Isolation and identification of pathogenic biofilm-forming bacteria invading diabetic wounds

Mohamed Salah[1*], Gamal Badr [2], Helal F Hetta[3], Walaa A Khalifa[4], Ahmed A. Shoreit[1]

[1]Botany and Microbiology Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt.

[2]Zoology Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt.

[3]Medical Microbiology and Immunology Department, Faculty of Medicine, Assiut University, 71516 Assiut, Egypt.

[4]Department of Internal Medicine, Faculty of Medicine, Assiut University, 71516 Assiut, Egypt.

* Corresponding author : mosalah@aun.edu.eg

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ABSTRACT

Diabetic foot ulcer (DFU) is the most serious diabetic complication. Gangrene is causing by the successive bacterial infection invading diabetic wounds and may lead to limb amputation for the diabetic patient. The bacteria inhabiting the wound exhibit the ability to forming biofilm, which is a protected mode of growth that allows cells to survive under harsh environments while also dispersing to colonize new niches. The main objective is to clarify the reasons of forming the bacterial biofilm, as well as, the multidrug resistant bacteria. The swab was used method for sample collection and bacterial isolation; different culture media as (BHI medium, MSA and TSA) were used for getting pure cultures from the pathogenic bacteria invading the diabetic wounds. Crystal violet was applied to detect the biofilm formed by these bacteria. Staphylococcus species are the most prominent bacteria invading the diabetic wounds forming a stable biofilm resistant to many antibiotics, one hundred bacterial isolated were recovered from diabetic foot ulcer belonging to five genus, namely, *Staphylococcus* sp., *Klebsiella* sp., *E.coli*, *Pseudomonas* sp., and *Bacillus* sp. using phenotypic analysis was confirmed by phylogenetic analysis.

INTRODUCTION

Diabetic wound infection is the major risk caused by bacteria. Not all bacteria detected in the diabetic wound considered as infection. The main criteria to term and considering the infection is not only to find these bacteria have the ability to colonize the wound [1], but also detect an immune response as a reaction to this bacteria wound

invasion, this called infection. Any other bacteria contaminate the wound or colonize with multiplying in number without a host response cannot be considered as infection [2, 3]. The most familiar symptoms appear in the wounded site to consider as infected wound for example, heat, redness, swelling, pain, cellulitis, increased exudation, or abnormal granulation tissue generated [4]. Indeed, using swab method is the most rapid test that can facilitate the treatment of the infection before it coming risk or complicated. Also using swab for detection the reason for any previous described symptoms associated with diabetic wounds could lead to misleading because, it might be the anaerobic bacteria. These bacteria cultured from swab [5]. Diabetic wound infection (DWI) was thought to be one of the most common, severe and dangerous complications of diabetes. According to previous literature, more than 60% of DW were infected with pathogenic bacteria [6]. The most important and serious risk with 50% is an amputation of limps or toes, because of DWI, in contrast to the wounds within diabetic foot ulcer without infection [7, 8]. The wound without bacterial infections being without risk [9, 10]. Interestingly, the important factor that must put in great attention is the biofilm formed by bacteria. This matrix act as an impregnable fortress against the antibiotics [11]. The formation of biofilm is complex, but according to the literature, it follows a few common steps: initial adherence to the host or wound surface, followed by micro-colony formation, maturation and formation of the biofilm's architecture, and subsequently diffusion of the extracellular matrix (biofilm). The current study aimed to monitor the distribution of the pathogenic bacteria with the diabetic patient with different ranges of ages, investigating the susceptibility rate of the isolated strains towards various antibiotics and differentiation of the whole pathogen according to their ability to forming biofilm.

MATERIALS AND METHODS

Collection of clinical samples
The study was designed and performed from February to December 2019; the clinical samples were isolated from diabetic patient with diabetic foot ulcer (DFU) of 50 total number of patients, from Assiut University Hospital's Diabetic Foot and Endocrinology Center, Assiut, Egypt.

