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RESEARCH ARTICLE

Screening of the normal bacterial flora in the gut of Aedes aegypti Mosquito in Saudi Arabia

Running title: Gut Bacteria in Aedes aegypti Mosquito

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ABSTRACT

Aedes aegypti (Culicidae), the mosquito that causes yellow fever, is regarded as a significant disease agent vector. The current study sought to learn more about mosquito mid-gut bacteria and their impacts. Mosquito specimens were gathered in Jeddah city (Saudi Arabia), and their gut bacterial flora was then analyzed. The collected mosquitoes exhibit all the characteristic features of A. aegypti, according to a microscopic study. Utilizing the partial mitochondrial cytochrome oxidase (COI) gene analysis, molecular testing established the identity of A. aegypti as a close relative of previously recognized Aedes species submitted in the GenBank, particularly those from Kenya.[23] Unfortunately, based on the results of RT-PCR, not all of the Aedes mosquitoes under study had dengue virus (DENV) present. On nutrient agar plates, the mid-gut bacteria were isolated.[42] Gram-positive 34 isolated bacteria were identified at the molecular level using the 16S rRNA gene and were divided into the two genera Bacillus and Lysinibacillus within the family Bacillaceae.[4] To our knowledge, this study is the first to examine the normal existence of bacterial flora in the gut of DENV-free mosquitos in Saudi Arabia. It is advised to conduct more research to determine how these bacteria affect the transmission of harmful pathogens carried by mosquitoes.[5] Additionally, further research into the antibacterial and anticancer activities of metabolites extracted from the mid-gut bacteria may help in the development of unique drugs.

Keywords: Aedes aegypti; Microbiota; Molecular analysis;[0] Gram stain

INTRODUCTION

Since they are carriers of numerous pathogenic agents that cause numerous infectious diseases that can be fatal, such as malaria, yellow fever, dengue fever, Zika, and many other serious illnesses that may infect humans and other vertebrates, mosquitoes have been regarded as one of the most annoying arthropods to humans over the past century (Resh and Cardé, 2009; Kraemer et al., 2019). Because they bite, other mosquito species are seen to be annoying because of their bites (Mullen and Durden, 2009). Around the world, there are roughly 3000 species and subspecies of mosquito.

Due to its wide range and impact on public health, Aedes aegypti (A. aegypti) (Diptera, Culicidae) is a well-known mosquito (Bhatt et al., 2013; Gómez et al., 2022). As the carrier of numerous arboviruses, such as Dengue, Chikungunya, Yellow fever, and Zika virus (Brady et al., 2012; Powell, 2018; Weetman et al., 2018), it is well known for its significance in medicine.[13] Adult females are the ones who spread pathogenic agents and require blood meals for the deposition of their eggs (Ponlawat and Harrington, 2005).[48] According to the World Health Organization (WHO, 2022), it is one of the most rapidly spreading mosquito-borne viral diseases globally.

[13] One of the most important pathogenic agents transmitted by A.[0] aegypti is DENV which causes dengue disease which is the highest mortality threat due to viral infection in more than half of the world's population (Goswami et al., 2012; Betanzos-Reyes et al., 2018; Gabiane et al., 2022). In several Saudi cities, dengue fever is regarded as a severe public health issue.[24] Aedes mosquito bites are the primary method of human-to-human transmission of the dengue virus (Guha-Sapir and Schimmer, 2005). Although millions of dollars are spent on vector control programs, Aedes aegypti, the dengue virus vector, has not yet been successfully eradicated (Reiner et al., 2016; Caragata et al., 2019; Hossain et al., 2022).

Recently, researchers concentrated on learning more about the gut microbiome of mosquitoes, which is crucial to their survival as it participates in numerous physiological and metabolic processes such as blood digestion, egg development, and fecundity (Fouda et al., 2001). They acknowledged the significance of the microbiome in viral development and the mosquito susceptibility to a particular infection, which can also be employed in managing vector competence (Muturi et al., 2017). However, research on the stomach microbiome of the common

A. aegypti mosquito is scarce.[7] The current study's goal is to discover the bacterial strains found in the mid-gut of mosquitoes.[41]

MATERIALS AND METHODS

Collection of mosquitoes

All samples have been received from King Abdulaziz City for Science and Technology which were collected by black hole traps from different residential areas in Jeddah city during the spread of DENV cases in September and October of 2021. The morphological characteristics were used to identify the mosquitoes using the keys previously described by Darsie (1999), Becker et al. (2020a,b), and Alzahrani et al. (2021).[22] The specimens were preserved in 95% ethanol at 4 ºC and used for the molecular identification of mosquitoes.

