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Original Article

Insight into multidrug-resistant microorganisms from microbial infected diabetic foot ulcers

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ABSTRACT

Aim: This study was undertaken to inspect the preponderance of multidrug-resistant (MDR) microorganisms in microbial infected diabetic foot ulcers (DFUs) in north Egypt. Moreover, their later impact on the patients and previous antibiotic therapies were taken into consideration.
Methods: To accomplish this goal, twenty-two of diabetic foot patients with purulent wounds were enrolled in this prospective study. These wounds were swabbed and the antibiotics susceptibility patterns of most virulent bacteria and yeast were studied. Furthermore, bacterial and yeast strains were identified using 16S rRNA and 16S rRNA nucleotide sequences, respectively, and following their phenotypic characteristics employing the VITEK 2 system.
Results: Microbial profiles showed a predominance of monomicrobial infections (77.3%), while polymicrobial infections were found in 22.7%. A total of 24 bacterial isolates (15 Gram-positive and 9 Gram-negative) and four yeast isolates were perceived. Four bacteria were selected based on their resistance toward more than six of empirical antibiotics. They were identified and deposited in GenBank as *Acinetobacter baumanni* MT3 (KY421195), *Staphylococcus aureus* MT1 (KY421197), *Klebsiella pneumonia* MT2 (KY421196), and *Staphylococcus aureus* MT4 (KY421198). On the other hand, one strain belonged to yeast was opted and identified as *Candida albicans* MT5 (MG851796).
Conclusion: These findings might effectively help to avert the severe complications of diabetic foot infections (DFIs) besides our endeavours to find new antimicrobial wound dressings.

1. Introduction

Diabetes mellitus became a common metabolic disorder, which is significantly expanding at an alert frequency worldwide menacing the public health. It is provoked by the incapability of diabetic patients to generate insulin to regulate carbohydrates metabolism [1]. One of the serious episodes of diabetes is the materialization of DFUs that arise due to several pathological factors, such as neuropathy, peripheral vascular disease and high plantar pressures [2]. Furthermore, diminishing in microvascular circulations obstruct the direct contact of phagocytes impairing the host immune response, and hinder antibiotics access to the wound spot, which consequently prompts the development of microbial infections [3]. These extrinsic and intrinsic factors make diabetic foot patients liable to microbial infections. DFIs are the most serious complications of DFUs that might cause tissue maceration and sepsis, thereby lead to septic gangrene obliging amputation of the foot. Diabetic patients are 25 times putting at risk to lose a leg compared to the healthy patients. Moreover, approximately 70% of them could amputate their leg to improve the wound healing process particularly with DFIs [4]. This infection can be easily observed, where microorganisms colonize over the wound bed

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developing multi-layered microbial communities protected by their secreted polysaccharides as an extracellular biofilm [5]. Biofilm restrains wound healing process, and the microbial infections treatment become very problematic to handle, as the wound get impenetrable by the antimicrobial agents, and the competence of immunological system is impaired [6]. Microbial profile of DFIs varies from hospital to hospital, patient to patient and even from country to another one [7]. Retrospective studies have demonstrated DFIs with the prevalence of different microorganisms as polymicrobial infections in nature implicating *S. aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus* sp., *Enterococcus* spp., *K. pneumoniae, Proteus* sp., anaerobic bacteria, and fungi [8–10]. To manage DFIs, antibiotic therapies should be implemented to avert the cross-contamination and accelerate the wound healing process of infected ulcers. Selection of empirical antibiotics is based on the pathogenic organisms and the antimicrobial susceptibility patterns; however, the application of inadequate antibiotics or low concentrations of antimicrobial agents could develop and distribute MDR microorganisms. Beta-lactam, ciprofloxacin and carbapenems antibiotic groups are frequently used to tackle the bacterial infections in the hospitals, but the mutation of such bacteria generates antibiotics resistant bacteria to this group that could be further extended with abuse of several antimicrobial agents [11]. Prior reports have recommended the proper identification of microorganisms accompanied DFIs to opt the effective antibiotics; otherwise, the treatment would be too harsh to control [12]. Egypt has a higher rate of diabetic populations than other countries that consider a feared alarm and the limb amputation occurs in great ratios accordingly [13]. Moreover, large populations in Egypt are suffering from DFIs with significant variations of microorganisms and their susceptibility to the standard antibiotic therapies administered in the hospital. However, to the best of our knowledge, one study used conventional microbiological approaches to examine the microbial profile of DFIs in Egypt has been published so far [14]. In light of this fact, the current study aims to investigate the prevalence of MDR microorganisms in DFIs after applying the broad-spectrum antibiotics to manage this infection. Moreover, the output of these infections was monitored via following up the diabetic patients after collecting the samples and recorded their cases in relation to the isolated organisms to substantiate our anticipation. Most virulent microorganisms would be identified utilizing biochemical and molecular techniques to use them in prospective future studies for developing effective antimicrobial wound dressings. These might be adequate for treatment of such specific case to boost the wound healing process.

2. Materials and methods

2.1. Samples collection

This research was implemented throughout 22 diabetic foot patients in Borg El-Arab hospital, Alexandria, Egypt, and the analysis were carried out in the City of Scientific Research and Technological Applications, Alexandria, Egypt.

Informed consents were obtained from all Diabetic foot patients before the use of their wound samples to investigate the incidence of multidrug-resistant microorganisms. The wound specimens were collected and handled in accordance with the Research Ethical Committee, which was published by the National Health and Medical Research Council policies and the recommendations of Ministry of Health and Population, High Committee of Medical Specialties, Egypt. All protocols used in this study have been evaluated and approved by the local committee at New Borg El-Arab hospital, Alexandria, Egypt. This study was implemented in the frame of the collaboration between the City of Scientific Research and Technological Applications, Alexandria, Egypt and New Borg El-Arab hospital, Alexandria, Egypt.

Prior to sample collection, each diabetic foot wound was cleaned with a sterile saline solution (0.85%), and the wound was subsequently swabbed using a sterile cotton-tipped swab to isolate the most existing microorganisms. These wounds were severely infected comprising purulence and manifestations of inflammation. Once the sample was collected, it was labelled with the patient identification number, date and the collection time. The samples were instantly transported under aseptic conditions to the microbiological lab for further examinations.

