

Short Communication:

Bacillus endolithicus and *Bacillus paramycoides*: New isolates from housefly *Musca domestica* in Saudi Arabia

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Abstract. *El-ghwas DE, Al-Nasser AS, Al-Sheikhy AA. 2021. Short Communication: Bacillus endolithicus and Bacillus paramycoides: New Isolates from housefly Musca domestica in Saudi Arabia. Biodiversitas 22: 4209-4215.* Housefly “*Musca domestica*” Linnaeus is a common insect widely distributed all over the world. It is one of the domestic insects found associated with humans and animals. The present study investigated the bacterial diversity associated with *Musca domestica* samples collected from different places such as food courts and trash cans near fast-food restaurants in Makkah Province from October 2019 to December 2019. Eighteen pure isolates of bacterial strains were isolated and identified by Gram staining. Most of the bacterial isolates were Gram-positive except for two species, which were Gram-negative. The VITEK system was used to identify randomly isolate no. 5, 7, 17, 18, and 29. The results revealed that they belonged to the genus *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp., and *Micrococcus* sp. respectively. Due to the most isolated strains were *Bacillus* sp., so 16S RNA was used to genetically identify novel isolated strains 5 and 7. Isolate no. 5 showed the highest similarity (99 %) with *Bacillus endolithicus*, and isolate no. 7 showed the highest similarity (99 %) with *Bacillus paramycoides*. This is the first record of *Bacillus endolithicus* and *Bacillus paramycoides* to be isolated from the house fly *Musca domestica* L.

Keywords: *Bacillus endolithicus*, *Bacillus paramycoides*, Identification, Isolation, *Musca domestica*

INTRODUCTION

The common housefly *Musca domestica* Linnaeus can be found in human residences, hospitals, food processing factories, food markets, restaurants, poultry, livestock farms, and different domestic areas or buildings. It is a nuisance to humans, poultry, cattle, and other farm animals (Zahn and Gerry 2020). As a result of their lifestyle and behavior, flies are considered mechanical and biological vectors of pathogenic microbes (Kobayashi et al. 2020). One of the most dangerous features of the housefly is its popularity in food. Since they are attracted to the decaying plants and animal organisms, flies come in contact with ecosystems, litter, and animal waste (Park et al. 2019). Areas with a lot of manure or compost, such as kennels and areas without human sanitation, are major areas for the increase of houseflies and simultaneous detection of bacteria (Meerburg et al. 2007). However, the efficacy and distribution of species in animal droppings or manure vary greatly between sites and across host forces (Himathongkham et al. 1999). Therefore, flies may come in contact with and swallow highly variable amounts of bacteria during their contact with animal waste (Ahmad et al. 2011). Sulaiman et al. (2000) isolated various pathogenic organisms from the gut of flies *M. domestica* including *Salmonella* and *Shigella* species. Flies are usually found in nasty places and later transmit these bacteria into food and water.

Furthermore, Park et al. (2019) investigated the internal and external microbiota of 400 samples of house flies from three different environments (cow farms, homes, and hospitals) in Belgium and Rwanda. They reported that whatever the country or habitat, house flies ported a high potential of various bacterial microbiota. They declared that the bacterial communities on the external body were much more diverse than the internal populations from the intestinal gut. Moreover, Rosef and Kapperud (1983) isolated 161 fragments of *Campylobacter fetus* subsp. *jejuni* from domestic flies. They found that 50.7% and 43.2% of their carriers were found in poultry and pig farms, respectively. Therefore, they concluded that flies play a key role in transmitting *Campylobacter* disease to humans by transmitting the virus from animals to humans. In addition, Rajendhran and Selvaraj (2003) identified fifteen species of pathogens in the mouth, and the external body of flies *M. domestica* collected from three different natural areas of Madurai, India. The five species most closely associated with flies were *Streptococcus* sp., *Staphylococcus aureus*, *Shigella* sp., *Escherichia coli*, and *Salmonella* sp.

