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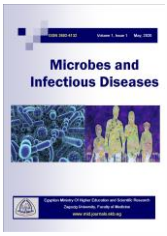

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Original article

Measuring the minimum biofilm eradication concentration for bacterial isolates from diabetic foot infections

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ABSTRACT

Background: Diabetic foot infection (DFI) is considered the most common cause of diabetes-related hospitalization. Diabetic foot ulcers are subjected to bacterial colonization with biofilm forming organisms which are difficult to eradicate. The aim of this study was to identify the spectrum of bacteria associated with DFI and their ability to form biofilm, to evaluate differences in antibiotic susceptibility pattern between planktonic and biofilm phases, and to determine the antibiotics which are active on the organism in the biofilm phase. **Methods:** The study comprised 50 patients with DFI. A deep swab was collected from each patient and cultured. All isolates were identified and screened for biofilm formation. Biofilm forming isolates were further subjected to minimum biofilm eradication concentration (MBEC) assays to determine resistance to different antimicrobials while in the biofilm phase. **Results:** Seventy-one isolates were identified, (14.1%) were Gram positive cocci, (83.1%) were Gram negative bacilli, and (2.8%) were Candida species. The most frequently isolated organism was Klebsiella spp. (18/71, 25.4%), followed by Proteus spp. (14/71, 19.7%). The prevalence of biofilm forming isolates was 38%. All the studied isolates showed MBEC higher than the MIC for all tested antimicrobials**. Conclusions:** The substantial discrepancy between MIC and MBEC results observed in this study emphasizes the lack of reliability of the routine antimicrobial susceptibility testing in case of biofilm formation. Among all tested antimicrobials; cefoperazone/sulbactam, gentamicin, and vancomycin demonstrated activity against bacteria in the biofilm phase.

Introduction

Diabetes mellitus (DM) is a serious health challenge that is rapidly expanding worldwide. It is one of the commonest chronic diseases all over the world, and it is continuously increasing in number and significance, it is estimated that in 2030 DM will be the seventh leading cause of death [1]. Patients with DM are at risk of complications all the time either macrovascular, microvascular or both (as in diabetic foot) [2].

Diabetic foot infection (DFI) is defined as colonization and invasion of a foot wound by pathogenic microorganisms leading to local tissue damage, this is favored by hyperglycemia [3]. Once the protective layer of the skin is broken, the deeper tissues are exposed to bacterial colonization, these bacteria may express many virulence factors

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including biofilm formation which may have a role in DFI chronicity [1].

Diabetic foot infection is mostly polymicrobial in nature, both Gram positive (*Staphylococcus aureus*, *Enterococcus* spp.) and Gram negative (*Pseudomonas aeruginosa*, *E. coli*, *Klebsiella* spp, *Proteus* spp, etc.) have been incriminated in DFI in addition to anaerobic bacteria [4-6].

Biofilm are aggregates of microorganisms in which cells are frequently embedded in a self¬-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface. About 65-80% of all bacterial infections are associated with bacterial biofilms which can be device- associated or non-device-associated infections. The non-device-associated infections include; chronic wound infections, lung infections in cystic fibrosis patients, chronic otitis media, chronic sinusitis, endocarditis and osteomyelitis [7-9]. An example of chronic wound infections is the infected diabetic foot ulcers (DFUs), which constitute a great clinical burden to patients and are characterized by the presence of microbial biofilms that result in delayed healing because it acts as a barrier which protects the organisms from phagocytosis and limits the diffusion of antibiotics [10,11].

Antimicrobial treatment can suppress the symptoms of infection by killing free-floating bacteria shed from the attached population, but often fails to eradicate bacterial cells still embedded in the biofilm [12]. After cessation of antimicrobials the biofilm can act as a nidus for recurrence of infection [9]. In addition, as antimicrobial concentrations which are sufficient to kill planktonic organisms are generally inadequate to kill biofilm organisms, especially those within core, selection of resistant subpopulations is a likelihood [10].

