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Larvicidal activities of local bacteria against *Aedes aegypti* larvae

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ABSTRACT

Since the beginning, mosquito-borne diseases like dengue fever, encephalitis, yellow fever, malaria, and filariasis have been caused by numerous medically significant pathogens and parasites, including viruses, bacteria, and protozoans. This indicates the necessity for the ongoing creation of new and effective mosquito-borne disease control strategies in Saudi Arabia and internationally. This investigation has tried to assess the potential larvicidal capacity of local bacteria isolated from the soil of the Rahat region of Makkah, Saudi Arabia for the bio-control of *Aedes aegypti* larvae, a main cause of dengue. The bacteria were identified using morphological and molecular characteristics. Bioassays were used to determine the pathogenicity of various strains against *A. aegypti* larvae. A total of 66 different bacteria were isolated. Overall, four (6.06%) of the 66 bacteria caused mortality in the *A. aegypti* larvae, and only two (*Brevibacillus centrosporus*, and *Cytobacillus firmus*) caused 100% mortality in 24 h. After 48 h, two isolates (*Escherichia fergusonii*1 and *E. fergusonii* 2) caused mortality of over 70%. The outcomes of this investigation exhibited that local isolates of bacteria in the soils of the Rahat region of Makkah, Saudi Arabia, have larvicidal ability. These bacteria have shown larvicidal effects on the larvae of *A. aegypti*. In conclusion, further studies are required to evaluate other mechanisms that contribute to the production of larvicidal toxins in these bacteria.

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Introduction

Saudi Arabia is a country in the Middle East. Every year, millions of Muslims around the world go to Mecca in Saudi Arabia for a religious pilgrimage known as the "Hajj." Pilgrims suffer significant health concerns during the Hajj, due to the enormous number of people present at this time, which increases the danger of getting infectious diseases [1]. In fact, this religious gathering draws attention to some of the world's most challenging public health and disease management issues. The possibility for infectious diseases and other health problems to spread during this time is a major concern for Saudi public health officials. Furthermore, the potential for new infectious diseases to become epidemics is a serious worry. The Saudi authorities are constantly refining and upgrading their disease prevention procedures for the administration of Hajj ahead of each Hajj season. In Saudi Arabia, mosquito species transmit several types of life-threatening mosquito-borne diseases. *Aedes* (Stegomyia) *aegypti* is the primary vector in the global resurgence of epidemic dengue fever, as well as a variety of other arboviruses that impact human populations worldwide, such as Zika and Chikungunya [2]. Dengue fever is a serious public health problem in Saudi Arabia and many other regions, and it is the world's second most common arbovirosis by the total number of people infected [3]. Dengue fever, which is spread by the *A. aegypti* mosquito [4, 5], was reported in Mecca and Jeddah in 2001 [6]. Rift Valley fever, which is spread by the mosquito *Aedes caspius*, is common in Saudi Arabia's southern and eastern areas [7]. As a result, Saudi Arabia is affected by several life-threatening mosquito-borne diseases, and the

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Saudi government is working hard to combat these diseases by reducing mosquito vectors. The Saudi Ministry of Health has designed and implemented comprehensive policies to reduce the risk of mosquito-borne disease, particularly during the Hajj season. So, insect control was carried out mainly through the use of chemical insecticides, but due to their lack of specialization, the resistance of insects to them, and their negative impact on the environment and human health [8], in addition to the fact that some types of them are toxic or carcinogenic, as well as the high cost of their production, it was necessary to turn attention to research about alternatives, such as biological control; It is an integrated strategy used to control pests and reduce their population by using natural enemies. The desirable characteristics of organisms used in biological control are that they are specialized and safe to use so as not to harm humans, animals, or crops, that control is carried out quickly to avoid widespread damage, and that the production process is inexpensive. Biological control of mosquito vectors has not been widely applied in Saudi Arabia, to our knowledge. Therefore, as a continuation of our interest in the control of mosquito-borne diseases in Saudi Arabia and elsewhere [9]. The larvicidal potential of bacteria isolated from soils of the Rahat region of Makkah, Saudi Arabia was evaluated in this study as the biological control of *A. aegypti* larvae.