Molecular identification of mosquitoes[21]

Following the manufacturer's recommendations, DNA was extracted using the QIAamp DNA Blood and tissues extraction kit (Qiagen, Germany).[44] NanoDrop ND-1000 Spectrophotometer (Thermofisher Scientific Inc, USA) was used to calculate the quantity and purity of DNA.[4] PCR was carried out to amplify the mitochondrial Cytochrome c oxidase subunit I (COI) gene using the primer set of COI-LC01490 F (5'-GGTCAACAAATCCATAAAGATATTGG-3') and /COI-HC02198 R (5' -TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) with the recommended thermocycling profile. All PCR products were tested on a 1.2%[6] agarose gel (Sigma-Aldrich, Missouri, USA) in 1×TAE buffer and post-stained with ethidium bromide against the GeneRuler 100 bp (Fermentas, Lithuania) and then examined using the image analysis system.[20] The amplified PCR products were sent to Macrogen Inc.'s DNA sequencing unit (Seoul, South Korea).[22] Using NCBI's BLAST program, sequences were found based on the partial COI gene. Using MEGA version 7.0,[20] a phylogenetic tree was constructed based on the Maximum Likelihood method with 1000 bootstrap replicates and the appropriate substitution models.

DENV Detection using Reverse-transcription PCR (RT-PCR)[30]

Following the protocol's instructions, viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germany).[30] RT- PCR was used for detecting and typing dengue viruses by

using the dengue virus universal primers of D1_F (5'-TCAATATGCTGAAACGCGCGAGAAACCG-3') and D2_R (5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3') (Ranjan et al., 2016) with the recommended thermocycling profile.[13] Then, a further serotype-specific PCR combining primer D1 with one of the following internal primers: TS1, TS2, TS3, or TS4 mentioned previously in Gupta et al. (2012) for typing.[22] The authority of public health in Saudi Arabia generously provided the positive samples after receiving KACST (IRB#20006) ethical approval. Gel electrophoresis has been performed as mentioned above.[23]

Bacterial flora screening

A total of 50 mosquito specimens were dried and then ground while adding 150µl of sterilized PBS.[5] Subsequently, the supernatant fluid was transferred into pre-prepared Petri dishes containing nutrient agar medium (Thermo Scientific, Oxoid, USA) as non-selective media and then incubated for 24 hrs at 33°C.[5] The growing colonies were picked up and streaked individually over NA plates (Sanders, 2012) and then incubated for 24 hrs at 37°C.[73] After that, the second round of purification was made following incubation at 37°C for 24 hrs.[91] On the next day, the single colonies were inoculated in labeled tubes containing 5 ml Nutrient broth using a sterile loop, and then tubes were incubated at 37°C for 24 hrs.[12]

Gram stain

The culture was spread with an inoculation loop to a thin film on a microscope slide and left to dry then fixed with heat by passing it over the flame 2-3 times. The staining process was started with a Crystal violet stain and then poured off with dist. H_2O. After that, gram iodine solution was used to cover the smear for 1 min followed by rinsing with dist. H_2O. A few drops of decolorizer (ethanol 95%) are added followed by rinsing with dist. H_2O. Smear was counterstained with Safranin solution for 1 min then washed off with dist. H_2O. The slide was examined under an Olympus B×61 microscope (Tokyo, Japan).

Molecular identification of bacterial flora

After 24 hrs of bacteria-broth incubation, the broth was divided into two equal volumes to extract the bacterial DNA of Gram-positive and negative separately.[5] Genomic DNA was extracted using the QIAamp DNA Blood and tissues extraction kit (Qiagen, Germany) using the recommended

protocol.[13] A different step in DNA extraction for Gram-positive bacteria is the need to lysis the cell wall, so it must be ensured that ethanol has not been added to AL Buffer.[6] PCR was performed to amplify the partial 16S rRNA gene from all the bacterial isolates by using primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 149R (5′-GGTTACCTTGTTACGACTT-3′) (De Lillo et al., 2006) with the recommended thermocycling profile.[22] Gel electrophoresis has been performed as described previously.[20] PCR products were sent to the DNA sequencing unit in Macrogen Inc. (Seoul, South Korea). Sequences were identified, and the phylogenetic tree was constructed using MEGA ver. 7.0 based on the Neighbor-Joining method with 1000 bootstrap replicates under appropriate substitution models.