2.2. Isolation of microorganisms

The collected samples were plated on Luria- Bertani agar (LB) (10 g peptone, 5 g yeast extract, 10 g NaCl and 15 g agar per 1 L distilled H_2O) to isolate the bacterial strains. For fungal strains, isolation was performed on YPD agar medium (20 g peptone, 10 g yeast extract, 20 g dextrose and 15 g agar per 1 L distilled H_2O) were utilized. After incubation for 24 h at 37 °C, pure bacterial and yeast isolates were obtained by re-growing individual colonies several times on their respective media. The strains were preserved in glycerol at −80 °C to avoid the unexpected mutations.

2.3. Antibacterial susceptibility studies

Antibiotic sensitivity tests of different bacterial isolates were performed using disc diffusion approach following the procedure described by **Hassan, Omer** [15]. The experiment was standardized by adjusting the turbidities of bacterial cultures to McFarland standards 0.5 in a sterile liquid LB medium employing spectrophotometer. Eleven antibiotic discs (Bioanalyse Co., Ltd., Ankara, Turkey) belong to seven antibiotic groups were chosen according to the previous reports, which demonstrated their prevalent applications for treatment of various wound infections [16]. The applied antibiotics can be categorized into different classes as follow: (I) Aminoglycosides: Amikacin 30 μg (AK), and Gentamicin 10 μg (CN), (II) Penicillins: Ampicillin 10 μg (AM), Oxacillin 1 μg (OX), and Ampicillin/Sulbactam 20 μg (SAM), (III) Cephalosporins: 2nd generation including Cefoxitin 30 μg (FOX) and 3rd generation including Ceftazidime 30 μg (CAZ), (IV) Carbapenems: Imipenem10 μg (IPM), (V) Fluoroquinolones: Ciprofloxacin 5 μg (CIP), (VI) Macrolides: Erythromycin 15 μg (E), and (VII) Sulfonamides or Folate synthesis inhibitors: Trimethoprim/sulfamethoxazole 25 μg (SXT). The results were interpreted and recorded for various bacterial isolates, which were derived from the antibiotics susceptibility tests guidelines provided by the manufacturer. Accordingly, the pathogenic bacterial isolates could resist at least six antibiotics were counted most resistant bacteria and picked out for further investigations. The selected bacterial isolates were identified on the basis of morphological characteristics including colony features, blood hemolysis and microscopic inspection of Gram-stained bacteria.

2.4. Antifungal susceptibility tests

Antifungal susceptibility assay of yeast strains was carried out toward fluconazole, voriconazole, caspofungin, amphotericin B, flucytosine, and micafungin using VITEK 2 automated microbiology system (bioMérieux, France). This system was adopted since it is a feasible and accurate system encompasses the specific antifungal agents for various strains. Yeast isolates were inoculated into YPD broth and incubated at 37 °C for 16 h. The cells were harvested via centrifugation for 20 min at 5000 rpm and washed twice by the sterile saline solution. The turbidities of yeast cultures were adapted to McFarland standards 0.5 in sterile saline solution using a Densimat densitometer (bioMérieux, France). YST card was employed for yeast strains and filled with the cell suspensions. Then, the cards were loaded into the VITEK 2 system, and the results were recorded. The gained data were analyzed following the manual instructions of the system.

2.5. Identification of most virulent microorganisms

2.5.1. Genetic identification using 16S rRNA and 18S rRNA nucleotide sequences

Selected bacterial and yeast isolates were identified using 16S and 18S rRNA nucleotide sequences. For bacterial strains, 16S rRNA genes were amplified utilizing genomic DNA as proposed by **Hassan, Haroun** [17]. The PCR reactions were carried out using 16S rRNA primers: 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCCGC-3′). The reactions were accomplished in 50 μl as follows: 1 μl of diluted DNA template (30 ng), 1 μl of 10 pmol of each primer, 0.8 μl of 12.5 mM (dNTP's), 5 μl of PCR buffer containing $MgCl_2$, 1 unit Taq polymerase, and water-free DNAse and RNAse were added up to 50 μl.

Thermal cycler was set up as follow: 3 min denaturation at 95 °C, followed by 35 cycles that composed of 1 min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C, and a final extension was carried out at 10 min at 72 °C. The amplified products were visualized and verified on 1.5% agarose gel using 100 bp DNA ladder (100–3000 bp), and the photo was snapped via a gel documentation system.

To amplify the 18S rRNA gene of yeast isolate, 18S primers were applied including NS1F (5′-GTAGTCATATGCTTGTCTC-3′), and NS8R (5′-TCCGCAGGTTCACCTACGGA-3′). The PCR reaction was implemented in 50 μl with the following components: 5 μl of 10X buffer, 1 μl of each primer (10 pmol), 1 μl of 10 mM dNTPs, 1 unit Taq polymerase and 1 μl of 50 ng DNA [18,19]. The PCR reaction was performed under the following conditions: a denaturation step at 95 °C for 5 min, followed by 35 cycles; 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 10 min. The amplified gene was investigated as previously described in the case of 16S rRNA.

2.5.2. Purification and nucleotide sequences of 16S and 18S rRNA genes

PCR products were purified from the agarose gel for DNA sequencing employing QIAquick Gel Extraction Kit (Qiagen, USA). The purified 16S and 18S rRNA genes were submitted to Sigma Company for identifying the nucleotide sequences, which was executed following the protocol of the enzymatic chain terminator technique.

2.5.3. Bioinformatic analysis and phylogenetic tree construction

The obtained nucleotide sequences of 16S and 18S rRNA were aligned against homologous sequences available in National Center for Biotechnology Information GenBank (NCBI GenBank) by Basic Local Alignment Search Tool (BLAST). The comparable sequences were retrieved from the GenBank, and the alignments were carried out by Clustal W employing MEGA5 software version 5.1 [20]. The phylogenetic trees were structured via applying neighbor-joining tree approach with 500 bootstrap values.

2.5.4. VITEK 2 system

To validate the molecular identification, the bacterial and yeast isolates were identified based on their phenotypic characteristics and biochemical tests employing the VITEK 2 system through its particular cards. The bacterial isolates were cultured into the nutrient broth at 37 °C for 16 h, while yeast isolate was grown in YPD broth following the previous method. The bacterial and yeast cultures were then prepared and adjusted to McFarland standards 0.5 as described above. Particular Biomerieux VITEK 2 cards including GN, GP, and YST cards were utilized to identify Gram-negative bacteria, Gram-positive bacteria, and yeast, respectively. The GN, GP and YST identification cards were constructed following the new biochemical methods and substrates. The cards were filled with the cell suspensions and loaded into the VITEK 2 system for strain identification. The results of biochemical tests were analyzed and the strains were identified accordingly.