Materials and Methods

Sample collection

For the current study, Soil samples were collected in February 2021 in sterile plastic bags from different sites in the Makkah region, Saudi Arabia (22°06'02.0 "N 40°01'36.6 "E and 22°05'48.7"N 40°01'32.6 "E), At the collection site, Temperature, pH range, and other physical properties were noted during the sampling. A pH meter was used to confirm the pH further in the laboratory. A total of six samples were collected and labeled as samples A to F. Each sample was air dried by spreading it on paper sheets at room temperature followed by sieving using a sieve with 0.2 mm size. One gram of the sieved sample was added to a tube containing 9 mL of sterile distilled water for serial dilution. An aliquot of 0.1 mL of the suspension was transferred to the surface of nutrient agar (NA) plates and spread with the aid of a spreader. The plates were incubated at 37°C for 4 days. The same procedure was repeated for each of the samples in duplicate.

Purification and preservation of bacterial isolates

All collected samples were serially diluted, and the plates were incubated for 48 h at 35 ± 2 ^{o}C; The cultivated bacterial colonies were counted as the colony forming unit (CFU)/g, and a single colony was streaked on NA until pure colonies were obtained. The purified single colony of bacteria was cultured in nutrient broth (NB) overnight and stored at −80 °C in NB broth containing 16% glycerol for preservation. The bacteria were kept in the Department of Biological Sciences until required for use. The bacteria were revived from the glycerol stock for use by culturing in an NB medium. A sterile loop was utilized to transfer the culture to a glass tube containing 10 mL of NB broth a day before the experiment. It was then cultured on an NA plate [10].

Identification of bacteria

Biochemical characterization of bacterial isolates

To differentiate between Gram-negative and Gram-positive bacteria, the Gram staining technique was used following the method described previously. The Oxidase test technique was used following the method described previously. The catalase test technique was used following the method described by Hayward [11].

Molecular identification of bacterial isolates

Nucleic Acid Isolation

The nucleic acid (DNA) from each of the bacteria was isolated by Zymo research "Quick-DNA™ Fungal/Bacterial Miniprep" kit according to the manufacturer's procedure. The quality and quantity of DNA measurements were evaluated using Denovix DS-11 Spectrophotometer.

Polymerase chain reaction

PCR was done by DreamTaq Green PCR Master Mix (2X) and the universal primers used are 27 Forward (5`AGAGTTTGATCMTGGCTCAG3`) and 1492 Reverse (5`TACGGYTACCTTGTTACGACTT 3`). The process is performed according to the manufacturer's instructions with a total volume of 25 ul. The PCR amplified the target DNA using Verti™ Thermal Cyclers.

Gel Electrophoresis

Agarose gel electrophoresis was used to confirm the PCR product size and quality before sequencing. The gel was prepared at 2% using Ultrapure Agarose cleaver scientific and 1X TBE buffer. It was stained with a SYBR-safe DNA stain (Invitrogen, California USA) before loading. To each well, 4µl of PCR product was loaded. A 100-1000 DNA ladder was used and run alongside the samples at 100 mV for 30 minutes. The size of the DNA fragments was visualized using a Gel Doc system imager system with a UV Trans-illuminator.

PCR purification

Amplicon purification was conducted using ExoSAP-IT™. According to the manufacturer's procedure, 5 µL of the PCR reaction product was mixed with 2 µL of ExoSAPIT™. The remaining primers and nucleotides were broken down during a 15-minute incubation at 37°C before the ExoSAP-IT™ reagent was inactivated during a 15-minute incubation at 80°C.