Moreover, Khamesipour et al. (2018) isolated 130 pathogenic organisms from the houseflies in which bacteria were the most frequent. Also, Haeidari et al. (2021) studied the bacterial diversity isolated from the external surface of house flies collected from the hospitals in Yazd Province of Iran. They declared the presence of three species responsible for nosocomial infections such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

The places from where the flies are collected are related to the microorganisms transmitted by these insects. Places such as hospitals and animal farms where antibiotic and growth stimulators are applied extensively showed flies carrying antimicrobial-resistant microorganisms (Nassiri et al. 2015; Nazari et al. 2017).

The kind and number of microorganisms that flies carry are closely related to the presence of the same organisms in the excreta and other wastes where flies develop and feed (Nichols 2005). An increasing problem in hospitals and other health care facilities is the involvement of houseflies in transmitting life-threatening antibiotic-resistant bacteria (Boulesteix et al. 2005; Macovei and Zurek 2006). Moreover, Heiden et al. 2020 investigated the bacterial fauna from 42 flies collected from a tertiary Rwandan hospital. They revealed that 48% (20/42) of the houseflies ported antibiotic-resistant bacteria and that all the strains were phenotypically multidrug-resistant (MRGN), including *E. coli* pathogenic lineage ST131, indicating the vital role of houseflies in spreading highly potent pathogens in medical locations and elsewhere. The present study aimed to investigate the bacterial diversity associated with housefly *Musca domestica* collected from and around restaurants and food court garbage to shed light on the importance of housefly control and maintaining a hygienic atmosphere to prevent the spread of diseases by this insect pest.

MATERIALS AND METHODS

Collection of housefly samples

Up to 20 adult houseflies, *Musca domestica* Linnaeus were randomly collected from October 2019 till December 2019 using an insect sweep net from various locations in Makkah Province, Saudi Arabia including food courts and trash cans near fast-food restaurants. Individual samples were placed in sterile test tubes and transported to the laboratory of the University of Jeddah for further analysis.

Isolation and purification of bacterial strains

All the media were prepared in an Erlenmeyer flask as directed by the manufacturer. Nutrient agar and Blood agar base medium were used as the media for the experiment. The media were autoclaved at 121°C for 15 minutes to sterilize them. Housefly samples were first destroyed by freezing them for 15 minutes at 4°C. The isolation of bacterial strains from the body of houseflies was then done using two methods: the first was carried out according to Nwankwo et al. (2019) with some modifications, in which 10 samples of houseflies were washed twice with distilled water for 1 minute each time and then soaked in 0.85 percent saline so that the bacteria could be isolated. Under aseptic conditions, the yield solution was transferred to nutrient and blood agar plates, spread with a glass spreader, and incubated for 48 to 72 hours at 35-37°C. The second approach was modified from that of Kassiri et al. (2012), in which 10 housefly samples were cleaned in 0.85% saline solution; then different body parts such as legs, antenna, wings, and head were cut and transferred to the surface of

nutrient agar and blood agar plates under aseptic conditions and incubated for 48 to 72 hours at 35-37°C. To ensure purity, the pure colonies were restreaked several times onto the surface of an agar plate on an isolation medium after the incubation period. Pure isolates were subcultured on nutrient agar medium slants and held at 4-5°C for further investigation (Atlas 1993).

Identification of the most potent bacterial isolates

The morphological characteristics of the cultured bacterial isolates were used to identify them, including colony morphology and Gram staining, as defined by (Baker 1967). The bacterial isolates were then classified using Bergey's Manual of Determinative Bacteriology 2005, 2009 and Automated Identification Systems (VITEK) based on their biochemical characteristics.