3. Results

3.1. Wound swabs and cultures analysis

In the current study, MDR microorganisms including Gram-positive and negative bacteria and yeast were isolated from various diabetic foot ulcer patients. A total of 22 swabs were collected from purulent wounds; thus, 24 pathogenic bacteria and four yeast isolates were picked out as pure strains. One or poly-microbial species had medical prominence were isolated from the festering wounds. Among 24 bacterial isolates, 15 (62.5%) bacterial isolates belonged to Gram-positive bacteria, while 9 (37.5%) bacterial isolates were Gram-negative as shown in Table 1. Therefore, the dominant bacteria in the infected wounds were Gram-positive bacteria. Furthermore, four (14.3%) pathogenic yeast isolates were isolated and observed in the same wound beds harbouring with the pathogenic bacteria as presented in Table 2. Tables 1 and 2 demonstrate that only one microbial species could be predominantly separated from each wound (77.3%). Conversely, the incident of mixed microbial species was perceived in 5 (22.7%) wound swabs that were mostly included two species representing 18% and 80% of the total collected wound swabs and poly-microbial wounds, respectively. In contrast, triple microbial strains were found only in one swab the represent 4.5% and 20% of the total collected swabs and poly-infected wounds, respectively.

3.2. Antimicrobial drug susceptibility patterns

To select the most virulent bacteria, antibiotic disk diffusion method was conducted using elven antibiotics, which belong to seven antibiotic classes as mentioned above. As can be seen in Table 1, the entire bacterial isolates were sensitive to imipenem except for isolate (No. 12). Imipenem revealed a slight inhibition zone (about 9 mm) that was interpreted as a resistance performance of isolate (No. 12), which exhibited impedance actions toward the whole antibiotic discs (Fig. 1). As a consequence of this untreatable infection, the patient's foot was further amputated. Ciprofloxacin exerted growth inhibition against the pathogenic bacterial isolates about 62.5% in relation to the total strains; however, the majority of them belonged to Gram-positive bacteria (twelve strains) with percentage up to 66.6%. Moreover, amikacin demonstrated the maximum inhibition percentage versus the studied strains up to 83.3%, whereas the prevalence of susceptible strains to gentamicin reached 62.5%. Table 1 highlights the incidence of resistant bacteria to the other empirical antibiotics as follow: ampicillin (54.2%), oxacillin (58.4%), ampicillin/sulbactam (50%), cefoxitin (25%), ceftazidime (62.5%), erythromycin (62.5%), and trimethoprim/sulfamethoxazole (37.5%). The most bacterial isolates revealed resistance toward at least four antibiotics, which belong to different antibiotic classes. Accordingly, these pathogenic bacteria consider MDR; however, the significant findings to emerge from the data are that the bacterial isolate no 1, 5, and 13 exhibited significant resistance to the major antibiotic discs, in addition to isolate no 12 as previously discussed (Fig. 1). Imipenem showed prominent activities against strains no 1, 5 and 13, which identified

Table 1
Antibiotic susceptibility patterns of bacteria isolated from purulent wounds of diabetic foot patients.

Bacterial code	Patient's number and history	Type of bacterial isolate	Antibiotic susceptibility pattern		
			Sensitive	Resistant	Intermediate
1	(1) Diabetic foot with purulent wound	G + ve	IMP	AK, CN, AM, OX, SAM, FOX, CAZ, E, and SXT	CIP
2	(2) Diabetic foot with purulent wound	G-ve	AK, CN, AM, FOX, IMP, E, and SXT	OX, SAM, CAZ, and CIP	–
3	(3) Diabetic foot with purulent wound	G + ve	AK, CN, OX, FOX, IMP, CIP, And SXT	AM, SAM, CAZ, and E	–
4	(4) Diabetic foot with purulent wound	G + ve	AK, CN, OX, SAM, FOX, IMP, and CIP	AM, CAZ, E, and SXT	–
5	(5) The diabetic foot was further amputated	G-ve	AK, and IMP	CN, AM, OX, SAM, FOX, CAZ, CIP, E, and SXT	–
6	(6) Diabetic foot with purulent wound	G + ve	AK, OX, FOX, CAZ, IMP, CIP, E, and SXT	CN, AM, and SAM	–
7	(7) Diabetic foot with purulent wound	G + ve	AK, SAM, FOX, CAZ, IMP, CIP, and SXT	CN, AM, OX, and E	–
8	(8) Diabetic foot with purulent wound	G + ve	AK, CN, SAM, FOX, IMP, E, and SXT	AM, OX, CAZ, and CIP	–
9	(9) Diabetic foot with purulent wound	G-ve	AK, CN, SAM, CAZ, IMP, CIP, and E	AM, OX, FOX, and SXT	–
10	(10) Diabetic foot with purulent wound	G-ve	AM, CN, FOX, CAZ, IMP, CIP, and SXT	AK, OX, SAM, and E	–
11	(11) Diabetic foot with purulent wound	G-ve	AK, AM, CN, SAM, FOX, CAZ, IMP, and CIP	OX, E, and SXT	–
12	(12) The diabetic foot was further amputated	G-ve		AK, CN, AM, OX, SAM, FOX, CAZ, IMP, CIP, E, and SXT	–
13	(13) The diabetic foot was further amputated	G + ve	AK, and IMP	CN, AM, OX, SAM, FOX, CAZ, E, and SXT	CIP
14	(13) The diabetic foot was further amputated	G + ve	AK, CN, OX, SAM, FOX, IMP, and CIP	AM, CAZ, E, and SXT	–
15	(14) Diabetic foot with purulent wound	G + ve	AK, CN, AM, OX, SAM, FOX, IMP, and E	CAZ, CIP, and SXT	–
16	(15) Diabetic foot with purulent wound	G + ve	OX, SAM, FOX, CAZ, IMP, CIP, E, and SXT	AK, CN, and AM	–
17	(16) Diabetic foot with purulent wound	G + ve	AK, CN, AM, OX, SAM, FOX, IMP, CIP, and SXT	CAZ, and E	–
18	(17) Diabetic foot with purulent wound	G + ve	AK, CN, AM, FOX, IMP, CIP, and SXT	OX, SAM, CAZ, and E	–
19	(17) Diabetic foot with purulent wound	G-ve	AK, AM, OX, SAM, FOX, CAZ, IMP, and SXT	CN, CIP, and E	–
20	(18) Diabetic foot with purulent wound	G + ve	AK, CN, AM, FOX, CAZ, IMP, CIP, and SXT	OX, SAM, and E	–
21	(19) Diabetic foot with purulent wound	G-ve	AK, AM, SAM, FOX, IMP, CIP, E, and SXT	CN, OX, and CAZ	–
22	(20) Diabetic foot with purulent wound	G + ve	AK, CN, AM, OX, FOX, IMP, CIP, and SXT	SAM, CAZ, and E	–
23	(21) Diabetic foot with purulent wound	G-ve	AK, CN, AM, SAM, CAZ, IMP, CIP, E, and SXT	OX, and FOX	–
24	(22) Diabetic foot with purulent wound	G + ve	AK, CN, OX, FOX, IMP, E, and SXT	AM, SAM, CAZ, and CIP	–