Cycle sequencing PCR

Using BigDye XTerminator(Applied Biosystem, USA), the cycle sequencing purification was carried out. The cycle sequencing PCR reaction used 45 µl of SAM solution. After thoroughly mixing the mixture, 10 µl of XTerminator (beads) were added. The plate was then sealed, vortexed for 45 minutes at 3500 rpm, then centrifuged for two minutes at 14,000 rpm. The upper layer was then gently deposited into each well of the optical 96-well plate using 30 µl. The plate was then spun down and sealed with Septa 96 well [12].

Sequencing Platform

Sequencing was performed using SeqStudio (Life Technologies, USA) and run following a long module.

Sequence Alignment

Sequence analysis was done by SNapGene version 6.0.2 to extract fasta format and alignment through an online tool, the NCBI blast.

Larvicidal activity

Obtaining and growing larvae in the laboratory

The larvae of *A. aegypti* mosquitoes have been grown. In the Department of Biology at King Abdulaziz University, the larvae were placed in a separate breeding room in which the appropriate environmental conditions for the life of mosquitoes were taken into account, such that the temperature was 26 ± 2 °C and the relative humidity rate was 10%, with average light (12:12) [13].

Blood feeding of *A. aegypti* mosquitoes

After growing the larvae and reaching the adult stage, *A. aegypti* females need blood meals to produce eggs, pigeons were used to feed the larvae with blood, thus obtaining eggs [14].

Susceptibility bioassays

Bacteria isolated from the soil samples were selected and evaluated for toxicity to the third-instar larvae of *A. aegypti* [15]. The susceptibility bioassays toward the larvae were carried out under controlled conditions. The larvae were obtained from the laboratory of King Abdulaziz University, at a mean temperature of 26 ± 2°C, and a 12 h photophase period. Bacterial suspensions were prepared for each isolate by growing the bacteria in NB at 28°C for five days until sporulation. All the bacterial content was then transferred to new tubes. The tissue culture dish was used for the bioassay test in triplicates. For each tissue culture dish, 10 third-instar larvae of *A. aegypti*, and 2 mL of the bacterial suspension were added in each well of the plates. The negative control well contains no bacterial suspension but rather sterile distilled water. The plates were maintained at mean temperature and larval mortality was observed after 24 h and 48 h of the addition of the bacterial suspension. Mortality as a result of the suspension was confirmed by counting the living and dead larvae in each well. A larvae is considered dead when the larvae do not move when touched with a sterile stick.

Phenotypic antimicrobial susceptibility

In compliance with the clinical and laboratory standards institute instructions, the Kirby-Bauer disk diffusion method was used to determine the antimicrobial profile of the bacteria tested (Oxoid, Basingstoke, UK). The following antibiotics were used; amikacin (AK) (30µg), penicillin G (PG) 10 Unit, piperacillin (PRL) (100µg), cephalothin (CP) (30µg), ciprofloxacin (CIP) (5µg), erythromycin (E) (15µg), ampicillin (AP) (10µg), imipenem (IMI) (10µg), clinamycin(CD) (2µg), cotrimoxazole (TS)

(25µg), ceftazidime (CAZ) (30µg), aztronam (ATM) (30µg).

Results and discussion

Physiochemical parameters of collected soil samples

The soil samples collected from different sites were subjected to different physiochemical parameters. The collected soil samples had a pH in the range of 6.44 to 7.54 (Table .3.1). The lowest pH recorded from the soil samples from the Rahat region is 6.44 ± 0.05. In contrast, the sample from other places has the highest pH of 7.54 ± 0.05. The average and permissible pH value for soil samples from the Rahat region is 6.99± 0.05. Among the other physiochemical parameters, such as the temperature of all the samples was between 27 ^{o}C to 32 ^{o}C. However, the sample from R-A1 had the lowest temperature of 27 ^{o}C compared to all the samples, while the temperature of the sample collected from R-F1 was the highest. In six samples, the soil's colors were due to a combination of various chemicals. The physical characteristics of the soils vary according to the blending of multiple forms of compounds derived from different environmental conditions.