Genetic identification of bacterial strains no. 5 and 7

Individual colonies from randomly selected isolates 5 and 7 were inoculated in 3 mL of LB media and grown in a shaker bath at 37°C for 16 hours to confirm the identification of bacteria. DNeasy columns were used to remove DNA from each strain (QIAgen). The following primer collection was used to amplify the 16S RNA gene: SPIR F: 5' GAGTTTGATCCTGGCTCAG 3' and SPIR R: 5' AGAAAGGAGGTGATCAGCC 3' (Rainey et al. 1992). The amplification was carried out with 2 mM MgCl₂, 200 mM dNTPs, 0.4 M of each primer, 1.25 units of Taq polymerase (Invitrogen), and a cycle programmed at 95°C/3 min, accompanied by 30 cycles of 95°C/30 s, 50°C/1 min, and 72°C/2 min, with a final extension of 10 minutes at 72°C. The DNA band was cleaned from the agarose with a QIAquick Gel Extraction kit (QIAgen) and cloned into DH5- competent cells in a pGEM-T Simple vector (Promega) (Invitrogen). QIAprep Spin Miniprep kit was used to purify the plasmids (QIAgen). Big Dye Terminator Sequencing kit was used to sequence the inserted DNA using the vector primers T7 and SP6 (Applied Biosystems). Ethanol precipitation was used to extract the unincorporated labeled nucleotides, which were then sent to Sigma Company for processing. In our lab, the Sequencher software was used to analyze, edit, and align the resulting electropherograms (Gene Codes Corporation). BLASTn ([http:// www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)) was used to compare nucleotide sequences to a nucleotide database (Clark et al. 1974).

RESULTS AND DISCUSSION

After 24 to 72 hours of incubation at 35-37°C, 90 % of the Petri dishes contained bacterial growth while 10 % of the samples did not contain bacterial growth; therefore, those samples were excluded, as illustrated in Figure 1. Finally, 18 pure isolate strains were isolated. Nutrient agar medium, as well as blood agar medium, gave the same type of bacterial growth. Thus, the pure bacterial cultures were plated and maintained on the nutrient agar medium for further study.

Identification of isolated bacterial strains

Morphological characteristic

The 18 bacterial isolates were identified to genus level by colony morphology, texture, and Gram staining according to Bergy's manual of systematic bacteriology 2005, 2009. As illustrated in Table 1, all the bacterial isolates were classified as Gram-positive except isolates no.18 and 20, which were Gram-negative. The morphology of the colonies varied in color, size, and mucus formation. The cell shapes obtained were cocci, monococci, pairs, and rod in Gram-positive samples and rod shape in Gram-negative samples. Finally, the result demonstrated that the bacteria isolated from houseflies belong to four genera which are *Bacillus* sp. (14), *Pseudomonas* sp. (2), *Micrococcus* sp. (1), and *Staphylococcus* sp. (1). Most of the bacteria isolated were Gram-positive except for a few which were Gram-negative. Also, most isolates belonged to *Bacillus* sp.

Automated identification systems (VITEK)

To confirm the genus level of bacterial isolates under study, Automated Identification Systems (VITEK) was used. *Bacillus* sp. Isolate no. 5, 7, 17, 18, and 29 were chosen randomly for the confirmation process. The results indicated that isolate no. 5 and 7 were Gram-positive, rod-shaped, and the colony was spherical. On the other hand, isolate no. 17 and 29 were Gram-positive and cocci, while isolate No. 18 was Gram-negative and rod-shaped. Also, the results proved that isolate no. 5 and 7 belonged to the genus *Bacillus* sp., isolate no. 17 belonged to *Staphylococcus* sp., isolate no. 29 belonged to *Micrococcus* sp. and isolate no. 18 was *Pseudomonas* sp.