Microorganisms and culture condition
All samples taken from diabetic wounds using swab methods [12], were transferred into sterile Brain heart infusion (BHI) broth medium to be cultured in the laboratory. The composition of BHI medium is (Heart infusion powder 12.5 g/l, BHI powder 5 g/l, protease peptone 10 g/l, dextrose (Glucose) 2 g/l, sodium chloride 5 g/l, disodium hydrogen phosphate 2.5 g/l and the final pH was adjusted at 7.4±0.2). All samples were cultured in Tryptic soy agar (TSA) medium that composed of (Tryptone 17 g/l, Soya peptone 3 g/l, sodium chloride 5 g/l, dextrose (Glucose) 2.5 g/l, dipotassium hydrogen phosphate 2.5 g/l, agar 15 g/l and the pH adjusted to 7.3±0.2) for 24 hours at 35±2 ºC [13]. The formed isolated colonies were streaked to be purify into Mannitol salt agar (MSA) medium with the composition of (protease peptone 10 g/l, sodium chloride 75 g/l, D-Mannitol 10 g/l, phenol red 0.025 g/l, agar 15 g/l and the final pH (at 25°C) was adjusted to 7.4±0.2) for detection of Gram positive bacteria. Macconkey agar medium [Peptones (meat and casein) 3 g/l, pancreatic digest of gelatin 17 g/l, lactose monohydrate 10 g/l, bile salts 1.5 g/l, sodium chloride 5 g/l, crystal violet 0.001 g/l, neutral red 0.03 g/l,

agar 13.5 g/l] for Gram negative bacteria. Eosin methylene blue agar (EMB) medium with a composition of [Peptone 10 g/l, dipotassium hydrogen phosphate 2 g/l, lactose 10 g/l, eosin Y 0.4 g/l, methylene blue 0.065 g/l, agar 15 g/l] for *E.coli* culture detection.

The cultures were incubated under aerobic and static condition overnight at 37ºC. All single colonies formed were further purified to obtain a pure isolate for further microbiological studies [14].

Susceptibility test

For detection the rapid susceptibility of the tested isolated towards different antibiotics (VA: Vancomycin 30mcg; CTX: Cefotaxime 30mcg; CX: Cefoxitin 30mcg; OX: Oxacillin 5mcg; MET: Methicillin 5mcg), Mueller Hinton agar (MHA) medium was used [15]. After streaking of the pure isolate, the antibiotics discs were put using sterile forceps then the plates were put in the opposite position and incubated overnight at 37ºC. The clear zone formed by the antibiotics was detected and this represents a susceptibility of the isolates toward the antibiotics [16].

Detection for bacteria biofilm formation using Microtitre plate method

Staining with crystal violet and using microplate method is one of the first methods used to measure biofilm biomass [17] all steps in this assay were described below.

a. Storage of bacterial strains for biofilm testing

A few colonies from the overnight incubated agar medium are emulsified in appropriate broth supplemented with 10–15% glycerol to obtain a suspension greater than 2 on the McFarland scale, and after labelling, immediately frozen at -70 °C [18].

b. Inoculum

Prior to assaying for biofilm production, strains from the stock culture were transferred, onto TSA (Tryptic Soy Agar) incubate overnight to 24 h aerobically at 35°C–37°C. After verifying purity of the strain, a few colonies with identical morphology are suspended in 5 ml BHI (Brain Heart Infusion) broth without shaking for 18 hr±30 min [19]. The turbidity of the bacterial suspension is adjusted to match turbidity comparable to that of the 0.5 McFarland standard ($\sim 10^8$ CFU/ml). It is preferable to perform this step by using a photometric device.

The stationary phase culture should be vortexed for at least 1 min. Subsequent 1:100 dilution of this suspension will result in the final testing inoculum. Another point of interest in preparing inocula is to avoid inoculation of pre-existing cell clusters [20], since they may lead to false-positive results. Therefore, prepared cell suspensions must be vortexed [19].

c. Medium for biofilm cultivation

Composition of the medium is probably the most important factor influencing the ability of bacteria to produce biofilm under *in vitro* conditions [17] . This was investigated in a number of studies and the results showed that BHI is sometimes better than TSB (Tryptic Soy Broth) [21, 22]. To identify biofilm positive isolates, use of BHI + 2% glucose + 2% sucrose [23] was recommended which was used in this study.

d. Cultivation of biofilm

The wells of the microtiter plate were filled with 180 µL of BHI+2% glucose+2% sucrose. Thereafter, a 20 µL quantity of previously prepared bacterial suspensions is added to each well then, the resulted suspension is vortexed. The negative control wells contain broth only: 200 µL of BHI + 2% glucose + 2% sucrose per well. To reduce the possibility of contamination, this step should be performed in a biological safety cabinet.

Since phenotypic expression of biofilm formation is highly susceptible to various *in vitro* conditions, in order to minimize errors and provide reliable analysis of the data obtained it is essential to perform testing of each strain at least in triplicate (three wells per strain). In addition, each test should be carried out three times. Six wells should be used for the negative control. Up to 30 strains may be tested per one 96-well microtiter plate. The bottom of the microtiter plate may be U-shaped, V-shaped, or flat. Although on rare occasions U-bottomed microtiter plates were used for biofilm quantification [24] , flat-bottomed polystyrene microtiter plates were most frequently used [21, 25, 26] and it was used in our study. The inoculated plate should be covered with a lid and incubated aerobically for 24 h±30 min [27] at 35°C–37°C under static conditions. To reduce the possibility of contamination, this step should be performed in a biological safety cabinet. Since phenotypic expression of biofilm formation is highly susceptible to various *in vitro* conditions, in order to minimize errors and provide reliable analysis of the data obtained it is essential to perform testing of each strain at least in triplicate (three wells per strain). In addition, each test should be carried out three times. Six wells should be used for the negative control. Up to 30 strains may be tested per one 96-well microtiter plate.

e. Washing

There are several ways to measure bacterial growth, but the easiest way is to measure the turbidity in wells using a microtiter-plate reader [28, 29]. It is important to measure turbidity without previous shaking of the microtiter plate to ensure that the integrity of the biofilm is not disturbed prior to washing [28, 30]. After incubation, the contents of the wells were decanted into a discard container. Each well is washed three times with 300 µL of sterile phosphate-buffered saline (PBS; pH 7.2). PBS should previously be warmed at room temperature. Following every washing step, the wells were emptied by flicking the plates. Prior to fixation of the biofilm, the plates were drained in an inverted position. As far as the washing technique is concerned, there is an obvious variety of methodologies applied. We found that careful pipetting does not compromise integrity of the biofilm and recommend washing using micropipettes and emptying by flicking as a simple and effective method. However, turning the microtiter plate upside down will not empty the wells because of capillary and intermolecular forces. It is necessary to splash the content out of the microtiter plate though this may result in the formation of aerosol and possible contamination of the environment and should therefore be carried out very carefully**.**

f. Fixation

After washing, the remaining attached bacteria should be heat-fixed by exposing them to hot air at 60 $^{\circ}$C for 60 min [31].

g. Staining

The modified Christensen's method [32] includes resolubilization of the dye and measures the biofilm formed both on the bottom and walls of the well. The adherent biofilm layer formed in each microtiter plate well is stained with 150µl crystal violet which is used for Gram staining (2% Hucker crystal violet) for 15min [33] at room temperature. Although crystal violet stains only bacterial cells and not the slimy material, its use is acceptable because the former washing steps wash off all non-adherent cells, so only the resting adherent cells will be stained. After staining, the stain was aspirated with pipette. The washing was continued until the washings are free of staining. Each well was washed 3-4 times by pipetting water until the negative control became colorless. The

plate was air dried at room temperature 150µl of 95% ethanol were added gently per well. The addition of ethanol enables indirect measurement of bacteria attached both to the bottom and walls of the wells. Biofilms are as diverse as the microorganisms which produce them. Floating biofilms, or pellicles, that form at the liquid-air interface of standing cultures represent one type of biofilm [34] . ethanol should be gently added to the wells, and shaking of the microtiter plate to speed up the process of resolubilization is prohibited. The plate was covered with lid and left at room temperature for 30 min without shaking.

h. Measurement of results

The optical density (OD) of each well stained with crystal violet was measured at 620 nm using a microtiter-plate reader. The problem of common OD readers is that they measure the OD only at one point in the middle of the well. Thus, if the thickness of the biofilm at that point significantly differs from the rest of the well, the measurement will not be accurate.

However, homogeneous resolubilization of the dye bound to the bacterial cells in the biofilm layer achieved by the recommended protocol enables indirect but precise measurement of the biofilm production.

Molecular identification of Staphylococcus **species** *using 16S r RNA*

a. DNA isolation

The genomic DNA was extracted from the *Staphylococcus* sp. AUMC b-331 using the genomic DNA Prep kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions after glass bead beating to disrupt the cell walls. The extracted DNA was then used as a template for PCR to amplify the 16S rRNA gene. A universal bacterial primer set of 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3 ′) was used to amplify the nearly complete 16S rRNA gene [35].

b. PCR amplification and DNA sequencing

The PCR amplification was performed in a 25 µl reaction volume containing 10–50 ng of the template DNA, 0.4 µM of each primer, 0.75 U of EF-Taq DNA polymerase (SolGent, Daejeon, Korea), 0.2 mM of each d NTP (SolGent, Daejeon, Korea), and 1×EF-Taq reaction buffer (SolGent, Daejeon, Korea). The thermo cycling conditions included an initial denaturation step at 95 °C f or 15 min followed by 30 cycles at 95 °C for 20 seconds, 50 °C for 40 s , and 72 °C for 1.5 min with a final extension step at 72 °C for 5 min. The PCR product was separated by gel electrophoresis on 1.5 % agarose containing ethidium bromide with a 0.5× Tris-acetate-EDTA (TAE) buffer, and visualized using a UV illuminator. [35]. 60µL of the PCR product was then purified using a SolGent PCR purification kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions. The amplified 16S rRNA gene was sequenced using an ABI Big Dye Terminator (v 3.1)

cycle sequencing kit (Applied Biosystems, Foster City, Cal., USA) and an ABI 373 0XL DNA analyzer (Applied Biosystems, Foster City , Cal., USA).

c. Phylogenetic analyses

The 16S RNA dataset included 33 sequences, of which one sequence was obtained in this study for *Staphylococcus* sp. AUMC b-331, 31 sequences downloaded from GenBank for the nearest strains of *Staphylococcus* including the available type species, and one sequence for *Streptomyces brasiliensis* NBRC 12596 as the outgroup. DNA sequences of *Staphylococcus* sp. AUMC b-331 were assembled using the DNASTAR computer package (DNA star version 5.05). Assembled sequence of *Staphylococcus* sp. AUMC b-331 was aligned with those downloaded from GenBank using MAFFT [36]. Alignment gaps and parsimony uninformative characters were treated by BMGE [37]. Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0 [38]. The robustness of the most parsimonious trees was evaluated by 100 bootstrap replications [39]. The best optimal model of nucleotide substitution for the ML analyses was determined using Smart Model Selection (SMS) version 1.8.1 [40]. The phylogenetic tree was visualized using Figtree version 1.4.3. The resulting tree was edited using Microsoft Power Point (2016) and saved as TIF file [41].

Data analysis

Data was analyzed using (GraphPad Prism version 5) Statistical Software.

RESULTS

The samples were collected carefully under aseptic condition under supervision of specialized doctor in DFU. **Figure 1** demonstrate the collecting of sample from wound of diabetic patient using sterile swab. The data presented in **Table 1** were monitored the bacterial cultures identified using microscopic examination and the patient ages. The data demonstrated that the patient ages of 50 to 59 with diabetic wounds were highly susceptible to the bacterial contamination and pathogenicity.

Figure 1. Isolation of pathogenic bacteria from diabetic wound using swab method.

Table 1. Number of DFU isolates with distribution according to patients' ages

Age (Years)	No. of sample and (%) of wound contamination	Number of isolates					
		Staphylococcus sp. (%)	*Klebsiella* sp. (%)	*Pseudomonas* sp. (%)	*Bacillus* sp. (%)	*E.coli* (%)	Total no.
40-49	16 (15)	11 (17.7)	2 (20)	1 (7.1)	0	5 (45.5)	20
50-59	18 (41)	25 (40.3)	7 (70)	5 (35.8)	0	2 (18.2)	39
60-69	11 (22)	16 (25.8)	1 (10)	7 (50)	2 (66.7)	1 (9.1)	27
70-79	3 (14)	6 (9.7)	0	0	0	3 (27.2)	9
80-89	2 (8)	4 (6.5)	0	1 (7.1)	1 (33.3)	0	6
Total	50	62	10	14	3	11	100

In contrast to the patient ages between 80-89 illustrated the least susceptibility towards contamination. The data provide evidence with a great abundance of *Staphylococcus* sp. in all clinical collected samples with a percentage 62% of total screened bacterial isolates. Followed by *Pseudomonas* sp. with 14% abundance, 11% *E.coli*, and 10% *Klebsiella* sp. the least abundance bacteria in the screened clinical isolates was *Bacillus* sp. with only 3% of the total number of isolates.

The tested organisms showed various susceptibility towards the antibiotics used in our current study. *Staphylococcus* species demonstrated highest susceptibility towards OX, followed by VA and all species showed resistance against CTX. Some of *Klebsiella* sp. illustrated susceptibility towards VA, CTX and CX, with a resistance against OX and

MET. Amazingly, all of the isolated *Pseudomonas* sp. showed resistance against the whole antibiotics used except one isolate shown a susceptibility towards CTX. Whereas *Bacillus* sp. isolates showed resistance towards CTX and CX. Additionally, *E.coli* isolates demonstrated susceptibility towards all tested antibiotics. All data with percentages are shown in **Table 2**.

Table 2. Rate and percentage of the susceptibility of pathogenic bacteria towards the antibiotics.

Organism	Number of isolated bacteria	VA 30mcg	CTX 30mcg	CX 30 mcg	OX 5mcg	MET 5mcg
		Number (%) of isolates susceptible to antibiotics				
Staphylococcus sp.	62	16 (25.8)	ND	7 (11.3)	20 (32.2)	8 (12.9)
Klebsiella sp.	10	4	3	4	ND	ND
Pseudomonas sp.	14	ND	1 (7.1)	ND	ND	ND
Bacillus sp.	3	1 (33.3)	ND	ND	1 (33.3)	1 (33.3)
E.coli	11	2 (18.1)	8 (72.7)	6 (54.5)	1 (9.1)	2 (18.1)

All tested isolates were tested for biofilm formation after purification for all isolates. The bacterial ability for biofilm formation was detected by using the Microtiter plate assay and the optical density was measured using (Thermo Electron Corporation,

Finland) ELISA reader at 620 nm. The OD with isolates which gave >1 and up to 2.9 considered as biofilm forming bacteria. From the represented data in Figure 2, about 74% of *Staphylococcus* sp. isolated were detecting biofilm, 66.6% of *Bacillus* sp. isolates demonstrated for biofilm formation, 60% of *Klebsiella* sp. isolates were biofilm positive, and 35.7% of the total number of *Pseudomonas* sp. showed susceptibility for biofilm formation. Only 27.2% of tested *E.coli* isolates considered as biofilm forming.
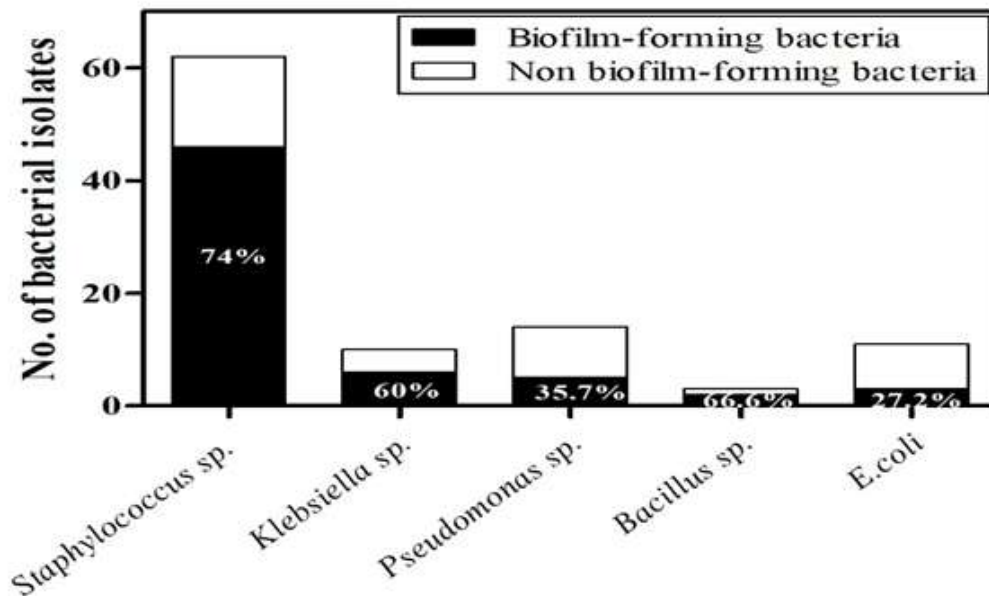
Figure 2. Occurrence of biofilm and non-biofilm-forming bacteria recovered from diabetic foot wound.

Phylogenetic analysis of 16S RNA dataset was employed to determine the taxonomic status of the *Staphylococcus* sp. AUMC b-331 relative to other members belonging to genus *Staphylococcus*. The entire 16S RNA dataset comprised 33 sequences. The maximum parsimony dataset consisted of 1508 characters with 1386 constant characters (no gaps, no N), 90 variable characters which were parsimony-uninformative (6.5% of constant characters), and 52 characters were counted as parsimony informative (3.8% of constant). General Time Reversible (GTR) was the perfect model for substitution of nucleotides. The dataset for maximum parsimony yielded consensus of seven best trees with a tree length of 183 steps. The best scoring ML tree with the final ML optimization likelihood value of -3504.69383 and tree size of 0.17195 was selected to represent and discuss the phylogenetic relationships among taxa. In the phylogenetic tree, the strain *Staphylococcus* sp. AUMC b-331 consistently located within the subclade including *Staphylococcus haemolyticus* JCM 2416 and *S. haemolyticus* SM 131 (type strain), endorsing high bootstrap value of 68% ML/65% MP **Figure 3**. Therefore, it can be identified as *Staphylococcus haemolyticus*.
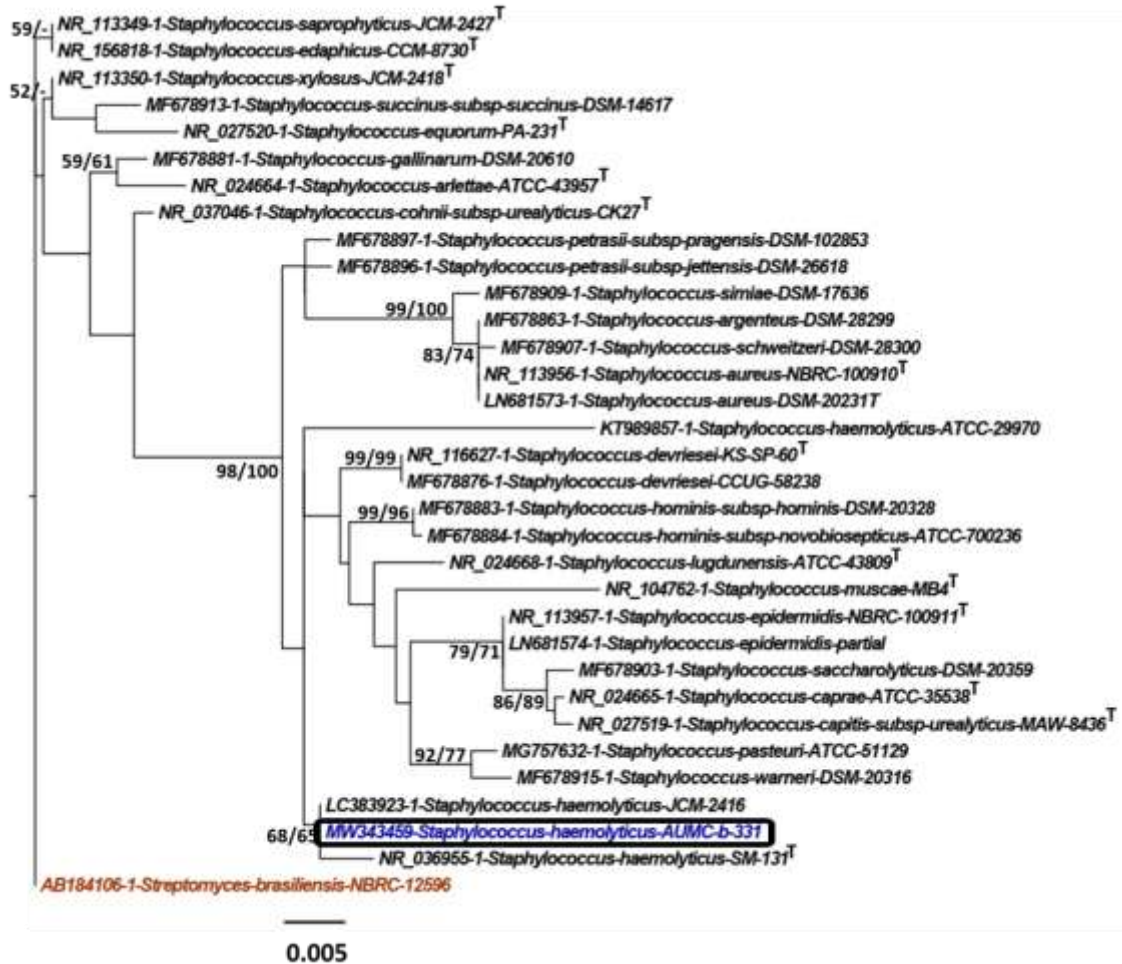
Figure 3. Phylogenetic tree generated from MP analysis, the neighbor-joining tree based on 16S rRNA gene sequences showing the positions of the isolate Staphylococcus sp. AUMC b-331(surrounded by black line) and related strains. The tree is rooted to Streptomyces brasiliensis NBRC 12596 as out group (in red color). Sequences derived from type materials were indicated with superscripts [T].

DISCUSSION

This study provided that the wounds of patients with uncontrolled diabetes with age range between 50 to 59 years were the highest susceptible to wound contamination and infection. These results are in agreement with recent published literatures studying the distribution of DFU with the patient ages [42, 43]. Patient with ages between 50 to 60 with DFU has a great risk and highly susceptible to wound infection may be due to the immunosuppressed as a result of diabetic complication [44]. The bacteria invade the wounds via various mechanisms, generally by attached within the skin section at the wounded area, begin to colonize and multiply exploits the immune-suppressed and the

high glucose levels [45]. This study illustrated the presence of various pathogenic bacteria mainly *Staphylococcus* species and others related to family Entrerobacteriaceae. The most prominent bacteria inhabiting diabetic wounds was *Staphylococcus* sp., as agreed with the line of literature in this field of study [46-49]. Although, the use antibiotics with various effects, spectrum and doses have the ability to eliminate and reduce the pathogenic bacteria invading the diabetic wounds, but the excessive and successive use of those synthetic antibiotics generate the multidrug resistant bacteria. These pathogenic bacteria have the ability to genetically modified to produce polymer matrices, which are primarily made up of exopolysaccharides, protein complexes, and extracellular DNAs called biofilm [50]. Consequences, lead to resistance to the antibiotics as shown in Table 2, moreover this gave the ability to those bacteria to overcome the effect of Vancomycin; which have the significant ability to kill and inhibit the bacteria growth [51]. Differs from the previous studies [52, 53], vancomycin which regarded as a first-line treatment for severe MRSA infections. In this study, the use of vancomycin for the clinical isolated bacteria demonstrated inhibition for only about 25% of the total *Staphylococcus* species tested. In the treatment of DFI, there is no standard antimicrobial agent has been shown to be superior to others [54]. This point of view was provided with the data resulted from the biofilm assay which reported that most of the staphylococcus species more the 70% have the ability to produce biofilm. Therefore, these bacterial isolates showed the ability to remain viable and active even after the antibiotic treatment. The phylogenetic analysis for the most biofilm-forming bacteria from all 100 recovered isolates, illustrated a probability to be *Staphylococcus haemolyticus*. This agreed with the data describing the virulence factors and risks associated with *St.haemolyticus* in the previous studies [55-57]. Substantially, the risk factor of diabetic wound infection is to delay in detection and the delay in treatment may cause further complications that may lead to limb amputation. In the future, the new strategies, able to overcome the multi-drug resistant bacteria and reduce the amputation and surgical techniques to fight these pathogenic bacteria. At the same time, it may add significant incredibly interesting opportunity for future investigation on bacterial biofilm.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The ethical committee of the Faculty of Medicine, Assiut University, Egypt, approved the study. All participants gave informed consent.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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