RESULTS

Morphological species identification

The targeted mosquito is the small to medium-sized Aedes aegypti (Figure 1), which has a distinctive silvery-white scaling pattern on its head, scutum, legs, and abdomen. The distinctive pattern of the species is formed by the lyre-shaped pattern of white scales on the scutum. A pair of membranous wings with narrow scales on all the veins and toothed claws of the fore- and mid-tarsi. Piercing and sucking proboscis is long dark scaled, the palps are shorter than proboscis in females while they are the same length in males. Sparsely hairy antennae in females but the males have plumose antennae.

[32]Molecular identification of mosquito species

The partial sequences of the COI gene were amplified via PCR reaction.[30] PCR products were deposited in GenBank under the accession numbers OQ023868, OQ023869, OQ023969, OQ024016, OQ024046, OQ024067, OQ024185, and OQ024190. One family (Culicidae) was identified for the recovered mosquito species. All the GenBank entries that matched our COI sequence under the highly stringent criteria (99.71-99.13% identity, 100% query coverage, and E-value 0.0[18]) were assigned to the species of Aedes.[2] In ML analysis (Figure 2), the taxa of Aedes were grouped in a distinct clade with high bootstrap values of 100.[79] Dendrograms confirmed the association of our specimen with the Aedes group, with special reference to the previously deposited sequences in the GenBank for A. aegypti (MW255873, MW255872 MH538701, MW255845, and KU186990) that were collected from Kenya.[2]

DENV Detection

All examined mosquito specimens had failed to demonstrate the presence of dengue virus in comparison to the positive control which contains the dengue virus, all samples were considered negative for dengue (Figure 3).

Bacterial flora mosquitoes

After 24 hrs of incubation, different morphological colonies appeared (Figure 4).[24] 34 isolates were classified according to their reactivity to the Gram stain, where the Gram-positive group was detected as shown in Table (1), and appeared in violet under the microscope.[0] The partial 16S rRNA gene was targeted and amplified using PCR to identify the bacterial flora (Figure 5).[4] PCR products for nine Gram-positive bacteria were isolated and deposited in the GenBank database under the accession numbers OQ071618 - OQ071627. One family (Bacillaceae) was identified for all bacterial flora isolated in this study. All sequences were matched with a dataset on GenBank with seven species embedded within the genus Bacillus and two sequences in the genus Lysinibacillus. In ML analyses (Figure 6), the taxa of Bacillus and Lysinibacillus were closely related to each other with high bootstrap values.

DISCUSSION

Mosquitoes are tiny arthropod organisms that have destroyed global health since ancient times (Mullen and Durden, 2009). According to Pise et al. (2022), mosquitoes are considered a severe hazard because they propagate deadly diseases that kill billions of people each year. One of the most important species that contributed to tampering with global health scales is A.[0] aegypti which is reported as a vector of many arboviruses diseases such as Dengue, Chikungunya, Yellow fever, and Zika virus (Resh and Cardé, 2009; Brady et al., 2012; Bhatt et al., 2013; Sofi et al., 2022).

In this study, both morphological and molecular techniques were used to identify the collected mosquito species to obtain our aim which is screening the bacterial normal flora present in A. aegypti gut.[27] Based on the morphological characteristics of the gathered mosquitoes which include small to the medium-sized dark body with unique silvery white scaling patterns of body parts, pair of membranous wings, piercing and sucking proboscis where the palps are shorter than proboscis in females, and sparsely hairy antennae in females, all of these features proved

that the collected species related to A. aegypti. The same morphological characteristics were described by previous studies by Darsie (1999), Becker et al. (2020a,b), and Alzahrani et al. (2021).[32]

Moreover, molecular analysis based on the partial COI gene sequences confirms the morphological identification of A. aegypti specimens. This is inconsistent with Kumar et al. (2007) and Alajmi et al.[32] (2021) reported that the COI gene is considered an effective taxonomic gene used to identify insects in general and insects of complex species. All obtained sequences were identical and showed 99.71-99.13% identity with different strains of the A. aegypti in the GenBank which was collected from Kenya, which is compatible with Ali et al. (2016) proved that A.[0] aegypti entered Saudi Arabia through African pilgrims and continued to circulate in the western region of Saudi Arabia. Genetic diversity within closely related mosquito species is also recorded herein, this agreed with El-Badry et al. (2016), Khater et al. (2021), and Noureldin et al. (2022) showed the same point between local mosquito species and their phylogeny.