Genetic identification of most potent bacterial strain

Molecular analysis of 16S RNA was used as the confirmation test with the most potent bacterial isolates 5 and 7, which were widely distributed. The 16S rRNA was amplified by PCR using universal forward and reverse primers. The DNA of the two isolates 5 and 7, was then isolated and purified from the agarose gel and sequenced on an automated sequencer. The sequence data of the isolates were then analyzed by comparison with its GenBank database. The obtained results revealed that the sequence of isolate no. 5 showed the highest similarity (99 %) with *Bacillus endolithicus* as illustrated in Figure 2. On the other hand, the sequence of isolate no. 7 showed the highest similarity (99 %) with *Bacillus paramycoides*, as illustrated in Figure 3.

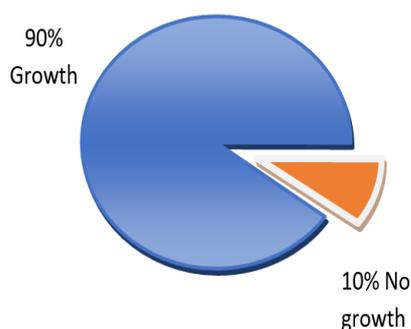


Figure 1. Infection percentage in 20 samples of *Musca domestica*
Table 1. Morphological characteristic and Gram stain of bacterial isolates

Sample no.	Gram stain	Colony color and cell shape
1	+	White/ cocci
2	+	White/ rod
3	+	White/rod
4	+	White/rod
5	+	yellow/rod
7	+	White mucus/rod
10	+	White/rod
11	+	White/rod
12	+	White/rod
17	+	Smooth, glossy, opaque yellow/Staphylococcus
18	-	White hairy/rod
20	-	Yellow/rod
21	+	White/rod
28	+	White Creamy/rod
29	+	Yellow, smooth/cocci
40-(10)	+	White/rod
40-(11)	+	White/rod
41-(12)	+	White/cocci

Discussion

Houseflies transmit microorganisms in homes, hospitals, and farms. They spread these microorganisms when they sit on pollutants for ovipositing their eggs, where various parts of their body (legs, parts of the mouth, and wings) get contaminated with various pathogenic microorganisms (Bahrndorff et al. 2017; Manandhar and Gokhale 2017). These reports agree with the findings of the present study, in which different strains of the virus were isolated from the outside and various body parts of sample houseflies that were collected (n = 20). The presence of these bacterial species may be related to the various places from where the samples of houseflies (food courts, cans of garbage in fast-food restaurants in the province of Makkah) were collected, which could be loaded with strong bacteria.

The separation process was composed of two solutions based on the surface of the outer body of the housefly *M. Domestica* and parts of the mouth, legs, and wings. After the discovery of bacterial classification by morphological and gram stain technique, the results showed that the isolated bacteria belonged to the 4th generation: *Bacillus* sp. (14), *Pseudomonas* sp. (2), *Micrococcus* sp. (1), and *Staphylococcus* sp. (1). Most isolated bacteria were Gram-positive except for a few which were Gram-negative. Moreover, most of the separated ones belonged to *Bacillus* sp.

On the other hand, the identification to genus level was done by the biochemical tested automated identification systems (VITEK). Isolate no. 5, 7, 18, 17, and 29 were randomly selected for the confirmation process. The results proved that isolates 5, 7, 17, 18, and 29 were *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp., and *Micrococcus* sp., respectively.