Amikacin (AK); Gentamicin (CN); Ampicillin (AM); Oxacillin (OX); Ampicillin/Sulbactam (SAM); Cefoxitin (FOX); Ceftazidime (CAZ); Imipenem (IPM); Ciprofloxacin (CIP); Erythromycin (E); Trimethoprim/sulfamethoxazole (SXT).

Table 2
Antifungal susceptibility patterns of yeast isolated from purulent wounds of diabetic foot patients.

Yeast code	Patient's number and history	Antifungal susceptibility pattern		
		Sensitive	Resistant	Intermediate
1	(6) Diabetic foot and purulent wound	Fluconazole, Voriconazole, Caspofungin, Amphotericin B, Flucytosine, and Micafungin	–	–
2	(12) The diabetic foot was further amputated	Fluconazole, and Amphotericin B	Voriconazole, Caspofungin, and Micafungin	Flucytosine
3	(13) The diabetic foot was further amputated	Fluconazole, Voriconazole, Caspofungin, Amphotericin B, Flucytosine, and Micafungin	–	–
4	(16) Diabetic foot and purulent wound	Fluconazole, Voriconazole, Caspofungin, Amphotericin B, Flucytosine, and Micafungin	–	–

at the initial threshold of antibiotic sensitivity on the basis of the antibiotics disc testing guidelines of the manufacturer. Moreover, amikacin revealed notable actions versus strains no 5, and 13. On the contrary, ciprofloxacin demonstrated intermediate performances against bacterial isolates no 1, and 13. It could be deduced from these findings that four bacterial isolates were resistant to almost all antibiotic discs expressing various antibiotics classes. As these four bacterial isolates demonstrated substantial virulence, we picked them for further identification via 16S rRNA sequencing approach and confirmed this identification employing the VITEK 2 system. With respect to the yeast isolates, strain no 2 showed a capacity to resist voriconazole, caspofungin, and micafungin, whereas, it exhibited intermediate resistance toward flucytosine. In contrast, fluconazole, and amphotericin B hindered the growth of this yeast. On the other hand, the other yeast isolates were susceptible to all antifungal agents (Table 2). Consequently, yeast no 2 was chosen for identification via 18S rRNA gene sequence and VITEK 2 system. The bacterial isolates no 1, 5, 12, and 13 were coded to be MT1, MT2, MT3, MT4, respectively, while yeast isolate was named MT5.

3.3. *Identification of bacteria and yeast isolates*

The bacterial and yeast strains were identified employing molecular approach. Strains MT1, MT2, MT3, and MT4 were identified utilizing 16S rRNA gene sequence, while 18S rRNA nucleotide sequences were used for strain MT5. The entire 16S rRNA genes were amplified from genomic DNA and about 1500 bp were obtained as illustrated in Fig. 2, and the whole gene size of 18S rRNA gene was amplified. The 16S rRNA nucleotide sequences were procured using the 16S rRNA forward primer. On the other hand, 18S rRNA nucleotide sequence was gained applying 18S rRNA forward and reverse primers. Nucleotide sequences of 1060 bp, 696 bp, 417 bp, and 639 bp were submitted to GenBank as partial 16S rRNA sequences for bacterial isolates MT1, MT2, MT3, and MT4, respectively. Similarly, nucleotide sequences of 1697 bp were deposited in GenBank as partial 18S rRNA sequences for Yeast isolate MT5. Various 16S rRNA nucleotide sequences were analyzed employing BLAST analysis and 16S rRNA sequences of strain MT1, MT2, MT3, and MT4 exhibited the utmost correspondence of 97% with *Staphylococcus aureus*, 93% with Kl*ebsiella pneumonia*, 92% with *Acinetobacter baumannii*, and

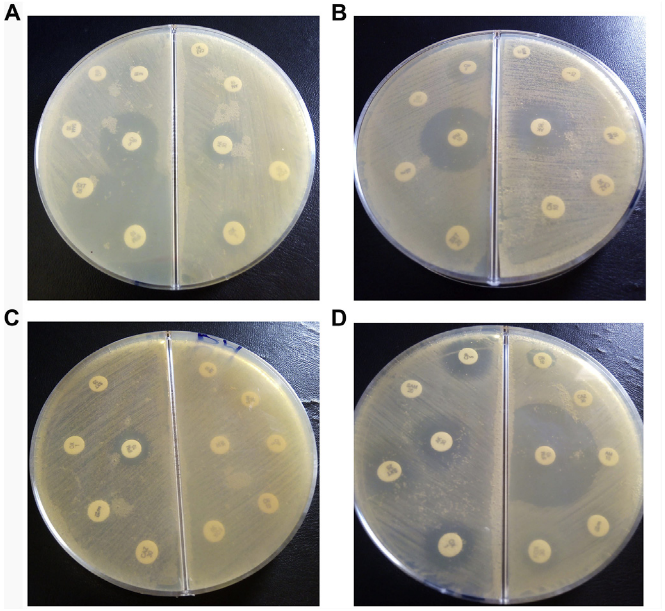

Fig. 1. Antibiotic susceptibility patterns of (A) bacterial isolate No. 1, (B) bacterial isolate No. 5, (C) bacterial isolate No. 12, and (D) bacterial isolate No. 13, which were isolated from purulent wounds of diabetic foot patients.