[75]Reverse Transcription PCR testing on all mosquitoes used in this investigation produced negative Dengue virus results.[0] This result is consistent with the most recent statistical report announced by the Ministry of Health (MOH) in Saudi Arabia (2019), which indicated a decline in the number of dengue cases recorded (Melebari et al., 2021).[1] However, previous studies reported that the emergence of dengue fever disease in Saudi Arabia synchronized with the presence of A.[1] aegypti mosquitoes, as it has been the vector of the disease since 1994 in the Jeddah region (Fakeeh et al., 2001; Brady et al., 2012; Alhaeli et al., 2016; Al Sheikh et al., 2017; Organji et al., 2017).[3] The negative PCR results of DENV in the collected mosquitoes could be attributed to two factors, firstly is the strict control measures launched by the Saudi MOH, and secondly is the lockdown all over the Kingdom during Corona epidemic may be a factor in decreasing the spreading of Dengue, which is consistent with Lemey et al.[26] (2020) and Khan et al.[57] (2022) as they fail to collect mosquitoes infected with Dengue during the coronavirus disease 2019 (COVID-19) pandemic period.

[34]Gut microbiota is represented as the most famous biota that has been demonstrated in several insect species (Dillon and Dillon, 2004).[3] Our findings based on culture-dependent assays to screen the microbiota associated with A.[84] aegypti's gut showed that all bacterial isolates from collected mosquitoes were Gram-positive, belonging to the Bacillaceae family.[28] According to

Valiente Moro et al. (2013), mosquito gut microbiota is predominantly obtained through vertical transition or environmental acquisition.[9] Based on the partial sequencing of the 16S rRNA gene, which has already been reported from the midgut of Aedes as well as other mosquito species (Pidiyar et al., 2004; Rani et al., 2009; Yadav et al., 2015), the most prevalent bacterial genera in our data were the Bacillus rather than Lysinibacillus.[51] This is consistent with the findings of De Lillo et al. (2006), Sogin et al., (2006), and Geng et al.[33] (2022), who indicated that 16S rRNA gene sequencing technology is the most widely used approach for detecting insect gut microbiota.

[27]Moreover, this study indicates that the microbial community associated with A.[1] aegypti samples is characterized by a limited bacterial diversity which agreed with Zouache et al. (2011) reported few genera in A. aegypti either by culturing or by PCR-based methods.[1] In agreement with our study, previous studies have been conducted to manipulate gut microbiota to control the spreading of many diseases (Moreira et al., 2009; Osei-Poku et al., 2012). This confirms our finding for the negative results of DENV in the examined mosquitos. According to Dong et al. (2009), Rodrigues et al. (2010), Apte-Deshpande et al. (2014), and Thongsripong et al. (2018), the mosquito's susceptibility to several pathogenic taxa is influenced by its microbiome. Additionally, they might prevent viruses like DENV and chikungunya from growing in mosquito vectors (Joyce et al., 2011; Ranasinghe et al., 2021).

[80]The limitation of this study is the use of nutrient agar to isolate the mid-gut bacteria which is biased since some bacteria require specific culture media and others can not be cultivated.[14] The use of the metagenomics approach in future studies will give a complete profile of the mid-gut microbiome.

CONCLUSION

In this study, the gut microbiota structure of A.[80] aegypti mosquitoes collected in Jeddah city, Saudi Arabia, is described for the first time. Moreover, the gut microbiota's functions of A.[1] aegypti remains poorly understood and more studies are still needed to understand the interaction of midgut microbiota and their role in A. aegypti susceptibility to infection with pathogenic agents, particularly Dengue virus.[29]

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[1]

Conflict of interest

The author(s) declare that they have no conflict of interest regarding the content of this article.

[26]

Data Availability Statement

All the datasets generated or analyzed during this study are included in this published article.