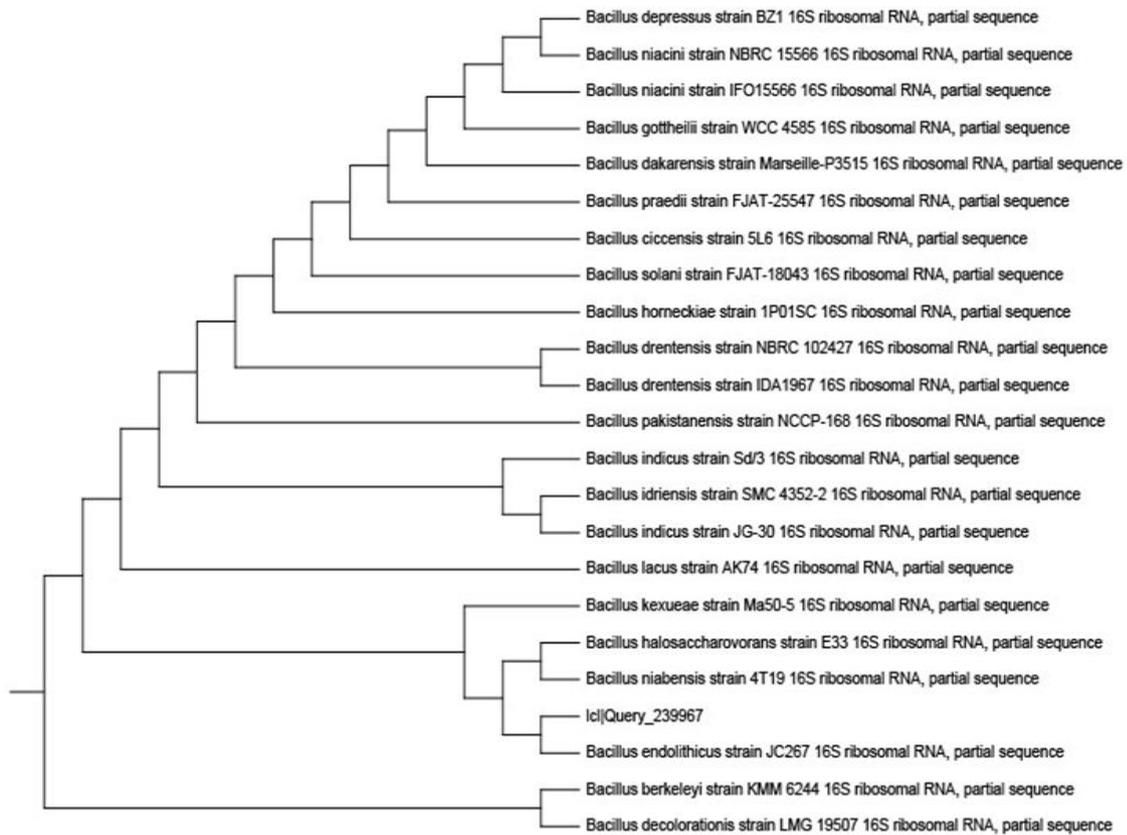


Figure 2. The phylogenetic tree of isolate no. 5 (*Bacillus endolithicus*) and other *Bacillus* sp. constructed using the neighbor-joining method

