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Isolation and Identification of Local Soil Bacteria with Larvicidal Activities Against *Aedes aegypti* Larvae

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Abstract: Mosquito-borne diseases caused by infected mosquitoes like dengue fever, Zika fever, Chikungunya and malaria are spread to humans through biting causing disease epidemics all over the world. This indicates the necessity for new mosquito-borne disease control strategies in Saudi Arabia and internationally. This study evaluated the potential of local bacteria isolated from the soil of the Rahat region of Makkah, Saudi Arabia for the biocontrol of *Aedes aegypti* larvae, a major agent for the transmission of dengue fever. Soil samples were collected from the Rahat region, Makkah for the isolation of bacteria. The bacteria were evaluated for larvicidal activity against the larvae of *A. aegypti*. The bacteria that show toxicity to the larvae were identified using morphological and molecular characteristics. Four different strains of the bacteria with a toxicity towards *A. aegypti* larvae were isolated and identified. Two of the isolates (*Brevibacillus centrosporus* N8 *and Cytobacillus species* N7) caused 100% mortality in 24 h while the other two isolates (*Escherichia coli N3 and Escherichia coli N4*) caused at least 70% mortality. The findings of this study revealed the larvicidal activity of soil bacteria from the Rahat region, Makkah and could be a potential candidate for the biocontrol of mosquito vectors.

Keywords: Larvicidal, *Aedes Aegypti*, Biocontrol, Soil Bacteria, Dengue Fever

Introduction

In the Kingdom of Saudi Arabia (KSA), a variety of mosquito species transmit many sorts of life-threatening mosquito-borne diseases caused by pathogens and parasites due to the wide distribution of mosquito vectors in the Kingdom (Ahmed *et al*., 2011). Different species of mosquitoes are found in the kingdom and so far, verified ones include culicidae, anophelines, and culcines (Alahmed *et al*., 2019). Several cases of mosquito-borne diseases exist in KSA and malaria has been endemic in the kingdom for more than a century (Al-Seghayer *et al*., 1999). Among the diseases spread by mosquito vectors in the kingdom include dengue fever caused by dengue virus (Alhaeli *et al*., 2016).

Dengue Fever (DF) is a mosquito-transmitted viral disease that occurs worldwide with economic and health implications. The first case of DF was recorded in KSA in the early 90s in the coastal city of Jeddah and the country is endemic for the disease (Fakeeh and Zaki, 2001). Many factors predispose the kingdom to DF which includes weather conditions that support the transmission of the disease such as humidity and temperature (Campbell *et al*., 2013, 2015). Another factor relevant in the transmission of the disease and increasing its risk in the kingdom is high population density (Khormi and Kumar, 2012). High population density is unique in KSA due to large number of migrant workers and religious pilgrims (Alhaeli *et al*., 2016).

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The disease is widespread and endemic in the holy city of Makkah, located about 70 km away from Jeddah. There was an outbreak in the city in the year 2004 that affected both locals and visitors (Khan *et al*., 2008). The city is at high risk of dengue fever due to water scarcity and its unique feature and relevance as the site for holy pilgrimage for Muslims. Annually, the city received millions of pilgrims from many countries across the globe who usually visit the kingdom for religious rituals. Due to multiple constructions works in the city, it received thousands of migrant workers from countries across the Middle East, North Africa, and Asia where DF is endemic (Alhaeli *et al*., 2016). These factors were attributed to the distribution and transmission of the various genotype of the virus in the kingdom (Altassan *et al*., 2019) making the city a hotspot for the transmission of DF through mosquitoes. A recent rise was observed in the number of cases of DF in the city, which was linked to the increase in rainfall which facilitates the disease transmission (Melebari *et al*., 2021). There are many incidences of local cases in the city, however, many incidences have been linked to pilgrims and migrant workers from other dengue fever endemic countries (Altassan *et al*., 2019).