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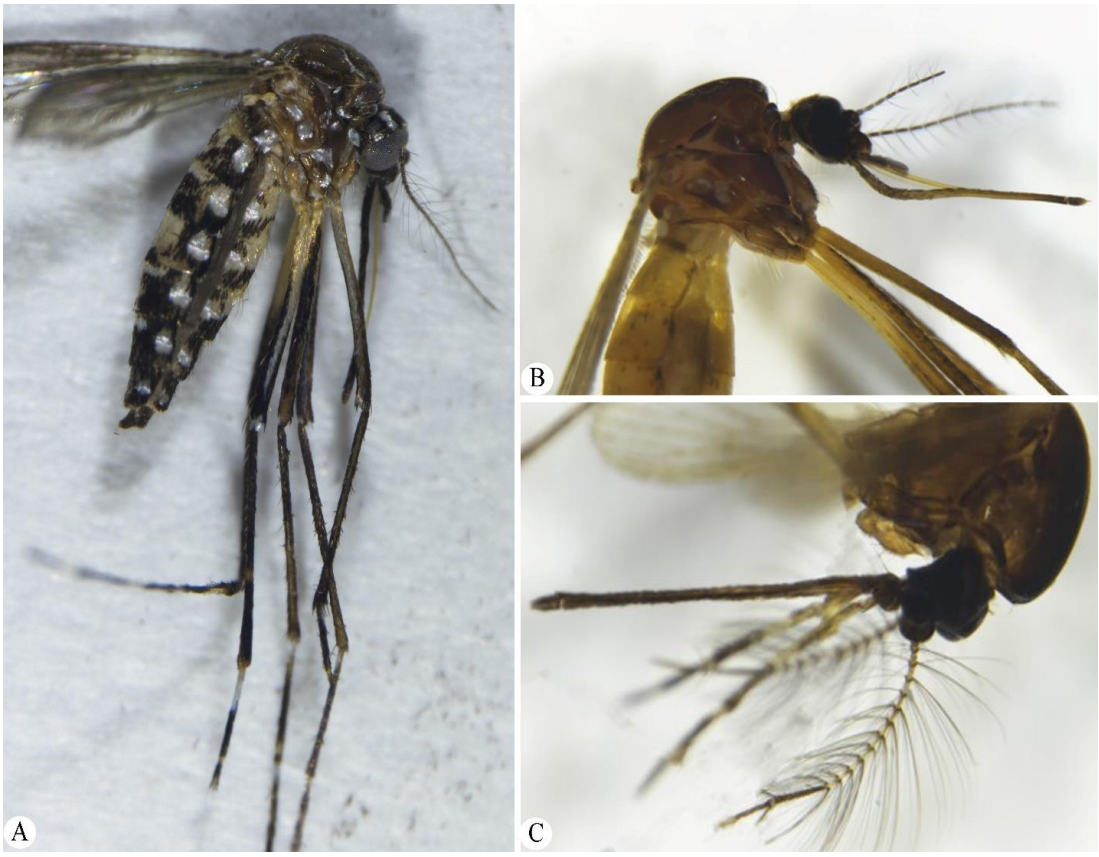

Figure 1. Micrograph of the adult of Aedes aegypti. (A) whole body. (B) male head. (C) female head.