The aim of this study was to identify the spectrum of bacteria associated with DFI, to assess the biofilm formation by the clinical isolates from DFI, to evaluate differences in antibiotic susceptibility pattern between planktonic and biofilm phases of these clinical isolates and to determine the antibiotics which are active on the organism in the biofilm phase.

Patients, Materials and Methods

A descriptive case series study was conducted on patients with type 2 DM presenting with infected DFU admitted to the vascular surgery department in Alexandria Main University Hospital AMUH from February 2019 to July 2019. An informed consent was taken from all patients. A detailed medical history including a record of age, sex, duration of the ulcer and duration of DM was taken. Ulcers were examined for signs of infection (swelling, exudates, surrounding erythema, cellulitis, bad odor, tissue necrosis and crepitation).

-Inclusion criteria: Only patients with infected DFUs were included in the study. Each patient was included only once.

-Exclusion criteria: Patients presented with uninfected ulcers (vascular or neuropathic) were excluded from the study.

Specimen collection and processing

A deep swab was taken from the depth of DFU after thorough rinsing of the ulcer with sterile saline. The swab was transported in amies transport medium (Oxoid,UK) to the Diagnostic Microbiology Laboratory in AMUHwithin 2 hours of collection. The specimen was inoculated onto blood and MacConkey's agar, incubated aerobically overnight at 37°C. An additional plate of Sabouraud dextrose agar (SDA agar) was inoculated and incubated for 48 hours. All isolates were identified according to the standard microbiological procedures including; colonial morphology, Gram stained film, and biochemical reactions [13]. The susceptibility of all isolates to antimicrobial agents was determined by the Standard Kirby–Bauer disc diffusion technique, (antimicrobial discs were obtained from Oxoid, UK), and interpreted in accordance with the guidelines established by the CLSI [14].

Biofilm detection using tissue culture plate (TCP) method:

All isolates were screened for biofilm formation by TCP method [15,16]. A 0.5 McFarland standardized suspension of each isolate was prepared in trypticase soya broth (TSB). For each isolate, 200 µl of suspension were inoculated into each of three successive wells of a sterile flat bottomed 96-well microtiter plate. Three wells containing uninoculated TSB were used as negative control. The plate was incubated aerobically overnight at 37°C then washing of the plate was performed three times using sterile phosphate-buffered saline (PBS) to remove planktonic cells. Methanol was added to

the wells for 15 minutes to fix any adherent bacteria. Then a washing step using PBS. Crystal violet was added for 15 minutes to stain the adherent biofilm layer, followed by a washing step (3 times using sterile distilled water). The plates were then left to air dry. Ethanol 95% was added to the dried wells for 30 minutes for re-solubilization of the stain. Finally, the optical density (OD) of each well was measured at 630 nm using ELISA reader (Stat Fax 2100 microplate reader) [16-19]. The average OD value of negative control wells as well as the three wells of each sample was calculated. The cut-off optic density (ODc) was determined as three standard deviations above the mean OD of the control (uninoculated TSB). Isolates were classified as non-biofilm producer, weak, moderate and strong biofilm producer according to the following equations:

OD sample (ODs) ≤ ODc = non-biofilm producer.

ODc < ODs ≤ 2ODc = weak biofilm producer.

2ODc < ODs ≤ 4ODc = moderate biofilm producer.

4ODc < ODs = strong biofilm producer [16,18].

Reference strains; *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922, were used as positive and negative controls respectively [15].

Biofilm forming isolates were subjected to: Measuring the minimum inhibitory concentration (MIC) of different antimicrobials and determining the minimum biofilm eradication concentration using the (MBEC) assay.

MIC was measured by VITEK 2 Compact system (Biomérieux), GP AST 67 card was used for Gram positive organisms, GN AST 71 for Gram negative fermenter organisms, N 222 for non-fermenters, and AST YS 08 for *Candida* [20]. For levofloxacin and cefoperazone/sulbactam antibiotics which were not included in the VITEK card, MIC was measured according to CLSI guidelines by broth microdilution technique [14]. In case of cefoperazone/sulbactam, the package insert was used as reference for interpreting the results [20].

MBEC assay

a- Biofilm formation: For inoculum preparation; bacterial colonies were collected from overnight culture plate and suspended in TSB to achieve 1.0 McFarland. The suspension was diluted 1:30 in TSB to obtain 1x107 CFU/ml, 150 µl of this dilution was added to each well of the microtiter plate. Sterile autoclaved PCR tubes were used as pegs that were inserted into the wells containing the bacterial suspension to provide a surface for biofilm formation, then plates were covered and incubated overnight at 37° C **(Figure 1)**.

Reference strains; *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922, were used as positive and negative controls for biofilm formation respectively [15].

b-Biofilm susceptibility testing: Serial double fold dilutions of the antimicrobials were prepared with cation adjusted Muller Hinton broth (CAMHB), starting with concentration of 512 µg/ml to 0.25 µg/ml and distributed into the rows of a microtiter plate. Pegs with biofilms formed on its surface were then transferred to the antibiotic plate, the plate was incubated overnight at 37˚C. After overnight incubation, pegs were removed from the plate and rinsed in PBS. Pegs were then transferred to a second recovery plate containing only CAMHB, mechanical disruption of biofilms was done by simply shaking the plate on a shaker for 2 minutes, then the plate was further incubated overnight at 37˚C. MBEC was defined as the minimal antimicrobial concentration at which bacteria fail to regrow after antimicrobial exposure. It was determined by checking turbidity visually in the wells of recovery plate [15,21].

For Gram positive biofilm forming isolates, MBEC values of levofloxacin, gentamicin, vancomycin, linezolid, doxycycline and clindamycin were determined while for Gram negative biofilm former, levofloxacin, gentamicin, cefepime, meropenem and cefoperazone/sulbactam were used.

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean and standard deviation. Significance of the obtained results was judged at the 5% level.

Results

A total of fifty patients with infected DFU were admitted to the vascular surgery department in AMUH during the study period. Their mean age was 55.9 ± 9.2 years and females constituted 52%. **Spectrum of organisms:** From the 50 specimens, 71 isolates were identified. The infection was mono-microbial in 30 specimens (60%), while in the other 20 (40%) it was poly-microbial. From the 71 isolates, 10 (14.1%) were Gram positive cocci, 59

(83.1%) were Gram negative bacilli, and two (2.8%) were *Candida* spp. The most frequently isolated organism was *Klebsiella* spp. (18/71, 25.4%), followed by *Proteus* spp. (14/71, 19.7%), whereas the least encountered isolates were; *Stenotrophomonas*, *Citrobacter*, and *Morganella* spp. (1/71). *Klebsiella* spp. was the most frequently encountered isolate from mono-infected ulcers (8/30, 26.7%), followed by *E. coli*, *Proteus* spp., and *Pseudomonas* spp. (4/30, 13.3% for each). On the other hand, *Acinetobacter* and *Citrobacter* spp. were the least frequently isolated (one for each, 3.3%). Of note, the two *Candida* isolates were identified to the species level as *Candida tropicalis* and *Candida parapsilosis* using the Vitek apparatus.

Organisms isolated from poly-microbial infection: The most frequently encountered combinations were *Proteus* & *Pseudomonas* spp., and *Proteus* & *Klebsiella* spp. (20% of polymicrobial infections for each combination), followed by *Klebsiella* & *Pseudomonas* spp. (15 %), then *E. coli* & *Enterococcus* spp. (10% of polymicrobial infections)

Results of biofilm formation: Out of 71 isolates, 16(59.2%) produced weak biofilms, 7 (26%) moderate strength, and 4 (14.8%) produced strong biofilms. The four strong biofilm producing isolates comprised one *Proteus* and three *Pseudomonas* isolates. The only isolated *Stenotrophomonas* strain was proved to be biofilm forming. Eight (72.7%) of *Pseudomonas* isolates were biofilm producers, whereas 57.1% (8/14 isolates) of *Proteus* produced biofilm. Each of *Acinetobacter*, *Candida* and coagulase negative staphylococci (CoNS) demonstrated 50% biofilm production. Lower percentages were detected among *Enterococcus*, *Staphylococcus aureus*, and *Klebsiella* 40%, 33.3%, and 22.2% respectively. On the other hand; *Citrobacter*, *E. coli*, *Enterobacter*, *Morganella* were negative for biofilm production

Comparison between resistance rates among non-biofilm and biofilm forming isolates by disc diffusion method: Regarding resistance rates to all tested antimicrobials, there was no statistically significant difference between non-biofilm and biofilm forming isolates while in the planktonic phase ($p > 0.05$).

The two CoNS isolates were methicillin resistant as well as the three *Staphylococcus aureus* isolates were MRSA.

Antimicrobial resistance of biofilm forming isolates in planktonic versus biofilm phase:

All tested antibiotics showed MBEC higher than the MIC values for all the studied isolates (up to more than 11 folds increase for most isolates) **(Figure 2)**. There was a statistically significant difference between the planktonic and biofilm phase of *Proteus* isolates regarding resistance to meropenem, cefepime, and cefoperazone/sulbactam (p=0.003, 0.02, and 0.008 respectively) **(Table 1,2)**. The four *Klebsiella* isolates were resistant to cefepime when tested in the biofilm phase, whereas none of these isolates showed resistance to the same drug while in the planktonic phase **(Table 3)**. This finding was statistically significant (p=0.02). The difference in antimicrobial resistance profile between the planktonic and biofilm phase of *Pseudomonas* isolates was statistically significant only with cefepime (p=0.03) **(Table 4,5)**. With the exception of gentamicin, all tested antimicrobials failed to eradicate the biofilm formed by the eight *Pseudomonas* isolates. On the other hand, out of the three isolates that were susceptible to gentamicin in the planktonic phase, one remained susceptible, one changed to intermediately susceptible, and the last turned resistant with an increase from MIC values of <1, 4, 4 µg/ml to MBEC values of 2, 8, >512 µg/ml respectively.

Cefoperazone/sulbactam was effective against S*tenotrophomonas* in the planktonic as well as in the biofilm phase, while the organism was susceptible to levofloxacin only in the planktonic phase.

According to the MIC values, CoNS isolate was susceptible to vancomycin, linezolid, doxycycline, and clindamycin. Nevertheless, when measuring the MBEC values, it turned resistant to these drugs except vancomycin, to which the isolate showed intermediate susceptibility.

The *Staphylococcus aureus* isolate demonstrated resistance to vancomycin, linezolid, doxycycline, and clindamycin only in the biofilm phase. When tested in the biofilm phase, the two *Enterococcus* isolates were resistant to all tested antimicrobials. However, in the planktonic phase susceptibility to some of the tested antimicrobials was detected.

Table 1. Comparison between MIC and MBEC values for each *Proteus* biofilm forming isolate (n=8)**.**

AB/isolate no.		1	2	3	4	5	6	7	8
Gentamicin	MIC	>16	>16	4	8	>16	>16	>16	8
	MBEC	>512	>512	>512	>512	>512	>512	>512	>512
Meropenem	MIC	<0.25	<0.25	<0.25	<0.25	<0.25	>16	<0.25	<0.25
	MBEC	>512	>512	>512	64	128	>512	512	>512
Cefepime	MIC	>64	<1	8	<1	<1	16	>64	<1
	MBEC	>512	>512	>512	128	256	>512	>512	512
Cefoperazone/ sulbactam	MIC	32	2	4	2	1	256	256	8
	MBEC	>512	>512	>512	>512	>512	>512	>512	>512

Table 2. Interpretation of MIC and MBEC results for *Proteus* biofilm forming isolates

Antibiotic	***Proteus* (n=8)**								**Test of significance (*p*)**
	MIC				**MBEC**				
	R	**I**	**S**	**SDD**	**R**	**I**	**S**	**SDD**	
Gentamicin	5	2	1	-	8	0	0	-	MCP=0.157
Meropenem	1	0	7	-	8	0	0	-	MCP=0.003*
Cefepime	3	0	4	1	8	0	0	0	MCP=0.026*
Cefoperazone/ sulbactam	2	1	5	-	8	0	0	-	MCP=0.008*

*There was a statistically significant difference between the planktonic and biofilm phase of *Proteus* isolates regarding resistance to meropenem, cefepime, and cefoperazone/sulbactam (p=0.003, 0.02, and 0.008 respectively).

Table 3. Interpretation of MIC and MBEC results for *Klebsiella* biofilm forming isolates

Antibiotic	***Klebsiella* (n=4)**								**Test of significance (*p*)**
	MIC				**MBEC**				
	R	**I**	**S**	**SDD**	**R**	**I**	**S**	**SDD**	
Levofloxacin	3	0	1	-	4	0	0	-	MCP=0.9
Gentamicin	1	0	3	-	4	0	0	-	MCP=0.14
Meropenem	1	0	3	-	4	0	0	-	MCP=0.14
Cefepime	0	0	3	1	4	0	0	0	MCP=0.02*
Cefoperazone/ sulbactam	2	0	2	-	4	0	0	-	MCP=0.4

* There was a statistically significant difference between the planktonic and biofilm phase of *Klebsiella* isolates regarding resistance to cefepime (p=0.02).

Table 4. Comparison between MIC and MBEC values for each *Pseudomonas* biofilm forming isolate (n=8).

AB/isolate no.		1	2	3	4	5	6	7	8
Levofloxacin	MIC	1	32	128	>256	128	8	>256	64
	MBEC	128	>512	>512	>512	>512	128	>512	>512
Gentamicin	MIC	4	>16	4	>16	>16	<1	>16	>16
	MBEC	>512	>512	8	>512	>512	2	>512	>512
Meropenem	MIC	4	>16	<0.25	<0.25	>16	<0.25	>16	8
	MBEC	>512	>512	>512	>512	>512	512	>512	>512
Cefepime	MIC	2	>64	8	<1	8	<1	>64	>64
	MBEC	>512	>512	512	>512	>512	128	>512	128
Cefoperazone/ sulbactam	MIC	4	>256	16	>256	8	4	>256	128
	MBEC	>512	>512	>512	>512	>512	>512	>512	>512

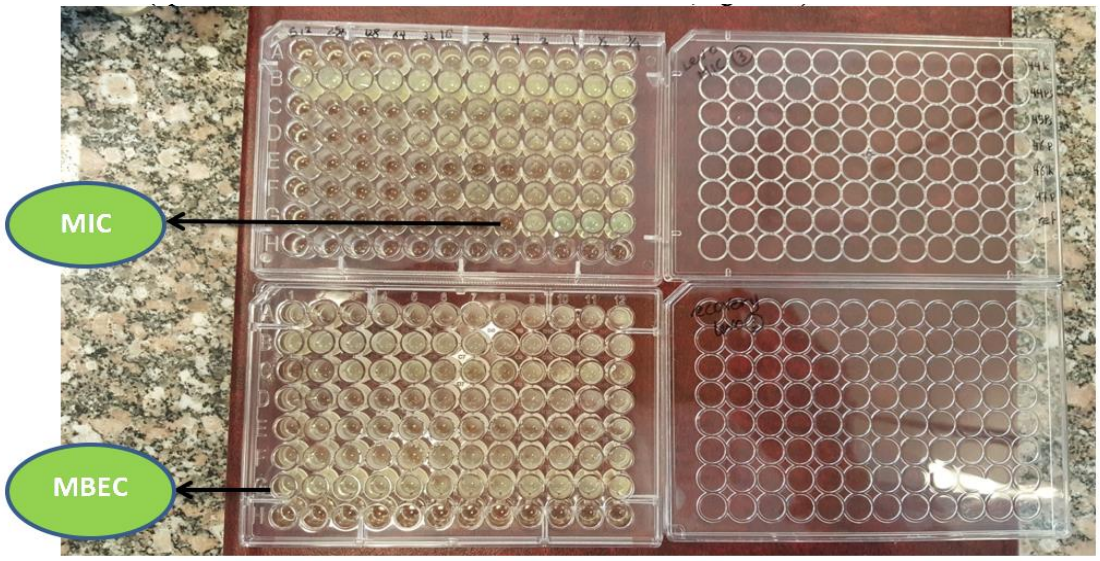
Table 5. Interpretation of MIC and MBEC results for *Pseudomonas* biofilm forming isolates

Antibiotic	***Pseudomonas* (n=8)**								**Test of significance (*p*)**
	MIC				**MBEC**				
	R	**I**	**S**	**SDD**	**R**	**I**	**S**	**SDD**	
Levofloxacin	7	0	1	-	8	0	0	-	MCP= 1
Gentamicin	5	0	3	-	6	1	1	-	MCP=0.35
Meropenem	4	1	3	-	8	0	0	-	MCP=0.07
Cefepime	3	0	5	0	8	0	0	0	MCP=0.03*
Cefoperazone/ sulbactam	4	0	4	-	8	0	0	-	MCP=0.08

* There was a statistically significant difference between the planktonic and biofilm phase of *Pseudomonas* isolates regarding resistance to cefepime (p=0.03)

Figure 1. Bacterial suspension with autoclaved pegs to act as surface for biofilm growth

Figure 2. MIC versus MBEC results of levofloxacin.



Discussion

Diabetes mellitus is one of the most challenging worldwide health problems. With the rising prevalence of DM, there is increasing problem of infections, particularly foot infections which is considered the most common cause of diabetes-related hospital admissions [22].

Diabetic foot infections have long been recognized to be poly-microbial in nature with Gram positive cocci being the most commonly isolated organisms [23]. However, the majority of studies conducted during the last two decades in western countries documented that unless antibiotics have been used prior, cultures from acute diabetic foot wounds grow a single pathogen [24].

In this study, 60% of the specimens revealed a single pathogen, while 40% were poly-microbial in nature, which is consistent with **Turhan et al.** [25]. On the contrary, **Jneid et al.** [6]; found that most of their samples (88.3%) were polymicrobial, which is well known for DFI. A limitation in our study is the lack of anaerobic culture, which excluded anaerobic bacteria that are not uncommon in DFIs [26], and may have also contributed to the predominance of the monomicrobial nature of our specimens.

The majority of our isolates (83.1%) were gram negative bacteria [*Klebsiella* spp. (25.4%), followed by *Proteus* and *Pseudomonas* spp. (19.7% and 15.5% respectively)], while gram positive bacteria constituted only 14.1% of isolates with Enterococcus the most prevalent Gram positive isolate, a finding which is not common in other studies [26,27]. The predominance of Gram negative organisms is consistent with some previous studies [25,28]. Indeed, the causative agent of DFI is influenced by several factors including; personal hygiene, duration of ulcer, state of glycemic control, ongoing or previous antimicrobial treatment and geographic origin of the patient which is one of the most important factors; in warmer countries (especially Asia and Africa), Gram negative bacilli are more prevalent compared to western countries [29,30].

The prevalence of biofilm- forming isolates in this study was 38% which is more or less in agreement with some other studies [18,28,31]. However, **Malik et al.** reported 67.9% prevalence of biofilm producing isolates from DFI [32]. The difference in the rate of biofilm production between different studies may be due to the variability of the prevalent bacterial species having different tendencies to produce biofilm also the incubation conditions may affect the result of biofilm formation. **Sanchez et al.** has reported that biofilm formation in *Staphylococcus aureus* increases with the duration of incubation, showing a significant increase at 72 hours incubation, while it was low at the end of the first 24 hours [33].

In this study, the most frequent biofilm forming isolates were *Pseudomonas* and *Proteus* spp. (8/27 isolates for each), followed by *Klebsiella* spp. (4/27 isolates), however, this finding may be affected, to some extent, by the predominance of these species among our isolates (together, they constituted around 61% of isolates, 43/71).

In order to evaluate the genuine biofilm-forming tendency of each species, we calculated the percentage of biofilm forming isolates of each spp. from its own total. In this way, the highest rate of biofilm formation was detected with *Stenotrophomonas* spp. (100%), followed by *Pseudomonas* and *Proteus* spp. (72.7% and 57.1% respectively). **Bonaventura et al.** reported 88% prevalence of biofilm formation among *Stenotrophomonas* (44 out of 50 isolates) [34]. This is consistent with what is known about *Stenotrophomonas* of being a pathogen with low virulence, yet its ability to form biofilm is an important virulence factor.

A pure growth of *Candida* spp. was detected in two specimens (2/50, 4%). They were identified as *Candida tropicalis* and *Candida parapsilosis*, the former was found to be biofilm producer. A much higher prevalence was documented by *Kumar et al*, where, *Candida* spp represented 48.4% of the etiological agents of DFIs, with *Candida tropicalis* being the most frequently isolated spp. [35]. It was, therefore, realized that non-albicans *Candida* spp. with biofilm forming ability are emerging as an important cause of the problematic condition of DFIs [35,36].

All biofilm forming isolates showed MBEC values higher than the MIC for all tested antibiotics (up to more than 11 folds increase from < 0.25 to > 512µg/ml) which was reflected on the interpretation of antimicrobial susceptibility results from being susceptible in the planktonic phase (MIC) to resistant in the biofilm phase (MBEC). Similarly, in a study performed on Gram negative bacteria isolated from peritonitis patients, higher MBEC values, compared to MIC values, were reported with decrease in the number of susceptible organisms in the biofilm phase [15,21].

Apart from gentamicin, all other tested drugs on pseudomonas isolates demonstrated resistant MBEC values, even with isolates that were susceptible based on the MIC values. This was in agreement with **Sepandj et al.** [21] a finding which highlights the utility of aminoglycosides rather than beta lactams for eradicating Pseudomonas biofilm. It was also observed in this study that two out of the four Klebsiella isolates, despite being resistant to gentamicin in the biofilm phase, both demonstrated relatively low gentamicin MBEC values (32 and 64 µg/ml) compared to other tested antimicrobial agents. This may suggest gentamicin as a candidate for further evaluation for biofilm produced by Gram negative bacteria, particularly *Pseudomonas*, despite the previous reports regarding the limited activity of positively charged aminoglycosides on biofilm.

Regarding the eight biofilm producing *Proteus* isolates detected in our study, there was a statistically significant increase in MBEC values compared to MIC values for meropenem, cefepime, and cefoperazone/sulbactam. Similar observation was reported by **Abdallah et al.** regarding the effect of amikacin and imipenem on *Proteus* biofilm [37].

In our study, cefoperazone/sulbactam demonstrated good activity against the individual Stenotrophomonas isolate (MIC 8 µg/ml). Interestingly, the drug was also effective in eradicating *Stenotrophomonas* biofilm (MBEC 16 µg/ml). This finding recommends cefoperazone/sulbactam for further research regarding its activity against *Stenotrophomonas* biofilm. On the other hand, levofloxacin exerted an activity against the organism in the planktonic phase (MIC 0.25 µg/ml) but not in biofilm phase (MBEC 32 µg/ml).

In this study, cultures performed on DFIs revealed three MRSA isolates, of which, only one produced a biofilm. Despite being susceptible to vancomycin, linezolid, doxycycline, and clindamycin when tested in the free floating form, it was found to be resistant to all four drugs when tested in the biofilm state. This finding was in agreement with **Smith et al.** [38]. It is worth mentioning that doxycycline demonstrated a relatively low MBEC for the tested Staphylococcus aureus isolate compared to other tested antibiotics (32 µg/ml versus > 512 µg/ml).

On comparing the MIC and MBEC values for the CoNS isolate, it was found that the isolate turned from vancomycin susceptible, based on MIC result, to intermediately susceptible based on MBEC result, whereas, it turned from susceptible to resistant when tested against linezolid, clindamycin, and doxycycline. In the light of this finding, further investigations of the role of vancomycin in eradicating CoNS biofilm may be worthy. Additionally, doxycycline demonstrated the lowest MBEC values (16 µg/ml) compared to linezolid (128 µg/ml), and clindamycin (> 512µg/ml). Same observation was detected with MRSA isolate in this study, therefore, the effect of doxycycline on staphylococcal related biofilm infections may

require further exploration on a large number of isolates [39].

Both biofilm forming *Enterococcus* isolates were resistant to levofloxacin, gentamicin, vancomycin, and linezolid in the biofilm phase with very high MBEC values (>512µg/ml for most antimicrobials) similar results were achieved by **Zaborowska et al.** [40].

Conclusion

Although the MIC assay is considered the gold standard for antimicrobial susceptibility testing, it does not reflect the actual bacterial biofilm behavior. The substantial discrepancy between MIC and MBEC results observed in this study emphasizes the lack of reliability of the routine antimicrobial susceptibility testing in patients with biofilm associated DFIs. The results of the MBEC assay are more closely reflective of the clinical response. It was postulated by many researchers that the use of biofilm relevant susceptibility tests may improve patient outcomes by enabling correct antimicrobial regimens to be rapidly identified, reducing treatment failure and halting the spread of antimicrobial-resistant strains. The MBEC assay provides an alternative for determination of antimicrobial susceptibility of biofilm forming bacteria, however, its use routinely in the microbiology diagnostic lab is discouraged being tedious and time consuming. Nevertheless, it can be reserved to cases lacking clinical response to antimicrobial therapy or recurrent infection caused by the same organism.

Biofilms were resistant to antibiotic concentrations, up to more than eleven times higher than the concentrations needed to kill free living cells. Moreover, many strains failed to be eradicated by the highest concentration of the antimicrobial agent used (512µg/ml), which makes such infections extremely difficult to eradicate using mono-therapeutics. Additional therapeutic approaches are needed such as topical antibiotics, debridement to remove biofilm followed by more topical treatment, alongside systemic antibiotics to kill any planktonic cells dislodged. Furthermore, new modalities in treatment of DFI should be sought. Hyperbaric oxygen treatment, platelet-derived growth factors, and granulocyte colony-stimulating factor (G-CSF) are new promising lines of treatment for chronic DFI.

Among all tested antimicrobials only; cefoperazone/sulbactam, gentamicin, and vancomycin demonstrated activity against bacteria in the biofilm phase. This study highlights the importance of defining the clinical susceptibility of DFIs in-vitro using methods that are relevant to the biofilm phenotype in-vivo, and highlights how current planktonic-based antimicrobial susceptibility tests are often misleading.

Recommendations

The current insufficient evidence to recommend antibiotics on the basis of biofilm susceptibility testing is mainly attributed to lack of standardized antimicrobial breakpoints thus, new guidelines are required for bacteria in the biofilm phase. Also, detection of the effect of combination therapy on biofilm forming bacteria is required to improve the clinical outcome. Therefore, MBEC assay adopting different antimicrobial combinations could be designed. Clinical trials are still mandatory to correlate the MBEC results with clinical outcome.

Conflict of interest : Nothing to declare

Financial disclosures: Nothing to declare

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