99% with *Staphylococcus aureus*, respectively. On the other hand, 18S rRNA nucleotide sequences of yeast isolate showed the greatest resemblance of 99% to *Candida albicans* and *Candida gigantensis*. Consequently, GenBank allocated specific accession number for each deposited sequence as follow in parentheses: *Staphylococcus aureus* MT1 (KY421197), K*lebsiella pneumonia* MT2 (KY421196), *Acinetobacter baumanni* MT3 (KY421195), *Staphylococcus aureus* MT4 (KY421198), and *Candida albicans* MT5 (MG851796). Homologous sequences were retrieved from GenBank database analysis and the closest sequences were utilized for constructing the phylogenetic tree in a neighbor-joining form. Phylogenetic analysis validated that all the isolates belong to four groups of microorganisms via comparing the obtained sequences with the previously reported sequences (Figs. 3–5). As consequence, this analysis demonstrated a great similarity of the studied strains with their correspondence.

To substantiate the molecular identification and differentiate between the identical strains, bacterial and yeast isolates were identified using the VITEK 2 system to obtain faster and reliable results. Many substrates were applied to determine various metabolic activities comprising the conventional and advanced tests such as catalase, coagulase, oxidase, sugars fermentation, acidification, enzyme activities, and the impact of inhibitory substances (Table S1-S5). The strains MT2 and MT3 were identified employing GN card, which is specific for Gram-negative fermenting and non-fermenting bacilli. In the case of strains MT1 and MT4, GP card was applied to differentiate between Gram-positive cocci and non-spore-forming bacilli. On the other hand, yeast isolate was investigated utilized YST card for yeasts and yeast-like organisms.

With respect to GN card, 47 biochemical tests and 1 negative control well appraising carbon source utilization, enzymatic activities and resistance were implemented as shown in Table S2 and S3. As can be seen in Table S1 and S4, 43 biochemical tests were carried out in GP cards for identifying Gram-positive bacteria. Concerning yeast identification, 46 biochemical tests clarifying the physiological characteristics of yeast strain were applied as illustrated in Table S5. The results were extrapolated by means of the providing identification database to the system. It could be deduced from the results that strain MT1, MT2, MT3, and MT4 are *S. aureus* MT1, *K. pneumonia* MT2, *A. baumannii* MT3, and *S. aureus* MT4, whereas the yeast isolate was identified as *C. albicans* MT5.

4. Discussion

Impaired wound healing in diabetic foot patients considers a great challenge for human healthcare worldwide. The leading issues that hamper the wound healing of DFUs are peripheral vascular disease, neuropathy, and impaired immune response through neutrophil dysfunction, which render the diabetic patients predispose to microbial foot infections [8]. Prompt clinical diagnosis of the pathogenic microbes and treatment using adequate antibiotics are crucial to avert the further severe complications. However, abuse or overuse of the antimicrobial drugs might induce the wide spreading of MDR microorganisms [21]. In this prospective study, we sought to examine the prevalence of MDR microorganisms in DFIs in Alexandria, Egypt after antibiotic therapies were applied. Though many studies have been performed to unveil the critical issues associated with the materialization of MDR microorganisms, few articles have reported the impact of such strains on DFIs with time progress. Hence, we elucidated the antibiotics resistance of organisms and the consequences of wrong clinical diagnosis later.

Concerning the microbial profile of DFI pathogens, we observed the superiority of monomicrobial infections accounted for 77.3% of the total isolates, while the polymicrobial infections represented only 22.7%. These findings are in correspondence with reported

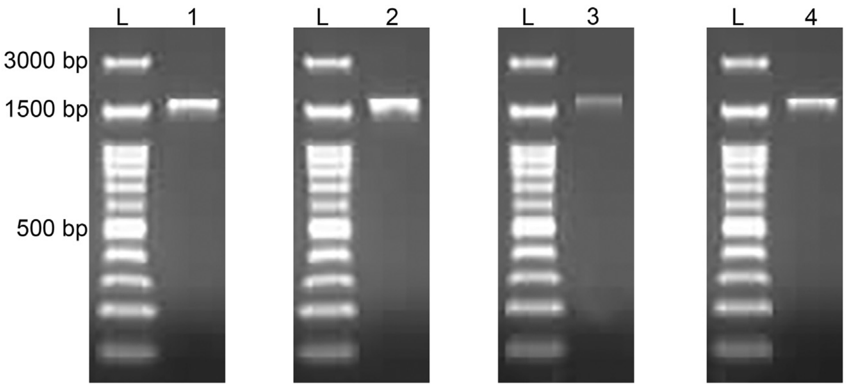

Fig. 2. Amplified 16S rRNA genes approximately 1500 bp of (1) bacterial isolate No. 1, (2) bacterial isolate No. 5, (3) bacterial isolate No. 12, and (4) bacterial isolate No. 13 comparing with (L) 100 bp DNA ladder.