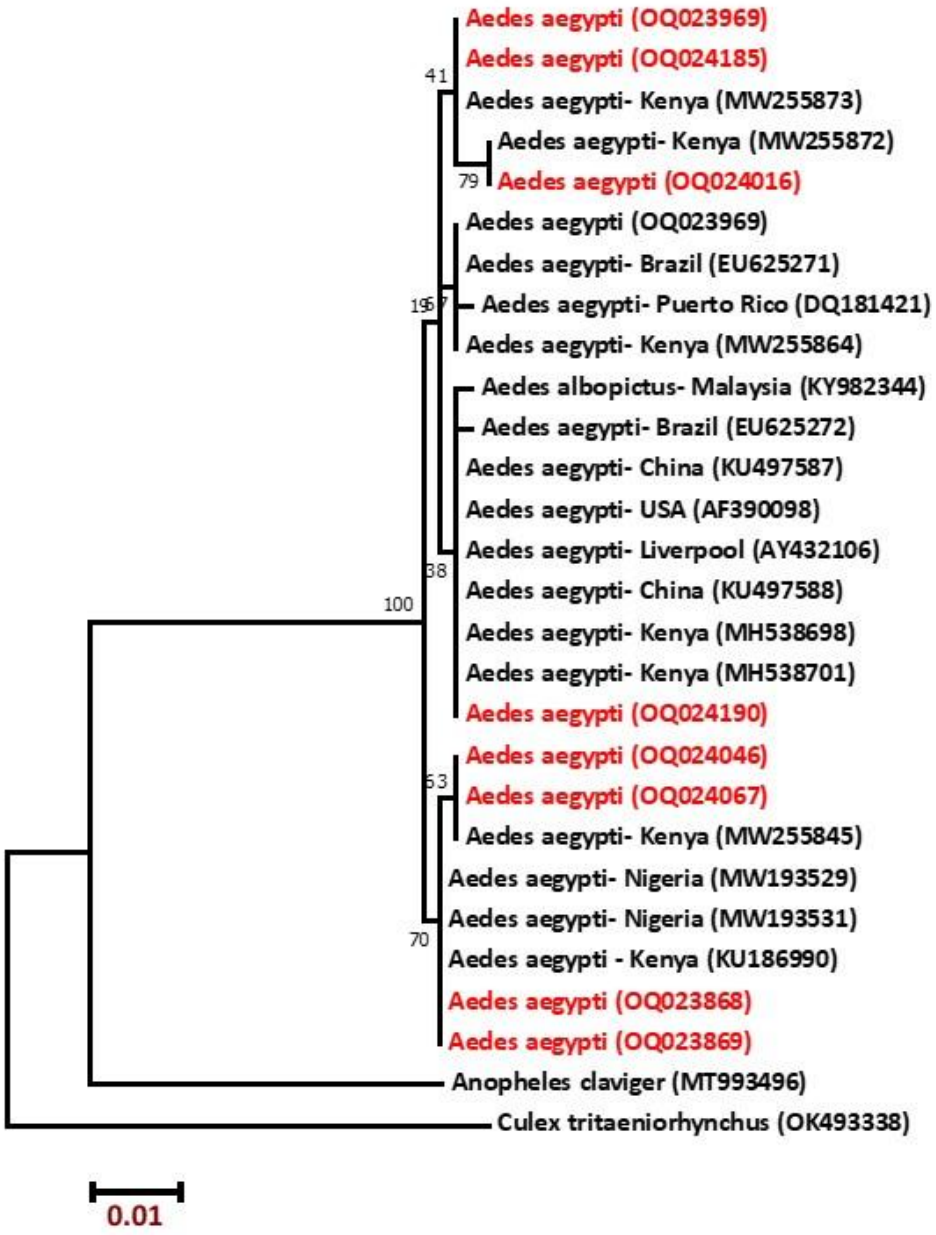

Figure 2. Molecular phylogenetic analysis of mt COI gene of mosquito specimens by Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-1147.40) is shown.

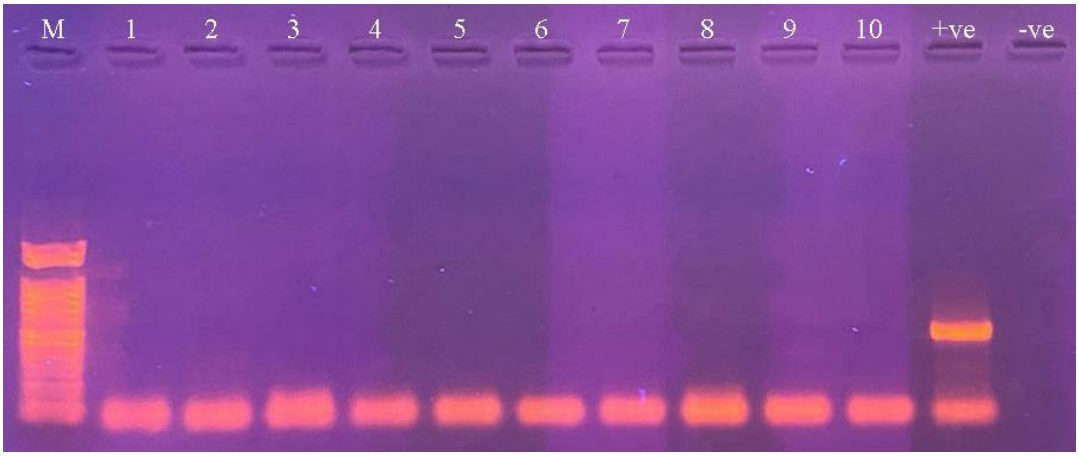

Figure 3. Agarose gel showing PCR products for the detection of dengue virus in different mosquito samples (Lanes 1-10). M: DNA marker, +ve: positive control, and -ve: negative control.