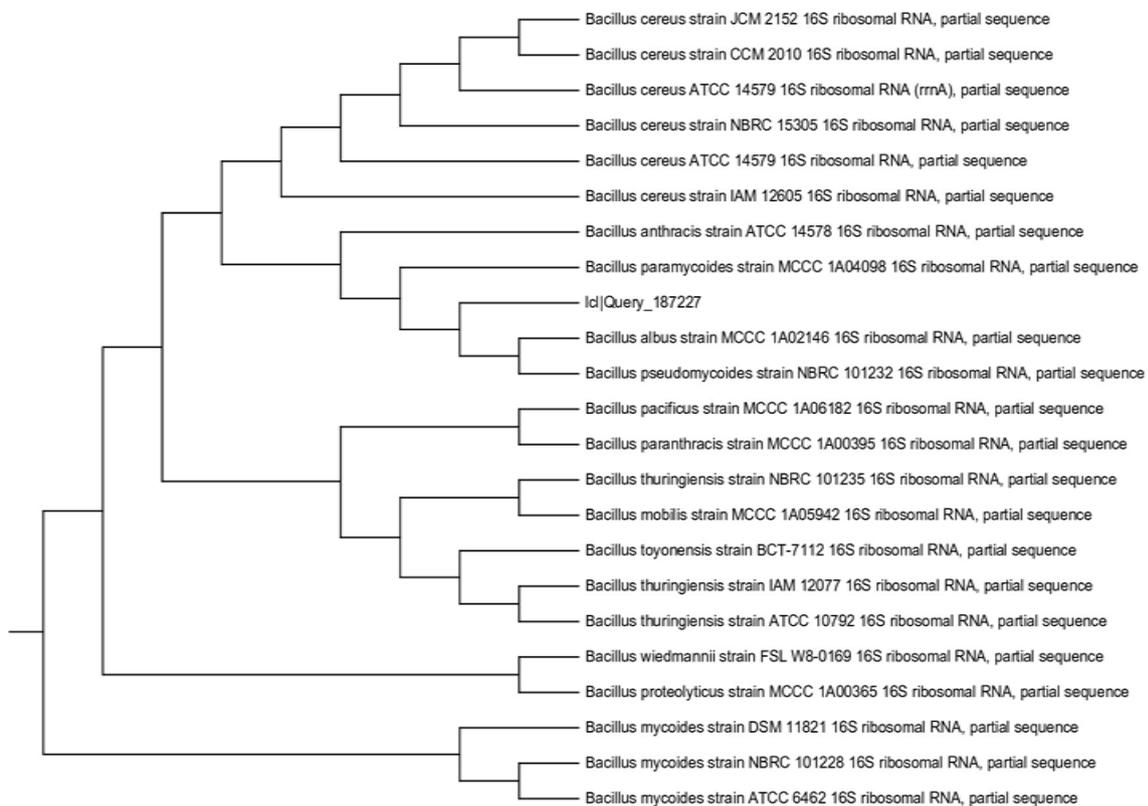


Figure 3. The phylogenetic tree of isolate no. 7 (*Bacillus paramycooides*) and other *Bacillus* sp. constructed using the neighbor-joining method

In addition, the molecular analysis of 16S RNA was used as the confirmation test for the highly potent bacterial isolates 5 and 7, which were widely distributed. The results obtained showed that isolate no. 5 showed the highest similarity (99%) with *Bacillus endolithicus*, while isolate no. 7 showed the highest similarity (99%) with *Bacillus paramycooides*. This is the first record of *Bacillus endolithicus* and *Bacillus paramycooides* obtained from the housefly *Musca domestica* L. The same methods of classification were used in previous studies (Songe et al. 2017; Ibrahim 2018; Khamesipour et al. 2018; Neupane et al. 2019; Akter et al. 2020).

Khamesipour et al. (2018) reported that the bacteria were significantly isolated from flies as they adhere to different parts of the body (mouth, wings, and legs). Förster et al. (2007) found *Bacillus* spp. on the surface of the body of houseflies. In addition, Nazni et al. (2005) recorded *Bacillus* sp., *Acinetobacter* sp., *Proteus* sp., and *Enterobacter* sp. from the surface of the body of *M. domestica*. On the other hand, Songe et al. (2017) discovered the most common infectious species of *Escherichia coli* from domestic houseflies, *Vibrio cholera* and *Bacillus anthracis*. Also, Ibrahim (2018) observed that 23 species of bacteria isolated from the surface of the body and intestines of houseflies were Gram-negative bacteria (*Escherichia coli*, *Salmonella* sp., *Pseudomonas* sp., *Shigella* sp., and *Klebsiella* sp.), while seven species were Gram-positive bacteria. (*Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp.). Also, it was reported that the majority of species isolated from houseflies collected from hospital areas were 78.6% *Staphylococcus aureus*, 66.4% *Salmonella* spp., and 51.4% *Escherichia coli*. Heiden et al. (2020) recorded many drug-resistant bacteria in houseflies, including *E. coli* pathogenic offspring ST131.

Current research has confirmed the ability of houseflies to control and transmit a wide variety of germs, such as *Pseudomonas*, *Bacillus*, *Micrococcus*, and *Staphylococcus*. Therefore, studies should be carried out on how to prevent houseflies from spreading infectious diseases.

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