The vector that is most important in the spread of DF is the mosquito *Aedes aegypti* which spreads the severe form of DF. Its success is dependent upon the nature of the environment such as water scarcity areas and densely populated areas without basic hygiene (Honório *et al*., 2009). *A. aegypti* is very common, widely distributed, and endemic in the city of Makkah (Ali *et al*., 2021). The life cycle of the vector takes about 7 to 10 days for the eggs to mature into adults. To prevent the spread of DF by *A. aegypti*, there is a need to understand its activity and control the growth of the vector and eliminate its breeding environment to reduce the larval population of the mosquito (Ali *et al*., 2021). Controlling the vector that transmits the disease can stop the transmission of the viral pathogens responsible for the disease. The insecticide is widely applied as a public health requirement in Saudi Arabia and many other places to control the vector. However, the use of insecticide has many setbacks. It is expensive, may have toxicity to humans and animals, and can impact the environment negatively (Al-Sarar, 2010). There is an increase in resistance of the mosquitoes to insecticides which is a major threat to the successful elimination of DF.

This necessitates the need for research on alternative control measures such as biological control. The biological control method has been applied to control mosquitoes including *A. aegypti* using predatory animals or pathogenic microorganisms (Katak *et al*., 2021). However, there is limited information on the reduction or elimination of the disease burden by the biocontrol agents and how it can be sustained (Huang *et al*., 2017). If this method succeeds and is applied to control the vector, it will help in the control of the disease in the holy city of Makkah which is crowded during the peak period in an environmentally acceptable way. Biological control of mosquito vectors has not been widely applied in Saudi Arabia, to our knowledge. Hence this study will investigate the potential of local soil bacterial isolates obtained from the soil of the Rahat region in Makkah, Saudi Arabia for the biological control of *A. aegypti* larvae. The aim is to discover an alternative way of controlling the mosquito vector and minimize or eradicate the application of insecticide chemicals in the future using local resources.

Materials and Methods

Sample Collection

For the current study, soil samples were collected in February 2021 in sterile plastic bags from different sites of Rahat Region, in Makkah city, Saudi Arabia (22°06'02.0"N 40°01'36.6"E and 22°05'48.7"N 40°01'32.6"E). The pH and temperature of the samples were recorded at the site of collection. The samples were dried in the laboratory and sieved with a 0.2 mm mesh.

Isolation of Bacteria and Preparation of Bacterial Suspension

A stock solution was prepared for each sample in duplicate by adding 1 gram of the sieved sample in 9 mL of sterilized distilled water followed by serial dilution and subsequent inoculation of 0.1 mL from dilution 10^6 in nutrient agar plates using the spread plate method. The plates were incubated for 48 h at 35±2°C. Distinct colonies that appeared after incubation were subcultured individually in nutrient agar plates to obtain pure culture isolates. Pure isolates of each of the bacteria were grown in tubes containing 10 mL nutrient broth and incubated with shaking (150 rpm) at 30°C for five days. The suspension was then transferred to new containers. Suspension from the bacteria isolated from the soil samples were evaluated for larvicidal activity against the third-instar larvae of *A. aegypti*.

Larvicidal Activity Assay

The insect larvae was obtained from the Dengue mosquito experimental station of King Abdulaziz University (KAU), Jeddah, KSA. At the department of Biological science at King Abdulaziz University, a culture of the larvae of *A. aegypti* mosquito's colony has been maintained for vector control studies. The Adequate conditions for temperature and humidity for the growth of mosquito were considered and applied in the breeding room as reported previously (Alotaibi *et al*., 2022). Average light was set at 12 h: 12 h light and dark conditions while the temperature was maintained at 26±2°C and the relative humidity rate at 10%. The larvae of the *A. aegypti* were grown until they reach the adult stage. The females were fed with the blood

of pigeons to enable them to produce eggs. For larvicidal activity, it was carried out in triplicates in 6-well tissue culture plates. For each plate, 10 third-instar larvae of *A. aegypti* were added to each well followed by the addition of 2 mL of the bacterial suspension to the wells except the control well. The control well contains only the larvae with distilled water without bacterial suspension. The plates were kept at the same conditions as described above and larvicidal activity was observed by counting the living and dead larvae in each well after 24 h of the addition of the bacterial suspension as described previously (Amazonas Maciel Magalhães *et al*., 2010). The results which were obtained in triplicates were averaged and the percentage mortality was calculated from the number of dead larvae out of the total larvae in the particular well. Only organisms that recorded at least 70% mortality after 24 h were considered for biochemical and molecular identification.

Identification of Larvicidal Bacteria

Among the isolates screened for larvicidal activity, 4 were selected because of their toxicity to the larvae of *A. aegypti*. The pure isolate of each of the 4 bacteria was identified first by Gram staining and then by oxidase and catalase tests using standard protocols (Reiner, 2010; Shields and Cathcart, 2010). All 4 bacteria were identified by 16S rRNA gene sequencing method. The DNA was extracted using Zymo research "Quick-DNA™ Fungal/Bacterial Miniprep"kit (cat# D6005) according to the manufacturer's procedure. After the extraction, PCR was carried out using universal primers used 27F(5`AGAGTTTGATCMTGGCTCAG3`) and 1492R (5`TACGGYTACCTTGTTACGACTT 3`). The process is performed with a total volume of 25 µL. The PCR amplified the target DNA using Verti™ Thermal Cyclers. The PCR program is as follows: One cycle of 95°C for 5 min followed by 28 cycles of 95°C for 45 sec and annealing at 58°C for 45 sec with extension at 72°C for 1 m 45 sec and a final extension step at 72°C for 10 min. After the PCR, the product size and quality were confirmed by agarose gel electrophoresis before sequencing. The PCR product was sequenced commercially at Alborg laboratories, Jeddah KSA. Sequences received from the company were subjected to BLAST for identification using a similarity identity of at least 99% for species identification using the highest total score, and search restriction to sequence from type material (Abdulrahman *et al*., 2022).

Phenotypic Antimicrobial Susceptibility Profile

The four strains were subjected to antimicrobial susceptibility testing using the Kirby-Bauer disk diffusion method. The method of Hudzicki (2009) was followed for the evaluation of the susceptibility of the organisms to commonly used antibiotics using commercially impregnated discs from Oxoid, UK. The antibiotics used are Amikacin (AK) (30 µg), Penicillin G (PG) 10 Unit, Piperacillin (PRL) (100 µg), Cephalothin (KF) (30 µg), Ciprofloxacin (CIP) (5 µg), Erythromycin (E) (15 µg), Ampicillin (AP) (10 µg), Imipenem (IMI) (10 µg), Clinamycin (CD) (2 µg), Cotrimoxazole (TS) (25 µg), Ceftazidime (30 µg), Aztronam (ATM) (30 µg).

Results

Soil Properties

The physicochemical parameters of the collected soil samples had a pH in the range of 6.44 to 7.54 while the average is 6.99±0.05. The temperature of all the samples was between 27 to 32°C as presented in Table 1.

Isolation and Purification of the Isolates Isolated

The results of the identification of the organisms presented in Table 2 shows that the organisms isolated have diverse features belonging to different genera. Comparing the 16S rRNA gene sequences with the NCBI database revealed that all the organisms have at least 98% similarity with their relative type strain. Among the identified bacteria, 2 of them (N3 and N4) have the same genus and species name but they are isolated from different sampling points with similarity above 99% with the type strain (*Escherichia coli* DSM 30083^T) and other related strains. The strain N7 has a similarity of 100% with the type strain *Brevibacillus centrosporus* DSM 8445^T while N8 has a little above 98% similarity with its type strain (*Cytobacillus Firmus* JCM 2512^T) and is therefore identified without the species name.

Larvicidal Activity of the Local Soil Bacterial Strains

The bacterial suspension from the four organisms caused mortality to the larvae of *A. aegypti* after 24 h. Suspension from two of the identified bacteria (*B. centrosporus* N7 and *Cytobacillus species* N8) have toxicity towards the larvae with 100% mortality after 24 h. The other two closely related strains (*Escherichia coli* N3 and *Escherichia coli* N4) resulted in 73 and 70% mortality respectively as presented in Fig. 1.

Antibiotic Susceptibility Test

The antibiotic susceptibility profile of the bacterial isolates is presented in Table 3. The results indicate that all 4 strains were 100% resistant to only two of the antibiotics which are Ceftazidime (CAZ) (30 µg) and Aztronam (ATM) (30 µg). However, all the strains are sensitive to the remaining antibiotics tested. They are sensitive to Amikacin (AK) (30 µg), Piperacillin (PRL) (100 µg), Imipenem (IMI) (10 µg), Cephalothin (KF) (30 µg), Ciprofloxacin (CIP) (5 µg), Ampicillin (AP) (10 µg), Cotrimoxazole (TS) (25 µg), Clindamycin (CD) (2 µg), Penicillin G (PG) 10 Unit, Erythromycin (E)(15 µg).

Table 1: Physicochemical parameters of soil samples at the time of collection

No	Sample code	Location	pH	Temperature (°C)	Organism isolated
1	RC-8	22°06'02.0"N 40°01'36.6"E	7.54±0.05	30°C	*Brevibacillus centrosporus* N7
2	RD-6	22°05'48.7"N 40°01'32.6"E	6.58±0.05	29°C	*E. coli* N3
3	RF-6	22°05'48.7"N 40°01'32.6"E	6.58±0.05	27°C	*E. coli* N4
4	RB-7	22°06'02.0"N 40°01'36.6"E	6.44±0.05	32°C	*Cytobacillus* sp. N8

Table 2: Identification of larvicidal bacteria from soil of Rahat region, Makkah, Saudi Arabia

Code. No	Gram reaction	Oxidase	Catalase	Closest type strain	Percent identity(%)	Scientific name number	Accession
N3	Negative	Negative	Positive	*Escherichia coli* DSM 30083^T	99.58	*Escherichia coli*	OP565043
N4	Negative	Negative	Positive	*Escherichia coli* DSM 30083^T	99.88	*Escherichia coli*	OP565044
N7	Positive	Positive	Negative	*Brevibacillus centrosporus* DSM 8445^T	100.00	*Brevibacillus centrosporus*	OP565045
N8	Positive	Positive	Negative	*Cytobacillus firmus* JCM 2512^T	98.12	*Cytobacillus species*	OP565046

Key: Superscript T = type strain

Table 3: Phenotypic antimicrobial susceptibility profile of tested bacteria

	Bacteria	AP	KF	CD	TS	PG	E	AK	CAZ	ATM	PRL	IMI	CIP
1	*Brevibacillus centrosporus* N8	S	S	S	S	S	S	S	R	R	S	S	S
2	*Escherichia coli* N4	S	S	S	S	S	S	S	R	R	S	S	S
3	*Escherichia coli* N3	S	S	S	S	S	S	S	R	R	S	S	S
4	*Cytobacillus* sp N7	S	S	S	S	S	S	S	R	R	S	S	S

Antimicrobial agents; Amikacin (AK) (30 µg), Penicillin G (PG) 10 Unit, Piperacillin (PRL) (100 µg), Imipenem (IMI) (10 µg), Cephalothin (KF) (30 µg), Ciprofloxacin(CIP) (5 µg), Erythromycin (E)(15 µg), Ampicillin (AP) (10 µg), Clinamycin (CD) (2 µg), Cotrimoxazole (TS) (25 µg), Ceftazidime (30 µg), Aztronam (ATM) (30 µg), S; sensitive, R; Resistant