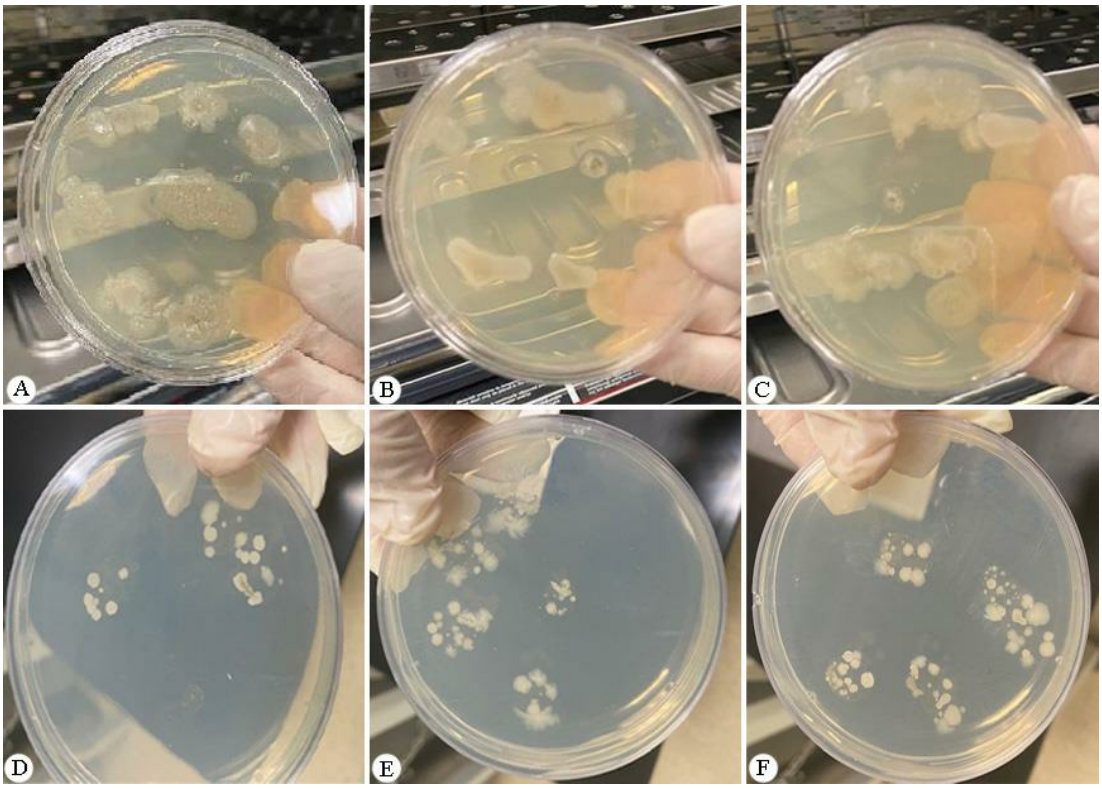

Figure 4. Colonies of mid-gut bacteria isolated from mosquitoes on NA plates.

Table 1. The isolated bacterial strains and their accession numbers in GenBank

Bacterial strain	Family	Accession numbers	Gram-positive/negative
Bacillus amyloliquefaciens	Bacillaceae	OQ071618	Gram-positive
Bacillus australimaris	Bacillaceae	OQ071625	Gram-positive
Bacillus paramycoides	Bacillaceae	OQ071621	Gram-positive
Bacillus cereus	Bacillaceae	OQ071622	Gram-positive
Bacillus aryabhattai	Bacillaceae	OQ071620	Gram-positive
Lysinibacillus macroides	Bacillaceae	OQ071627	Gram-positive
Lysinibacillus xylanilyticus	Bacillaceae	OQ071626	Gram-positive
Bacillus vallismortis	Bacillaceae	OQ071624	Gram-positive
Bacillus velezensis	Bacillaceae	OQ071624	Gram-positive