Table 1. Physiochemical parameters of the collected soil samples.

No	Code	GPS Coordinates	pH	Temperature	Source of soil	Date of collection
1	R-A1	22°06'02.0"N 40°01'36.6"E	7.02 ± 0.04	32 ^{o}C	Rahat region	2/ 2021
2	R-B1	22°06'02.0"N 40°01'36.6"E	6.44 ± 0.05	32 ^{o}C	Rahat region	2/ 2021
3	R-C1	22°06'02.0"N 40°01'36.6"E	7.54 ± 0.05	30 ^{o}C	Rahat region	2/ 2021
4	R-D1	22°05'48.7"N 40°01'32.6"E	6.58 ± 0.05	29 ^{o}C	Rahat region	2/ 2021
5	R-E1	22°05'48.7"N 40°01'32.6"E	6.90 ± 0.05	30 ^{o}C	Rahat region	2/ 2021
6.	R-F1	22°05'48.7"N 40°01'32.6"E	6.58 ± 0.05	27 ^{o}C	Rahat region	2/ 2021

Isolation and purification of the Isolates isolated

Six soil samples from 2 areas of the Rahat region were analyzed and 66 bacterial colonies were isolated from selected locations and purified by repeated streak culture on NA (Figure 1), of which, 14 (21.2%) were selected to complete this study to their bioactivities after the preliminary test. The 66 isolates were designated as (R-A1- R-A10), (R-B1- R-B12), (R-C1- R-C13), (R-D1- R-D6), (R-E1- R-E17), and (R-F1- R-F8) shown in (Table 2).

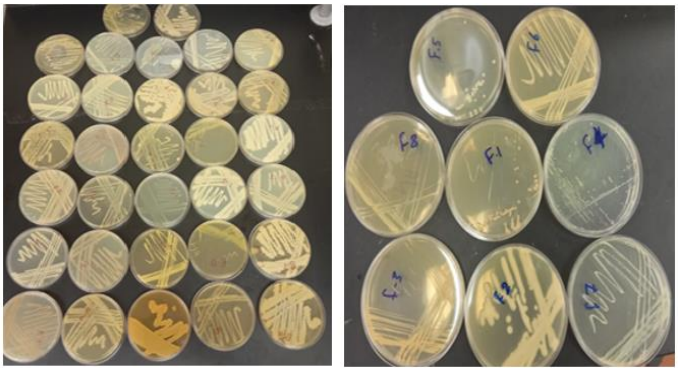

Fig. 1. Pure culture (Single colonies) of bacterial strains.

Selection of isolates according to their activity

Overall, four (6.06%) of the 66 bacteria caused mortality in the *A. aegypti* larvae, and two (*Brevibacillus centrosporus* and *Cytobacillus firmus*) led to 100% mortality in 24 h. After 48 h, two isolates (*Escherichia fergusonii*1, and *E. fergusonii* 2) caused mortality of over 70%.

Identification of bacteria

Biochemical characteristics

Biochemical characteristics test showed that among bacteria, isolates had a positive and negative reaction to gram stain, oxidase test, and catalase test (Table 3).

Table 2. Bacterial isolates were collected from various soils of Rahat region, Makkah, Saudi Arabia.