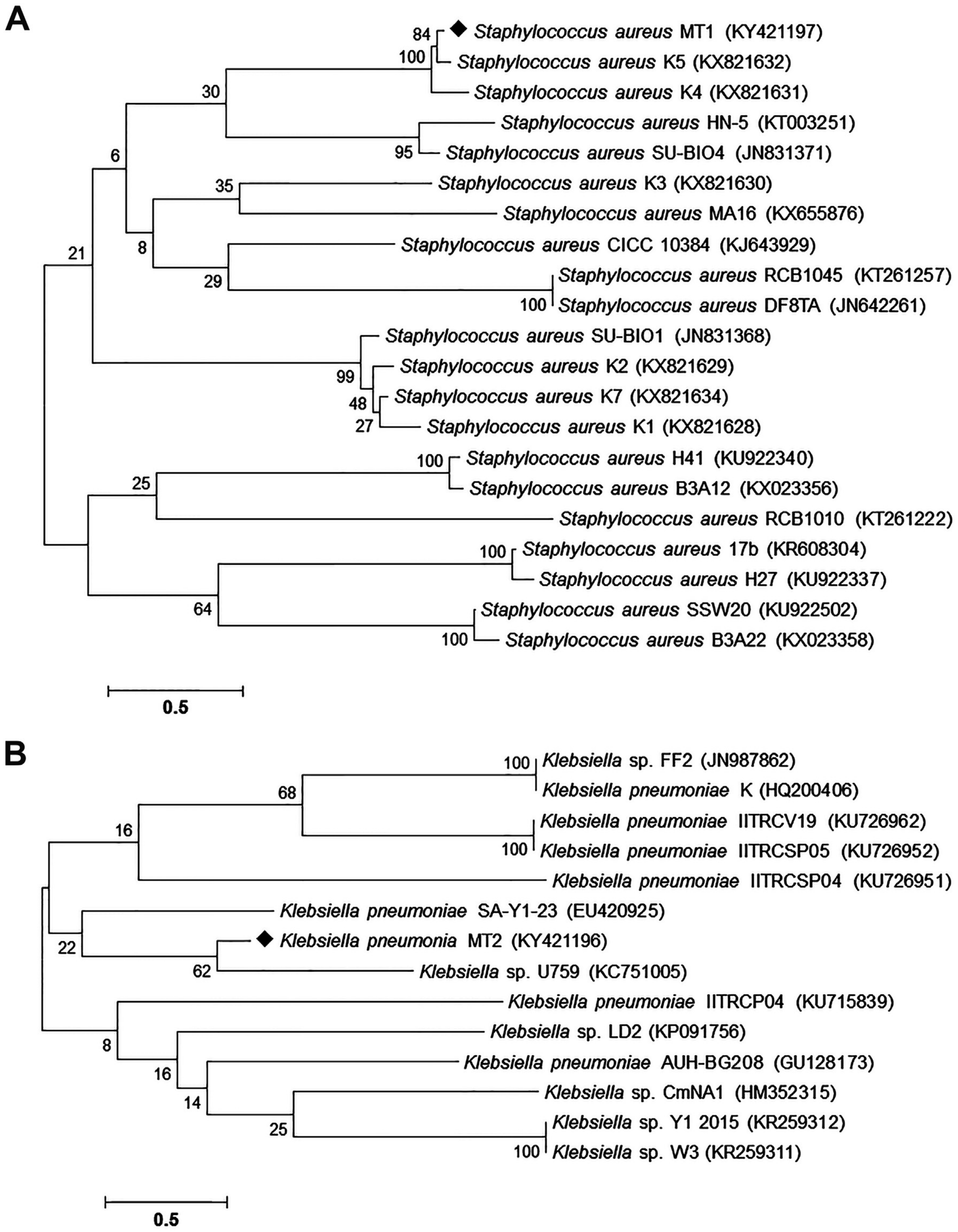

Fig. 3. Phylogenetic tree of (A) *S. aureus* MT1 (KY421197) and (B) *K. pneumonia* MT2 (KY421196) within the closest strains. The tree was structured via neighbor-joining tree approach including bootstrap values for 500 replicates. The accession numbers of the 16S rRNA nucleotide sequences are illustrated in parentheses.

results by **Turhan, Mutluoglu** [10] that showed monomicrobial and polymicrobial infections percent about 83.5% and 16.4%, respectively. Moreover, the present findings are supported by previous studies, which found the dominance of monomicrobial infections [22,23]. Similarly, **Banu, Noorul Hassan** [24] described that all DFI specimens produced monomicrobial isolates; however, another research reported 56.6% and 50% of monomicrobial infections [2]. These findings could be explained based on the duration of DFIs, ulcer grade and empirical antimicrobial agents. Furthermore, in the first episode of DFIs, the monomicrobial etiologies arise as common infections, and with the time progress, the polymicrobial infections emerge significantly. In this study, because of the prior use of several antibiotics to manage the DFIs, microorganisms could withstand the multidrug treatment were exclusively cultured. Other reports contradict these findings, which recorded the higher frequencies of polymicrobial than