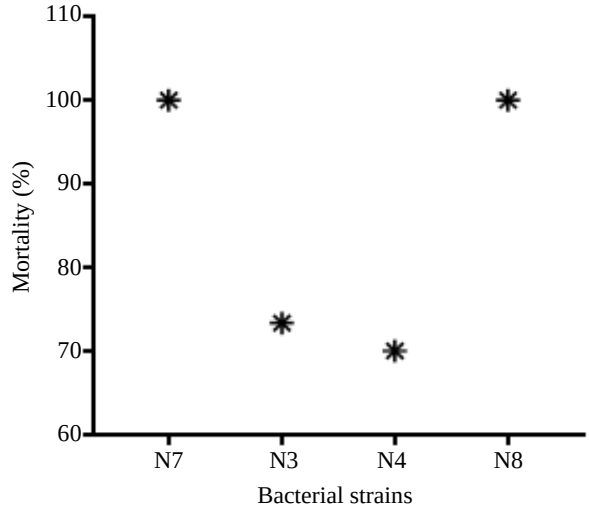

Fig. 1: Mortality caused by the bacterial strains against the third-instar larvae of *A. aegypti*. Key: N3: *Escherichia coli* N3; N4: *Escherichia coli* N4; N7: *Brevibacillus centrosporus* N7; N8: *Cytobacillus sp* N8

Discussion

Due to the public health importance of *A. aegypti* as a vector in the spread of DF globally, its control is a necessary step in the control of outbreaks due to DF. Soil is home to many microorganisms mostly bacteria that produce bioactive compounds with multiple activities (Yahya *et al*., 2021). The larvicidal activity of soil bacteria towards the larvae of *A. aegypti* is an important step in the subsequent application of the bacteria as means of biocontrol. Soil bacteria have been reported previously with toxicity to mosquito larvae (Radhika *et al*., 2011). In this study, the local bacteria isolated from the soil of the Rahat region from the holy city of Makkah demonstrate toxicity to the larvae of *A. aegypti* which could prove effective in the control of the spread of dengue virus. All the organisms identified with larvicidal activity have been reported from soil previously. Soil has been reported as a habitat for members of the genus *Escherichia*, *Brevibacillus*, and *Cytobacillus.* It is a reservoir and secondary environment for *E. coli* (NandaKafle *et al*., 2018) and the non-pathogenic strains of *E. coli* survive longer in the soil than the virulent ones due to interactions with both the living and non-living environment (Xing *et al*., 2019). Factors that support the survival and growth of *E. coli* include the ability of the soil to allow the multiplication of the bacteria based on nutrient availability (Byappanahalli and Fujioka, 1998). *B. centrosporus* is widespread and has been reported from soil occupying several niches (Hassi *et al*., 2012, Zhao *et al*., 2012). As a spore-forming and Gram-positive organism, it can survive under many conditions in the environment and colonize a range of environments including saline soils (Song *et al*., 2017). The other organism identified as *Cytobacillus species* which is related to the *Bacillus* genus are commonly found in the soil as well (Wei *et al*., 2016; Alyousif, 2022). Previously known as *Bacillus*

Firmus, several pieces of evidence have shown that this organism is distinct from members of the *Bacillus* genus and can survive in the soil for long periods, and has been reported from many types of soils (Patel and Gupta, 2020). This organism was identified in this study without the species name because the percentage similarity of the 16S rRNA gene sequence is 98% and, in most cases, the method is not much relied upon for species identification (Fox *et al*., 1992).

This use of beneficial bacteria as biocontrol agent is a strategy with many advantages (Soni and Keharia, 2021). Compounds from bacteria are natural and considered to be non-toxic, unlike their synthetic chemicals counterpart. The larvicidal activity of 100% mortality of the larvae within 24 h was achieved by *B. centrosporus* and *Cytobacillus species*. These two organisms belong to the same family and have been associated with the production of bioactive compounds with larvicidal activity against mosquitoes (Ramathilaga *et al*., 2012, Mani *et al*., 2015, Katak *et al*., 2021). The bioactive compounds produced by the organisms are effective against the larvae of a mosquito making them a suitable source of biocontrol agents of *A. aegypti* and in preventing the transmission of the dengue virus (Rajagopal *et al*., 2020). *B. centrosporus* is widely reported to produce bioactive compounds with several activities. The organism has shown antagonistic properties and bioactive compounds production relevant to biocontrol (Le Han *et al*., 2022). The potential of *B. centrosporus* as a biocontrol agent and a source of bioactive compounds has been acknowledged previously with its ability to inhibit the growth of phytopathogenic fungi and other Gram-positive bacteria (Saikia *et al*., 2011). Many of the compounds the organism produce have been shown to have beneficial activities that include antibacterial and anticancer (Samrot *et al*., 2015). It has been reported as an antifungal agent due to the production of antimicrobial peptides that is effective in the control of fungal pathogens in plants (Ahmed *et al*., 2018) and in the control of mycotoxigenic fungi (Joo *et al*., 2015). It can produce compounds that can prevent the growth of other organisms living in the same soil environment (Ray *et al*., 2020). The organism has proven to be effective in many biocontrol trial studies due to its ability to use different carbon sources and produces diverse bioactive metabolites (Ahmed *et al*., 2018, Ray *et al*., 2020; Li *et al*., 2021). *Cytobacillus species* is known to synthesize several compounds with antagonistic activity towards other organisms in the soil environment which gives it an advantage in outcompeting other organisms (Xu *et al*., 2022). The bioactive compounds produced by this organism have diverse roles including as a sustainable source of biopesticide and is one of the organisms recommended as a biocide due to the production of its bioactive compounds (Arakere *et al*., 2022) due to its nematicidal activity (D'errico *et al*., 2019) which makes the organism a potential biocontrol agent. It has been reported from Makkah in a previous study with potential antimicrobial activity (Alharbi *et al*., 2021). Other studies have reported the bioactive potential of the strain include the green synthesis of nanoparticles with antimicrobial activity (Sudarsan *et al*., 2021) and as a source of pigments (Hwang *et al*., 2022) and other high-value compounds for the management of diseases (Arun *et al*., 2022). Although larvicidal activity has not been reported previously for *E. coli*, the achievement of more than 70% mortality is significant for further investigation of the specific role of the organism in bioprospecting biocontrol agents. Many previous studies reported larvicidal activity evaluation using a similar method with the third-instar larvae of *A. aegypti* and recorded up to 100% mortality from different compounds (Amazonas Maciel Magalhães, *et al*., 2010; Dias and Moraes 2014; Rocha *et al*., 2015; Krzyzaniak *et al*., 2017). The use of bacterial suspension has also been reported previously in evaluating the insecticidal activity of soil bacteria against *A. aegypti* and A *albopictus* with mortality as high as 99% which is in agreement with the results of this study (Yooyangket, 2018).

Conclusion

In this study, we demonstrated that *B. centroporus* N7 and *Cytobacillus sp.* N8 isolated from soil have larvicidal activity against *A. aegypti* larvae which makes them potential to be applied as biocontrol agents in the control of Dengue fever. Further studies are required to understand the mechanism of action, optimize the production and characterize the larvicidal compounds produced by the bacteria. We plan to focus our subsequent research on local soil bacteria in the search for alternative biocontrol agents.

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Author Contributions

Saleh Mohammed Al-Maaqar and Naif Mohammed Alotaibi: Participated in all experiments and contribute to writing the first draft of the manuscript.

Idris Abdulrahman: Analyzed data, wrote the first draft and edit the final manuscript.

Amna A. Saddiq: Designed and coordinated sample collection process.

Turki Alshaikh: Analyzed the data and provide facilities for the experiments

Naser AL-Kenani: Coordinated and provide facilities for larvicidal activity.

Saber Abdelkader Saidi and Mohamed Mohamed Roushdy: Conceived the idea, planned, designed and coordinated the experiments.

Conflict of Interest

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

Ethics

This article is original and all materials were not published previously. The corresponding author confirms that all coauthors have read and approved the manuscript and that no ethical issues are involved.

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