No	Sampling Site	Isolate name	Activity	No	Sampling Site	Isolate name	Activity	No	Sampling Site	Isolate name	Activity
1	R-A1	R-A1	-	23	RC-1	RC-1	-	45	RE-1	RE-4	-
2	R-A1	R-A2	-	24	RC-1	RC-2	-	46	RE-1	RE-5	-
3	R-A1	R-A3	-	25	RC-1	RC-3	-	47	RE-1	RE-6	+
4	R-A1	R-A4	+	26	RC-1	RC-4	-	48	RE-1	RE-7	+
5	R-A1	R-A5	-	27	RC-1	RC-5	-	49	RE-1	RE-8	-
6	R-A1	R-A6	+	28	RC-1	RC-6	-	50	RE-1	RE-9	-
7	R-A1	R-A7	+	29	RC-1	RC-7	-	51	RE-1	RE-10	-
8	R-A1	R-A8	-	30	RC-1	RC-8	+	52	RE-1	RE-11	-
9	R-A1	R-A9	-	31	RC-1	RC-9	-	53	RE-1	RE-12	+
10	R-A1	R-A10	-	32	RC-1	RC-10	-	54	RE-1	RE-13	+
11	RB-1	RB-1	-	33	RC-1	RC-11	-	55	RE-1	RE-14	-
12	RB-1	RB-2	-	34	RC-1	RC-12	+	56	RE-1	RE-15	-
13	RB-1	RB-3	-	35	RC-1	RC-13	-	57	RE-1	RE-16	-
14	RB-1	RB-4	-	36	RD-1	RD-1	-	58	RF-1	RE-17	-
15	RB-1	RB-5	-	37	RD-1	RD-2	-	59	RF-1	RF-1	-
16	RB-1	RB-6	-	38	RD-1	RD-3	-	60	RF-1	RF-2	-
17	RB-1	RB-7	+	39	RD-1	RD-4	-	61	RF-1	RF-3	+
18	RB-1	RB-8	-	40	RD-1	RD-5	-	62	RF-1	RF-4	-
19	RB-1	RB-9	-	41	RD-1	RD-6	+	63	RF-1	RF-5	+
20	RB-1	RB-10	-	42	RE-1	RE-1	-	64	RF-1	RF-6	+
21	RB-1	RB-11	-	43	RE-1	RE-2	-	65	RF-1	RF-7	-
22	RB-1	RB-12	-	44	RE-1	RE-3	-	66	RF-1	RF-8	-

Table 3. Biochemical features of the isolated bacteria (A: *B. centrosporus*; B: *E. fergusonii*; C: *C. firmus*).

N.	Code	Bacteria	Gram reaction	Oxidase	Catalase
1	N3- RD-6	B	Negative	Negative	Positive
2	N4- RF-6	B	Negative	Negative	Positive
3	N7- RC-8	A	Positive	Positive	Negative
4	N8- RB-7	C	Positive	Positive	Negative

Molecular characteristics

The BLAST search at the NCBI database showed varying degrees (98.12 – 100%) of 16s rRNA gene sequence similarity of the isolated bacteria to a wide array of species belonging to *B. centrosporus, E. fergusonii, and C. firmus* as shown in (Table 4).

Antibiotic susceptibility test

The resistant profile of bacterial isolates has been shown in (Table 5). In this study, results indicate that both bacteria are 100% resistant to two antibiotics CAZ (30µg), and ATM (30µg). and all strains have sensitivity to AK (30µg), PRL (100µg), IMI (10µg), CP (30µg), CIP (5µg), AP (10µg), TS (25µg), CD (2µg), PG 10 Unit, E (15µg). Amikacin (AK; 30µg), penicillin G (PG;10 Unit), piperacillin (PRL; 100µg), imipenem (IMI; 10µg), cephalothin (CP; 30µg), ciprofloxacin (CIP; 5µg), erythromycin (E; 15µg), ampicillin (AP; 10µg), clinamycin(CD; 2µg), cotrimoxazole (TS; 25µg), ceftazidime (CAZ; 30µg), aztronam (ATM; 30µg), S; sensitive, R; Resistant.

Finding new strategies to prevent and defeat human pathogens is a critical issue in biomedicine [16-19]. In the screening assay, sixty-six bacterial cultures were tested for larvicidal activity against the *A. aegypti* mosquito, with four isolates proving to be effective. The effective isolates were identified as *B. centrosporus, E. fergusonii*1, *E. fergusonii*2, *and C. firmus*, based on their biochemical and molecular Features. Therefore, soils are a good source of bacteria that can be investigated for their biocontrol capabilities [20].

Bacillus sp., *Penicillium* sp., *Streptomyces* sp., and *Trichoderma* sp. are being investigated as biocontrol agents that could be used instead of chemical

compounds [21, 22]. Due to their known metabolic diversity, *Bacillus* species and related genera are promising candidates for discovering novel strains of bacteria and larvicidal metabolites. There are several studies demonstrated that the toxicity of the wild-type *Bacillus* sp. was determined towards *A. aegypti* larvae, such as *B. thuringiensis* and *Bacillus sphaericus* [23, 24].

Table 4. Genotypes of the selected four selected strains with 16s rDNA gene sequences were submitted to the NCBI GenBank database (A: *B. centrosporus*; B: *E. fergusonii*; C: *C. firmus*).

No	Code	Bacteria	Max score	Total score	Query cover	E value	Per. Ident	Acc. Len	Accession
1	N7- RC-8	A	1644	1644	99%	0.0	100.00%	1486	NR_112211.1
2	N3- RD-6	B	1729	1729	100%	0.0	99.58%	1542	NR_074902.1
3	N4- RF-6	B	1578	1578	100%	0.0	99.88%	1542	NR_074902.1
4	N8- RB-7	C	1493	1493	99%	0.0	98.12%	1477	NR_112635.1

Table 5. Phenotypic antimicrobial susceptibility profile of tested bacteria (A: *B. centrosporus*; B: *E. fergusonii*; C: *C. firmus*).

No	Code	Bacteria	AP	CP	CD	TS	PG	E	AK	CAZ	ATM	PRL	IMI	CIP
1	N8- RB- 7	A	S	S	S	S	S	S	S	R	R	S	S	S
2	N4- RF-6	B	S	S	S	S	S	S	S	R	R	S	S	S
3	N5- RF-5	B	S	S	S	S	S	S	S	R	R	S	S	S
4	N1- R-A4	C	S	S	S	S	S	S	S	R	R	S	S	S

In the present study, the isolates N8- RB-7, N4- RF-6, N5- RF-5, and N1- R-A4 were identified as *B. centrosporus*, *E. fergusonii*1, *E. fergusonii*2, *and C. firmus respectively*, they have activity against *A. aegypti* mosquito larvae among the isolates tested. However, there have been no previous reports of its mosquito larvicidal action.

Conclusions

This study identified some bacterial strains with larvicidal activity against *A. aegypti* larvae. These results are encouraging for the prospective development of innovative bioinsecticides for the management of medically significant mosquitoes. As the main conclusion, more studies are required to evaluate other mechanisms that contributed to the production of larvicidal toxins in these bacteria.

Study Highlights

- Four (6.06%) of the 66 bacteria caused mortality in the *A. aegypti* larvae, and only two (*B. centrosporus*, and *C. firmus*) caused 100% mortality in 24 h.
- After 48 h, two isolates (*E. fergusonii*1 and *E. fergusonii* 2) caused mortality of over 70%.
- The data are encouraging for the prospective development of innovative bioinsecticides for the management of medically significant mosquitoes.

Abbreviations

AK: Amikacin
AP: Ampicillin
ATM: Aztronam
CAZ: Ceftazidime
CD: Clinamycin
CFU: Colony forming unit
CIP: Ciprofloxacin
CP: Cephalothin
E: Erythromycin
IMI: Imipenem
NA: Nutrient agar
NB: Nutrient broth
PG: Penicillin G
PRL: Piperacillin
TS: Cotrimoxazole

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Conflict of interest

The present study was conducted without any conflicts of interest, according to the authors.

Ethical approval

This article does not contain any studies with animals or human participants performed by any of the authors.

Author Contributions

All authors: conceptualization, preparing the first draft, and editing.

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