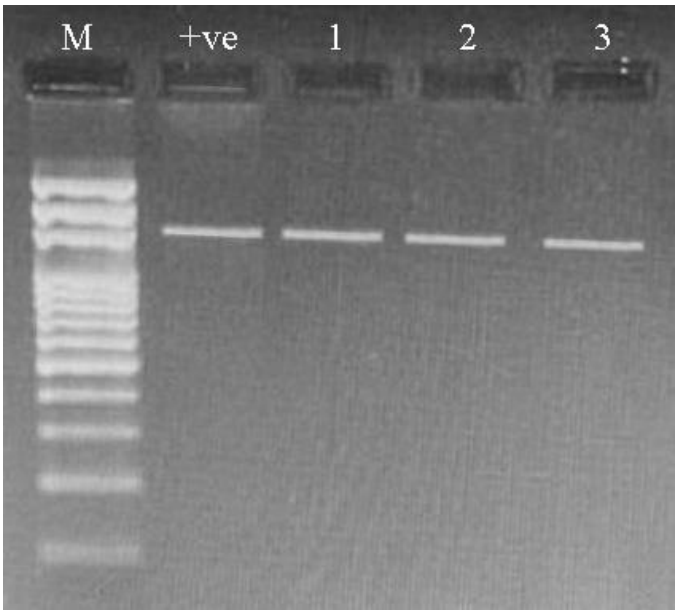

Figure 5. Agarose gel after loading the PCR products of 16S rRNA gene (Lanes 1-3). M: DNA marker, + ve positive control.

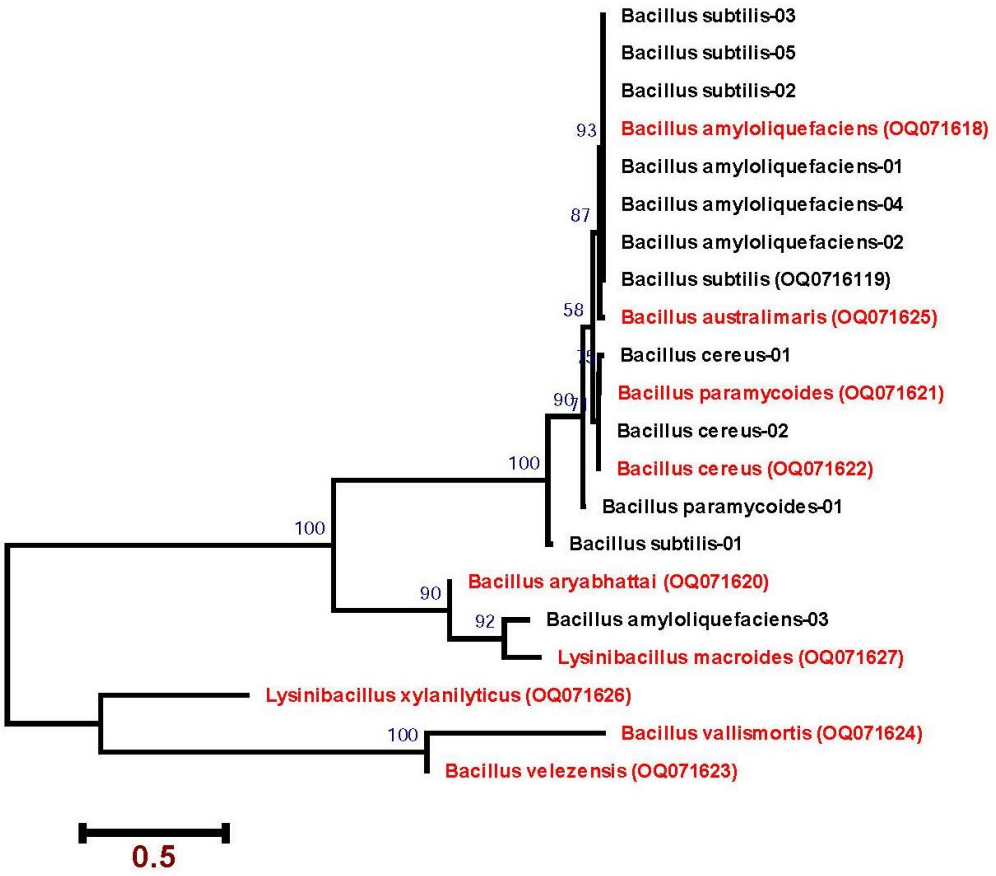

Figure 6. Neighbor-Joining tree of the 16s rRNA gene for bacterial flora. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches.