, B.coagulans* and *B.lentimorbus*.

The rates of isolation of *Bacillus* species from the mid-gut of Anopheles mosquito larvae are shown in Table 1. Table2 shows the biochemical properties of *Bacillus* species isolated from the mid-gut of *Anopheles* mosquito larvae. All *Bacillus* species were identified as Barrow and Feltham (1993).

Table 1: Prevalence rate of *Bacillus* species among *An.arabeinsis* mosquito larvae:

***Bacillus* species**	**Total No. of larvae**	**No. of Isolates**	**Percentage**
B.thuringiensis	30	10	33%
B.cereus	30	7	23%
B.sphaericus	30	7	23%
B.lentus	30	5	16%
B.brevis	30	4	13%
B.popilliae	30	3	10%
B.mycoides	30	3	10%
B.laterosporus	30	2	6.7%
B.pantithenticus	30	2	6.7%
B.alvei	30	2	6.7%
B.larvae	30	1	3.3%
B.polymyxa	30	1	3.3%
B.firmus	30	1	3.3%
B.badius	30	1	3.3%
B.megaterium	30	1	3.3%
B.circulans	30	1	3.3%
B.coagulans	30	1	3.3%
B.lentimorbus	30	1	3.3%

Table2: Characters and biochemical properties of *Bacillus* species isolated from the gut of *Anopheles arabiensis* mosquito larvae

Bacillus spp. / Test	*B.coagulans*	*B.circulans*	*B.megaterium*	*B.pantothenticus*	*B.badius*	*B.lentimorbus*	*B.mycoides*	*B.laterosporus*	*B.alvei*
Gram stain	+	+	+	+	+	+	+	+	+
Chains of cells	-	-	-	-	-	-	+	+	-
Motility	+	+	+	+	+	+	+	+	-
Spore positional shape	vx		ty	vx	vx	Vx	vx	vx	Vx
Swelling of cell body by spore	+	+	+	-	-	-	-	-	+
Growth in 10% NaCl	-	-	-	-	-	-	-	-	-
Carbohydrates Acid from Ass*									
Glucose	_	+	+	+	+	+	+	+	+
Cellobiose	-	-	-	-	-	-	-	-	+
Galactose	-	+	-	+	-	+	-	-	-
Mannose	-	-	-	+	-	+	-	-	-
Meilibiose	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	+	-	-	+
Salicin	-	+	-	+	+	-	+	+	+
Xylose	-	-	-	-	-	+	-	-	-
Arabinose	-	-	-	+	-	+	-	-	-
Manitole	+	-	-	+	-	-	-	-	-
Utilization of citrate	-	-	-	-	+	+	+	+	-
Urease	-	-	+	+	-	+	-	-	-
Indole	-	-	-	-	-	-	-	-	+
VP	-	-	-	-	+	-	+	+	+
Nitrate reduction	+	-	-	-	+	-	+	+	-
Hydrolysis of:									
Casein	+	-	+	-	+	-	+	+	+
Gelatin	+	-	-	-	+	-	+	+	+
Starch	-	-	-	+	+	+	+	+	+
Egg yolk reaction	-	-	-	-	+	-	+	+	-
Catalase	+	-	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
No. isolates tested	1	1	1	2	1	1	3	2	2

*T spores terminal, U spores central, V spores sub terminal/central; variable in position.

*X spores oval, Y, spores round.

*ASS: Ammonium Salt Sugar

Table 2: Contd.

Test \ *Bacillus spp.*	*B.polymyxa*	*B.larvae*	*B.firumus*	*B.brevis*	*B.popilliae*	*B.sphaericus*	*B.lentus*	*B.cereus*	*B.thuringiensis*
Gram stain	+	+	+	+	+	+	+	+	+
Chains of cells	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+
Spore positional shape	Vx	Vx	Vx	vx		Ty	vx	vx	Vx
Swelling of cell body by spore	+	+	+	+	+	+	-	-	-
Growth in 10% NaCl	-	-	-	-	-	-	-	-	-
Carbohydrates Acid from Ass*									
Glucose	+	+	+	+	+	-	+	+	+
Cellobiose	-	-	-	-	-	-	-	-	-
Galactose	-	-	-	-	+	-	+	-	+
Mannose	-	-	-	-	-	-	+	-	+
Meilibiose	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	+
Salicin	-	-	-	-	+	-	+	+	-
Xylose	-	-	-	-	-	-	-	-	+
Arabinose	-	-	-	-	-	-	+	-	+
Manitole	-	-	-	+	-	-	+	-	-
Utilization of citrate	-	-	-	-	-	-	-	+	+
Urease	+	+	+	-	-	+	+	-	+
Indole	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	+	-
Nitrate reduction	-	-	-	+	-	-	-	+	-
Hydrolysis of:									
Casein	+	+	+	+	-	+	-	+	-
Gelatin	-	-	-	+	-	-	-	+	-
Starch	-	-	-	-	-	-	+	+	+
Egg yolk reaction	-	-	-	-	-	-	-	+	-
Catalase	+	+	+	+	-	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
No. isolates tested	1	1	1	4	3	7	5	7	10

*T spores terminal, U spores central, V spores sub terminal/central; variable in position.

*X spores oval, Y, spores round. *ASS:Ammonium Salt Sugar

DISCUSSION

Anopheles arabiensis were successfully reared under standard insectary conditions at 28ºC, 80% humidity. Because of the high humidity and moistened environment, fungal sporulation can be occurred. Mosquito survival was determined daily. Any cadavers found were removed each day. Cleaning the insectary was necessary to eliminate fungal growth and to maintenance of mosquito's colonies.

A number of bacterial species isolated from the mosquito mid-gut larvae were both gram-negative and gram-positive; we discarded the gram-negative and focused only on the gram-positive bacteria, which were overall, spore-forming aerobic *Bacillus spp.* was predominant isolates.

To reach the goals of vector control strategies, this study must be achieved, that shows a wide diversity of new types of bacteria associated with mosquito microbes. The results of this study reported *Bacillus thuringiensis, B. coagulans B. sphaericus, B. mycoides B. larvae, B. popilliae, B. polymyxa B. lentimobus, B. lentus, B. cereus, B. firmus, B. megaterium, B. laterosporus, B. brev*e, *B. pantothenticus, B. circulan B. alvei* and *B. badius*.

Although *Bacillus thuringiensis, B.popilliae B.lentimorbus, B.larvae* and *B.sphaericus* were isolated from the dead insect and classified as insect pathogens (Burges, 1981 & Sneath, 1986), many other species of *Bacillus* were isolated in this study, these bacilli were: *Bacillus lentus, B.cereus, B.polymyxa, B.firmus, B.megaterium, B.circulans, B.laterosporus, B.brevis, B.badius, B.pantothenticus, B.coagulans,* and *B.mycoides*.

Our results substantiate Singer's (1973, 1975) findings, who reported the same results. He isolated *B.sphaericus*, *B.alvei*, *B.brevis* and *B.circulans* from the midgut of *Anopheles* mosquito larvae. Also, these results are very similar to those obtained by (Balaraman et al., 1979), which isolated *B.alvei*, *B.brevis* and *B.coagulans* from the mid-gut of mosquito and found them highly effective in controlling mosquitoes.

Recently, a new bacterium was isolated from the mid-gut of mosquito larvae and named *Janibacter anopheles* sp.nov by (Kämpfer et al., 2006). No similar bacteria could be isolated in our study.

In this work, the same results were obtained by (Lindh, 2007) who isolate *Bacillus cereus*, *Bacillus coagulans*, *Bacillus mucoides*, *Bacillus thuringensis*, *Bacillus megaterium*, from Anopheles mid-gut larvae.

In Sudan, *Bacillus*. *thuingiensis* was isolated from different locations, dead Anopheles mosquito and rearing bonds (Gorashi et al ., 2012).

In Iranshar, *Bacillus* was identified from both adult and larval of *An. culicifacies* mosquitoes (Chavshin et al., 2014).

Similar to (Soad, 1990) the commonest isolates of this study were *B.thuringiensis*, *B.alvei*, *B.polymyxa* and *B.alvei* were identified from mid-gut of *An.arabiensis* larvae, which probably explains its high prevalence as a dominant among mid-guts microbiota. On the other hand, a very low number of Bacillus isolates from mid-gut of laboratory rearing *An.arabiensis* mosquito larvae formed to be in Ethiopia as reported by (Berhanu et al., 2019).

Comparing the results of this study with other similar studies showed that some bacteria are spread among several important vectors For instance the genus Bacillus, which was isolated from *An. arabiensis*. Many previous studies indicated Bacillus species as insect pathogens like, *B. thuringiensis*, *B. popilliae*, *B. lentimorbus*, *B. larvae*, and some strains of *B. sphaericus* (deBarjac, 1981) This may be attributed to this species, the blood of insect larvae being an excellent food environment for bacterial reproduction, and sometimes for reproduction (Dulmage & Aizawa, 1982).

Albeit the number of isolates in this study is moderate, the data suggest that Gram-positive bacilli bacteria dominate the flora of *An. arabiensis*. This agrees with earlier results from culture-based studies on other species of Anopheles mosquito such as *An. gambiae* and *An. funestus Cx. quinquefasciatus* (Lindh, 2007) and of *An. gambiae* in the wild (Mali) (Tandina et al., 2016) All showing predominance for *Bacillus thuringiensis* bacteria in the mid-gut of anopheles larvae. This may be attributed to the fact that all the mosquito strains were grown under identical conditions with the same diet before sampling them for bacterial diversity studies.

On the other hand, in Sri Lanka *Bacillus fexus*, *B. megaterium*, *B. nealsonii* and *Leucobacter chironomi* were recorded among laboratory-reared *Ae. aegypti* and *Ae. albopictus* larvae. Hence, *Bacillus megaterium* and *B. licheniformis* have been identified from the mid-gut of *Ae. aegypti* larvae (Ranasinghe et al., 2021).

Identification and characterization of mosquito mid-gut flora is likely to contribute towards better understanding of mosquito biology including longevity, reproduction and mosquito pathogen interactions that are important to evolve strategies for vector control mechanisms.

Although a number of studies have been carried out to identify the bacterial microbiota of Anopheles mosquitoes, Furthermore, the gut micro-flora varied depending on the sugar and blood-feeding status of mosquitoes with reduced susceptibility of these mosquitoes to parasite development (Pumpuni et al., 1996). Moreover, mid-gut micro-floral diversity depends on the ecological niche and geographical locations of vector mosquitoes (deBarjac, 1981).

CONCLUSION

This study describes isolation and identification of micro-biota in the mid-gut of *An. arabiensis* from insectary. To our knowledge, it is the first study providing in-depth descriptions of the microbiota diversity in the mid-gut of Anopheles mosquitoes. Our findings indicated that Bacillus species had the dominant micro biota identified from all laboratory species reared Anopheles mosquitoes.

In addition, bacteria associated with *An. arabiensis* larvae can be explained to a large extent by the isolation examination of water of reared mosquito to corroborate the relationship between the microbiota in the mid-gut and the contaminant breeding site bacteria, and thus will be the target for future studies.

Abbreviations

WHO: world health organization
Bt: *B*acillus *thuringiensis*
pH: power of hydrogen

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Author Contribution
The authors contributed equally to establishing the research and design experiment topic.

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