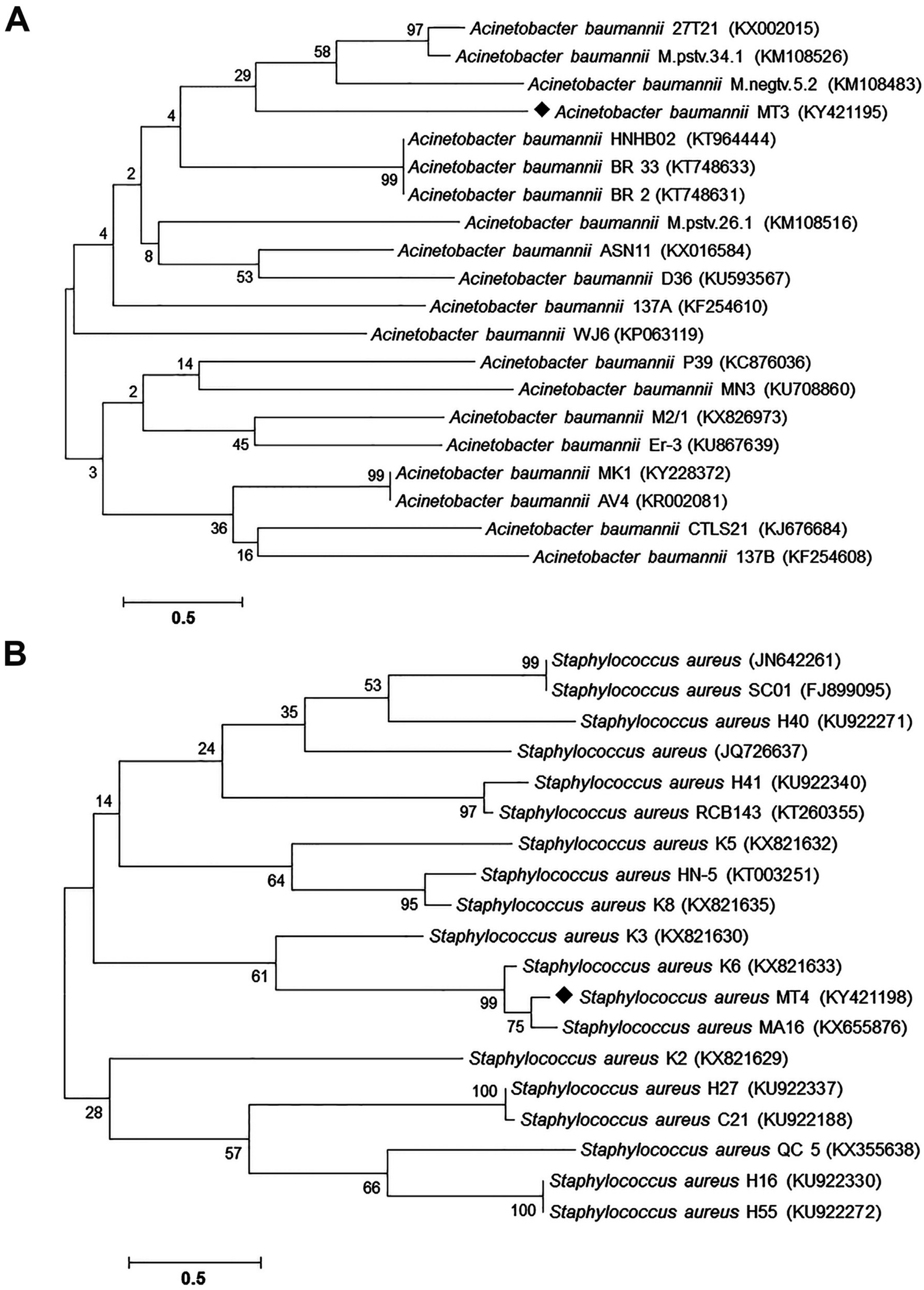

Fig. 4. Phylogenetic tree of (A) *A. baumanni* MT3 (KY421195) and (B) *S. aureus* MT4 (KY421198) within the closest strains. The tree was structured via neighbor-joining tree approach including bootstrap values for 500 replicates. The accession numbers of the 16S rRNA nucleotide sequences are illustrated in parentheses.

monomicrobial infections that accounted for 83%, 66%, and 55.7% [7,25,26]. This discrepancy could be explicated by the fact that most surveys depend on surveillance of microbiologist through isolation of the normal microbial flora and the pathogenic isolates without concern of patient's history, particularly the previous antibiotics scenario. Although previous investigations reported that Gram-negative bacteria developed as dominant in DFIs [10,16], our results demonstrated the prevalence of Gram-positive bacteria that

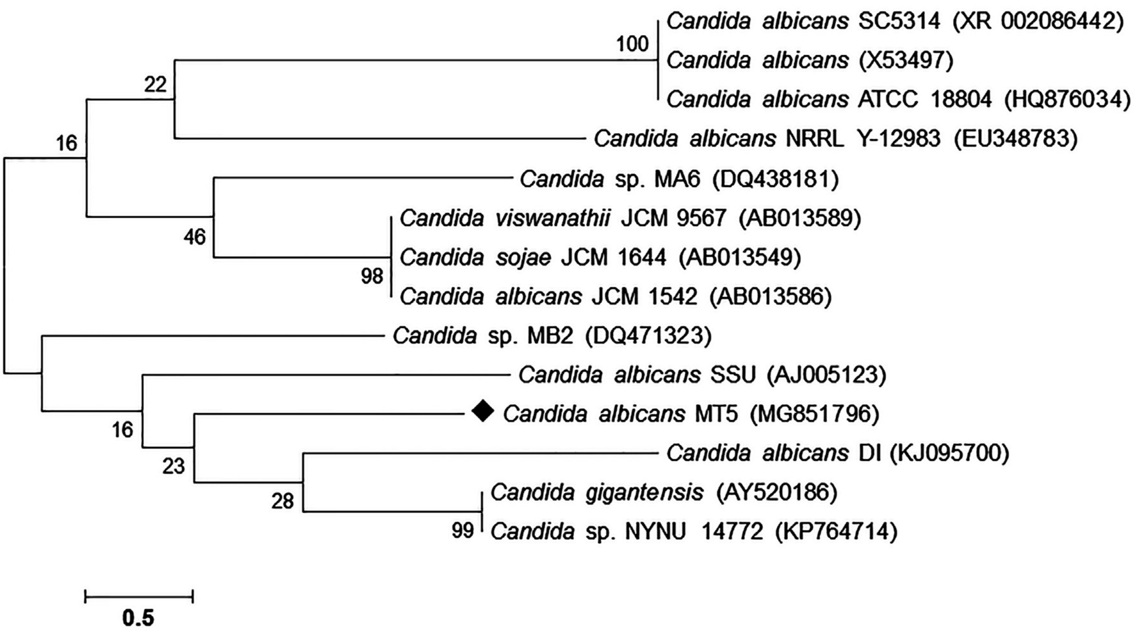

Fig. 5. Phylogenetic tree of *C. albicans* MT5 (MG851796) within the closest strains. The tree was structured via neighbor-joining tree approach including bootstrap values for 500 replicates. The accession numbers of the 16S rRNA nucleotide sequences are illustrated in parentheses.

match with the earlier reports [27,28]. The variations of MDR bacteria in DFIs might be ascribed to many factors; for instance, the demographic, difference in age, gender, the control of glycemia, ulcer evaluations, hospitalization duration and previous use of antibiotics treatment. Moreover, the hospitalization could significantly affect the presence and type of MDR organisms on DFIs, where patients are subjected to cross-infection by the colonization of nosocomial pathogens that resist most prescribed antibiotics and might be skin commensal.

It is worth pointing out in this context that the limited nutrient and moisture contents of normal skin have a crucial role via reducing the multiplications of fastidious bacteria, which can consequently decrease Gram-positive bacteria [29]. Conversely, purulent wound provides a rich environment that might promote the growth of a larger diversity of microorganisms mainly *S. aureus*, and *P. aeruginosa*. Earlier investigation in generated diabetic ulcer in animal model explained the extensive alterations of the bacterial type in DFIs on the basis of time progress and chronic and acute wounds [30]. The development of fungal pathogens in DFIs is one of the critical factors due to the fungal combating to the most conventional antibiotics and their intimate with such microbial infection is still not understood. **Chellan, Shivaprakash** [9] showed the dominance of fungal infections (27.9%) in deep tissues of diabetic lower limb wounds and *Candida parapsilosis* was the most common. In addition, another report demonstrated the percent of fungal infections only 9% of the cultured isolates [31]. On the other hand, our data pointed to the occurrence of a moderate percentage of fungal isolates (14.3%) of the whole microbial isolates compared to the previous results and found them mixed with bacteria.

Ampicillin, oxacillin, ampicillin/Sulbactam, cefoxitin, and ceftazidime account for the antibiotics with a β-lactam ring as main function group. Several Gram-negative bacteria particularly *Enterobacteriaceae* could produce extended-spectrum β-lactamases (ESBLs) to counter the action of these antibiotics and disintegrate their active group by binding and cleaving these bonds [32]. Our findings indicated that many extended-spectrum β-lactamases producers were generated including Gram-positive and negative bacteria. With regard to Gram-negative bacteria, we found out bacterial strains no 3, 8, 4, 3, and 4 were insusceptible to ampicillin, oxacillin, ampicillin/Sulbactam, cefoxitin, and ceftazidime, respectively. As a result of this occasion, DFIs became exceedingly complicated to manage the ESBL producers.

An earlier study proposed the recurrent treatment of bacterial strains β-lactam antibiotics could stimulate the mutation of β-lactamases in these bacteria expanding their activity versus the newly established β-lactam antibiotics through production of ESBLs [33]. In our research, this behaviour could be observed due to the previous persistent handling using broad-spectrum antibiotics. It is well known that carbapenems group are the backbone for the treatment of MDR bacteria in DFIs [34]. The carbapenems are broadly used for the treatment of serious infections triggered by ESBL-producers implicating *Enterobacteriaceae and K. pneumoniae* [35]. Likewise, *K. pneumonia* MT2 exhibited impedance against the used β-lactam antibiotics and other antibiotics except for amikacin and imipenem. In contrast, *A. baumannii* MT3 revealed insusceptibility to imipenem, which was typically mentioned for this bacterial genus by **Turhan, Mutluoglu** [10]. Moreover, a virulent *C. albicans* MT5 was found in the same DFI patient (No. 12) as a co-infected organism. Prior fungal inspection in DFIs illustrated the presence of pure fungal isolates (0.7%) and mixed fungal growth with bacteria (1.7%) representing various isolates involving *Candida albicans*, *Candida tropicalis*, *Candida* spp. *Aspergillus fumigatus*, and *Rhodotorula* sp [7].

Indeed, there is a close relation and mutual reaction of these two organisms that require upcoming accurate examinations to determine the contribution of each organism in DFIs. The future impact on this patient supports our assumption, in which, the patient's foot was amputated as a result of infections by uncontrolled microorganisms.

Furthermore, two bacterial strains belonging to *Staphylococcus* sp. were detected; one of them was sensitive only to imipenem and the other one showed susceptibility toward imipenem, and amikacin. These bacterial strains demonstrated resistance toward the rest of antibiotics including cefoxitin disk (30 μg); thus, they are methicillin-resistant strains according to the earlier study [36]. The identification of these strains utilizing 16S rRNA nucleotide sequences and phenotypic characteristics through VITEK verified our supposition that they are methicillin-resistant *S. aureus* (MRSA). These results are consistent with previous studies accomplished by **Mohammad, Abida** [22] and **Akhi, Ghotaslou** [36]. In a retrospective study at the Department of General Surgery, Al-Azhar University, Egypt, they successfully isolated Gram-positive bacteria included *S. aureus*, *Streptococcus pyogenes* and methicillin-resistant *S. aureus*, while Gram-negative were *Pseudomonas aeruginosa*, *K. pneumoniae*, and *Acinetobacter* sp [14]. Furthermore, recent studies concerning etiology of DFIs indicated to the materialization of MRSA strains and ESBL-producers such as *E. coli* and *Klebsiella* sp [37,38].

We found out most isolates were susceptible toward amikacin, which is in line with prior studies that contended its high efficacy [10,35]. Furthermore, surveillance of ciprofloxacin-resistant bacteria predisposes the patient to the high risk as this antibiotic is a broad-spectrum antibiotic that commonly used in hospitals.

This study has a remarkable flaw, in which, it is evident in the results the absence of anaerobes isolates. This materialized since the specimens were collected via swapping over the surface of pus wounds and the microbiological culturing approach were designed for obtaining the aerobes MDR organisms. This mainly was accomplished as the antibiotics therapy killed the vast majority of normal flora including non-pathogen and pathogen anaerobic microorganisms; however, the conventional microbiological cultures are still unfair to select only one physiological bacterial type. Accordingly, our screening would be expanded using molecular biology tools to determine the aerobic and anaerobic MDR microorganisms and to focus on the role of each microbe in DFIs. Latest studies recommend averting the common microbial cultures and identifying microorganisms by DNA sequencing technique that seems time-saving and precise approach to scrutinize the vast majority of obligate anaerobes organisms in wounds [16]. However, this report might be supportive to ameliorate the treatment strategy of DFIs as the treatment employing broad-spectrum antibiotics should prevent the persistent episodes DFIs.

Recent studies strongly suggested that molecular techniques, such as 16S rRNA amplification, identify a greater diversity of bacteria than standard culture methods [1]. Phylogenetic trees of the identified strains pinpoint their genus and potential species. However, the nucleotide sequences of *C. albicans* resemble with two genera on the GenBank, which required another approach to differentiate between them and validate the identification. This usually happens when full nucleotide sequences not in use for the homology analysis. Although identification similarities percentage for *K. pneumonia* and *A. baumannii* did not reach to the 98% as short nucleotide sequences were obtained from the gene sequencing, their genus and species could be clearly identified. For these reasons, the VITEK 2 system was employed to avoid any misidentification probabilities. The VITEK 2 system is widely applied in the hospital to expedite the identification process of microorganisms with maximum accuracy and the results might be reproduced. Generally, the GP, GN and YST cards of VITEK 2 can properly determine 96.5%, 96.8%, and 98.9% of the microbial isolates. Therefore, the misleading of microbial identification might be effectuated in the highest restricted range. Our future plan would focus on the preparing of antimicrobial wound dressing materials that could suppress these feared infections and boost the wound healing process in DFIs using these microbial isolates as reliable models.

5. Conclusion

Four bacterial strains could resist more than six antibiotics were isolated via swapping the pus wounds of DFIs. They were identified using molecular tools and phenotypic characteristics as *S. aureus* MT1, *K. pneumonia* MT2, *A. baumanni* MT3, and *S. aureus* MT4. Remarkably, the identified bacteria belong to a group of pathogenic bacteria, which termed as ESKAPE pathogens. We found out the *S. aureus* strains were methicillin-resistant; therefore, they belong to MRSA that account one of the most infectious organisms in DFIs. Imipenem demonstrated inhibition capacities toward the entire bacterial isolates except for *A. baumanni* MT3. Moreover, amikacin could significantly encounter about 83.3% of the total bacterial isolates. According to antifungal susceptibility patterns, one yeast isolate was opted and identified employing 18S rRNA nucleotide sequences and biochemical tests as *C. albicans* MT5. This study provided valuable information concerning DFIs in Alexandria, which might help to prevent further severe complications particularly the amputation of the extremity limbs.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsx.2019.01.044.

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