Microbial profiling of wound pathogens in isolates from an Egyptian hospital using a microarray chip

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ABSTRACT

Chronic polymicrobial infections represent diagnostic challenges for both molecular and culture methods. Chronic wounds, inducing chronic pain and reducing the mobility of individuals, have a notable effect on the quality of life. At any given time, 1% of the population is usually affected and, therefore, multiple treatments are also required. Identification of the etiology of such infections facilitates the appropriate treatment. In this study, 20 wound samples were collected from 20 patients with suspected wound infection. The panel of the current assay targets 12 clinically relevant aerobic pathogens, commonly associated with chronic wound infection. Microbial wound infections were detected by both usual polymerase chain reaction (PCR) and subsequently testing using the DNA chip. In the current study, the results of culture-free bacterial identification using the two methods of DNA analyses were compared. By molecular detection using PCR, seven different bacterial species were identified: *Citrobacter* spp. (100%), *Enterobacter* spp. (100%), *Klebsiella pneumoniae* (100%), *Pseudomonas aeruginosa* (90%), *Proteus* spp. (80%), *Escherichia coli* (60%), and *Staphylococcus aureus* (10%). Mixed microbial infections were detected in all samples indicating four, five, or six different bacteria, identified in each sample. Microarray detection in comparison to PCR indicated 100% matching. These results demonstrate the possibility of fast identification of wound infection pathogens even in a mixed culture in a very short time, which in turn facilitates the proceeding steps for proper treatment.

INTRODUCTION

One of the most common health problems is usually associated with wound infection (Giacometti *et al.*, 2000), commonly induced by the entry of the bacteria through contamination of breached skin. With the same importance as renal and cardiovascular disorders, wounds can also have a similar impact on the quality of life. The Global Wound Care Market report indicated that this sector has reached about 20 billion dollars and is estimated to exceed 25 billion dollars in 2023 worldwide (Weller *et al.*, 2020). For this reason, and the pressure induced on both patients and the medical system, efforts have been made with the aim of managing wound infections and their associated pathological conditions, which in turn could improve the quality of life and increase life expectancy. In recent decades, different approaches have been developed to improve the rate and quality of chronic wound healing (Nosrati *et al.*, 2021). Despite the multiple etiologies of wound infections, bacterial colonization usually characterizes their chronicity (Loesche *et al.*, 2017). Multiple variable organisms concerning wound infection were documented in previous studies (Melling *et al.*, 2001). Most wound culture isolation procedures implement swab cultures, the easiest trauma-free way, which avoid hazards of complications on the wound bed (Meyers, 2008). A variety of microbes such as bacteria, fungi, and parasites are associated with wound infection (Bowler *et al.*, 2001). Wound microbiome composition could also be influenced by ecological processes, which in turn induces the emergence of polymicrobial infections exhibiting both synergistic effect and enhanced tolerance to antimicrobials (Baishya and Wakeman, 2019; Kalan and Brennan, 2019). In addition, stable microbial communities are usually correlated with delayed healing (Loesche *et al.*, 2017; Sloan *et al.*, 2019). Moreover, genotypic effects could also be the possible way to explain the recently observed patient-specific immunological responses to the same microbial exposure (Deusenbery *et al.*, 2019). Both Gram-positive and Gram-negative

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bacteria are usually detected including *Escherichia coli*, *Proteus* spp., and *Klebsiella* spp., in addition to *Enterobacter* spp. and *Staphylococcus aureus*, which was also identified (El-Saed *et al.*, 2020; Gupta *et al.*, 2019). However, a challenging bacterium, *Pseudomonas aeruginosa*, exhibiting resistance to most antibacterial drugs, could also be detected (Bowler *et al.*, 2001). In addition, the presence of anaerobic bacteria at baseline infection could also be associated with worse healing outcomes (Min *et al.*, 2020). In contrast to the intestinal microbiota, normal skin is mainly colonized by only a few taxa including *Corynebacterium*, coagulase-negative *Staphylococci*, and *Propionibacterium* (Scharschmidt and Fischbach, 2013) in which sebaceous sites are dominated by lipophilic *Propionibacterium* species, while *Corynebacterium* and *Staphylococci* are preferentially abundant in moist areas (Costello *et al.*, 2009; Grice *et al.*, 2009; Oh *et al.*, 2014). Wound infections are classified into two major classes, skin and soft tissue infections, which are usually undistinguished upon the progression of infection. In addition, in these infections when untreated, infecting bacteria affect healing and may also produce other signs and symptoms. Moreover, wound infections are considered among the most leading nosocomial infections and are associated with increased morbidity and other medical expenses (Cutting and White, 2004). For assessing and diagnosing infection, the most common signs usually include redness, swelling, increased drainage, and increased pain. Therefore, microbial etiology is one of the fundamental steps required before starting the treatment protocols. Most microbial identification is usually based on the bacterial separation by an enrichment culture, which favors the selection of some microbes, while excluding other species. For this reason, bacterial identification is now shifting away from metabolic biochemical testing toward genetic or molecular identification (Espy *et al.*, 2006; Han *et al.*, 2011; Seng *et al.*, 2010). Molecular technologies have provided worldwide researchers with more rapid and sensitive tools to examine human microbiota, in comparison with the old culture-based testing (Bowler and Davies, 1999; Dowd *et al.*, 2008a). In most studies, universal primers for 16S rRNA genes are used, followed by specific identification approaches such as polymerase chain reaction (PCR) (Hill *et al.*, 2003) and denaturing gradient gel electrophoresis and sequencing (Dowd *et al.*, 2008a, 2008b; Hill *et al.*, 2003; Price *et al.*, 2009). In addition, these methods exhibited successfulness in the detection of bacteria in burn wounds (Pirnay *et al.*, 2000), blood (Rothman *et al.*, 2002; Wellinghausen *et al.*, 2004; Yang *et al.*, 2002), cerebrospinal fluid (Poppert *et al.*, 2005), joint fluid (Yang *et al.*, 2008), and heart tissue (Breitkopf *et al.*, 2005). Moreover, in most cases, each experiment can be executed in not more than a few hours (Ecker *et al.*, 2010). In the same respect, for developing new therapeutic strategies, the use of these techniques speeds up the early detection of infecting microorganisms assisting clinicians and pathologists to find out the appropriate treatment of hospitalized patients.

For the detection of pathogens, DNA probes are immobilized in microarray experiments, in which more than one copy of each target gene could be included (Cannon *et al.*, 2010; Shen *et al.*, 2015). However, the detection of wound-associated bacterial pathogens was rarely described in previous studies. The aim of this study is the detection of wound pathogens in mixed samples and their application in samples from Egyptian hospitals.

MATERIALS AND METHODS

Specimens collection and processing

Hospitalized immunocompetent symptomatic patients, almost all adults suffering from wound infection, were included in this study. Control samples were taken from nonhospitalized individuals and those free from any symptoms of wound infection. Samples were collected from 20 different patients admitted to one of the Mansoura hospitals in Egypt. This study was approved and funded by the Competitive Funding Projects Postgraduate Research and Cultural Affairs Sector, Mansoura University. The experimental protocol conducted in this study was approved by the Research Ethics Committee, Faculty of Pharmacy, Mansoura University, and conducted in accordance with the Declaration of Helsinki involving the use and handling of human subjects.

Extraction of genomic DNA and DNA from Standard Cultures

Different bacterial cultures obtained from wound samples were prepared in LB broth, followed by incubation aerobically at 37°C and observation for the presence of visible microbial growth. Genomic DNAs were extracted from 37°C 24 hours cultures of wound samples using the genomic QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. By using a Nanodrop instrument (OPTIZEN NanoQ, Mecasys, Daejon, South Korea), the concentration of gDNA was determined. Purified DNAs were stored at −80°C.

PCR Amplification and Labeling to Get Hybridization Targets

By starting from isolated gDNA as a template, nearly full-length 16S rRNA was amplified in a PCR reaction. Two different biotin-labeled primers were used: forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT). Each PCR reaction is composed of 25 μl containing 2 μl of template DNA, 0.5 μM of each primer, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 1 U Taq polymerase (Dream Taq Green DNA Polymerase, Fermentas), and to the final volume nuclease-free water. PCR reactions were carried out using Cycler 003 PCR Machine [A & E Lab (UK)] with 2 minutes initial denaturation at 94°C, followed by 35 cycles of heating at 94°C for 30 seconds, cooling to 52°C for 30 seconds in an annealing step, and heating at 72°C for 30 seconds in an extension step, and a final extension at 72°C for 10 minutes. The 1% agarose gel and electrophoresis-separated PCR products were visualized by staining with ethidium bromide and UV light exposure.

Strain-Specific Genes PCR Test

As described previously, amplification of both genomic DNA and standard DNA was carried out using primers (Biosearch Technologies) listed in Table 1. The diagnostic PCR screening consists of a panel of 12 taxa including *S. aureus*, *Staphylococcus pyogenes*, *Mycobacterium* spp., *P. aeruginosa*, *Enterobacter* spp., *Klebsiella pneumoniae*, *Haemophilus influenzae*, *E. coli*, *Enterococcus faecalis*, *Citrobacter* spp., *Proteus* spp., and *Clostridium* spp. PCR reactions began with heating at 94°C for 5 minutes, followed by 35 cycles starting with heating at 94°C for 30

Table 1. Different oligonucleotides used in this work to detect different species of bacteria.

Primer name		Sequence	Tm		Citation
Proteus mirabilis	*Tuf* F	TCTACTTCACACGTAG	41	240	(Anbazhagan *et al.*, 2010)
	Tuf R	TTCTAACAGCTCTTCA			
	Probe	TGTGGCTTCTGGAGCTAACGCGTTA	62		(Mao *et al.*, 2008)
Enterobacter spp.	F	CGAGAGCCTGGTGCTG	48	180	(Anbazhagan *et al.*, 2010)
	R	GATTGGCTGACCCAAT			
	Probe	ACTCTTGACATCCAGAGAACTT	52		This study
All bacteria	F	GAGTTTGATCCTGGCTCAG	51	312	(Ginige *et al.*, 2013)
	R	GCTGCCTCCCGTAGGAGT			
H. influenzae	F	ACTTTTGGCGGTTACTCTG	50	272	(van Ketel *et al.*, 1990)
	R	TGTGCCTAATTTACCAGCAT			
	Probe	GCGTATTATCGGAAGATGAAAGTGCG	59		This study
K. pneumoniae	F	TCTGGACCGCTGGGAGCTGG	59	399	(Cole *et al.*, 2009)
	R	TGCCCGTTGACGCCCAATCC			
	Probe	CACTTTCAGCGGGGAGGAAGGC	59		This study
E. coli	TEcol553	TGGGAGCGAAAATCCTG	47	219	(Maheux *et al.*, 2009)
	TEcol754	CAGTACAGGTAGACTTCTG			
	Probe	GGGAGTAAAGTTAATACCTTTGCTCAT	55		(Mao *et al.*, 2008)
S. aureus	F	TGCTGGTGGTACATCAAA	49	96	(Ruimy *et al.*, 2008)
	R	ACGGTCAATGCCATGATTTAA			
	Probe	AACATATGTGTAAGTAACTGTGCACATCTTG	59		(Mao *et al.*, 2008)
P. aeruginosa	F	CGAGTACAACATGGCTCTGG	53	116	(Feizabadi *et al.*, 2010)
	R	ACCGGACGCTCTTTACCATA			
	Probe	GGGAGGAAGGGCAGTAAGTTA	53		(Mao *et al.*, 2008)
Mycobacterium spp.	F	GAACGGCTGATGACCAAACT	53	72	(Luo *et al.*, 2010)
	R	ATCAGCGATCGTGGTCCTG			
	Probe	CGATCCGAACTGAGACCGGCTTTTAAGG	64		(Jin *et al.*, 2005)
E. faecalis	F	ATCAAGTACAGTTAGTCTT	44	940	(Rathnayake *et al.*, 2011)
	R	ACGATTCAAAGCTAACTG			
	Probe	AACTGAACGTCCCCTGACGGTATCT	59		(Mao *et al.*, 2008)
Citrobacter spp.	F	GCTCAACCTGGGAACTGCATCCGA	62	529	(Anbazhagan *et al.*, 2010)
	R	AGTTCCCGGCCTAACCGCTGGCAA			
	Probe	GTACTTTCAGCGAGGAGGAAGG	56		This study
Clostridium spp.	F	CGGTACCTGACTAAGAAGC	50	429	(Bartosch *et al.*, 2004)
	R	AGTTTGATTCTTGCGAACG			
	Probe	TAAAGGGAGCGTAGGCGGATGATTA	60		(Mao *et al.*, 2008)
S. pyogenes	F	GTCAACATGCAGCTACAGGA	49	256	(Louie *et al.*, 1998)
	R	AATACCAACATCAGCATCA			
	Probe	GCAGGTTTTGCCTCTCATATTAAAGTCTT	59		This study

seconds, then cooling to annealing temp (as listed in Table 1) for 30 seconds, and heating at 72°C for 30. Finally, each PCR reaction was ended with a final extension step at 72°C for 10 minutes.

Oligonucleotide Probes Design and synthesis

Segments of 20–30 mer oligonucleotides with similar lengths, melting, and GC content with the optimum BLAST hit were selected after comparison (http://www.ncbi.nlm.nih.gov/

BLAST/) with all available sequences in the GenBank database. All oligonucleotides used in this study were listed in Table 1.

Oligo Aarray Printing

In a 384-well printing plate, using ArrayIt spotting buffer, each printing oligo solution was prepared to a final concentration of 50 pmol µl⁻¹. Spotting was carried out onto microarray slides in triplicate for each probe (Scienion, Berlin, Germany) using the
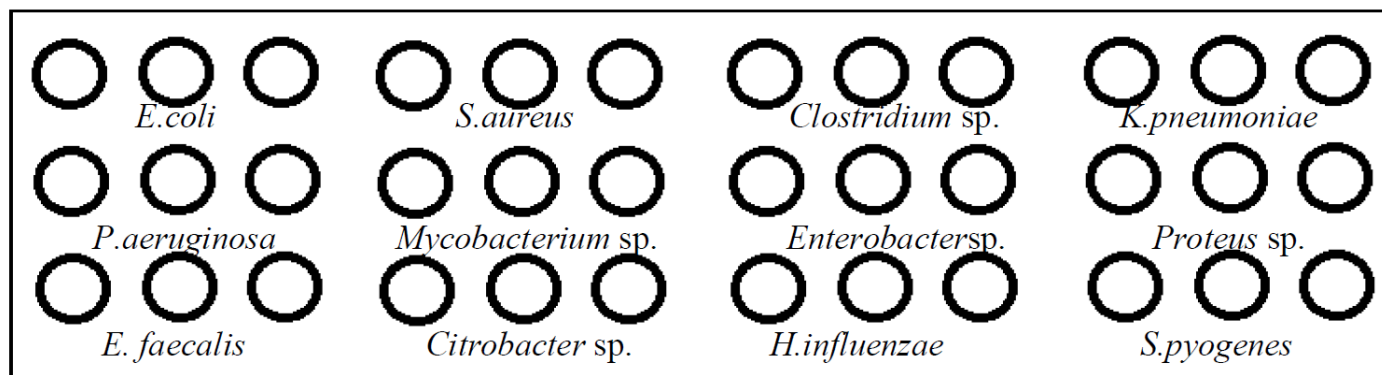
Figure 1. Different ssDNA probes spotted in the schematic representation of each microarray slide.

SpotBot microarrayer (ArrayIt, Sunnyvale, CA) spotting machine (Fig. 1).

Microarray Testing

Samples were labeled and then resuspended in a hybridization buffer (Scienion, Berlin, Germany). The method was carried out according to the recommendations of the manufacturer (Scienion, Berlin, Germany). In a final volume of 16, 8 µl of the PCR products was mixed with 8 µl of the hybridization buffer. The prehybridization step was carried out using 20 µl of the prehybridization solution (Scienion, Berlin, Germany). Labeled samples were then applied to a 42°C prewarmed microarray slide. Hybridizations were carried out in a hybridization station (ArrayIt, Sunnyvale, CA) using the hybridization mix, previously boiled for 2 minutes and cooled on ice. Hybridization was carried out by incubation at 42°C for 12 hours. Finally, at room temperature, using buffers I, II, and III (Scienion, Berlin, Germany), washing was carried out. Microarray staining was carried out by a streptavidin biotin color development system (Fermentas, Waltham, MA) using the manufacturer's instructions. For documentation of the results, images were acquired using the ArrayIt Microarray Scanner (ArrayIt, Sunnyvale, CA). Signal intensities were recorded using the Spotware software after subtracting the local background values from the per-sample median.

RESULTS

Results of bacterial identification

Twenty wound samples were analyzed by molecular testing. Overall, the most common organisms identified using molecular PCR testing were *Citrobacter* spp. (100%) in 20 samples, *Enterobacter* spp. (100%) in 20 samples, and *K. pneumoniae* (100%) in 20 samples, followed by *P. aeruginosa* (90%) in 18 samples, *Proteus* spp. in 16 samples (80%), and *E. coli* (60%) in 12 samples. However, *S. aureus* was rarely identified in two samples (10%). Other bacterial taxa such as *E. faecalis*, *H. influenzae*, and *Mycobacterium* spp. could not be detected in any isolate. Control samples were free from the tested pathogens. Concerning a mixed microbial infection, a total of eight wounds were found to have five different bacterial taxa; in addition, 10 of the total samples (50%) had six bacterial taxa. However, two of the total wounds (10%) had only four bacterial taxa (Table 2).

**Table 2.** Comparison between PCR and microarray results.

Comparison of PCR with specific primers and Microarray results			
Organism	**No of samples positive**		**Total number of patients with a positive result**
	PCR	**Microarrays**	
Mycobacterium spp.	0	0	0
S. aureus	2	2	2
P. aeruginosa	18	18	18
K. pneumoniae	20	20	20
H. influenzae	0	0	0
E. coli	12	12	12
E. faecalis	0	0	0
Proteus spp.	16	16	16
Enterobacter spp.	20	20	20
Citrobacter spp.	20	20	20
S. pyogenes	0	0	0
Clostridium spp.	0	0	0

Microarray Testing of clinical samples

In this study, by DNA microarray testing, the proportions of bacteria identified from the 20 positive samples were *Citrobacter* spp. (100%), *Enterobacter* spp. (100%), and *K. pneumoniae* (100%), followed by *P. aeruginosa* (90%), *Proteus* (80%), and *E. coli* (60%). However, *S. aureus* was rarely identified (10%). A mixed bacterial infection could be identified in all different samples as four, five, or six different bacteria were identified in each sample as shown in Figure 2. No difference could be observed when compared to the results obtained by PCR (Table 3).

DISCUSSION

Microbial molecular diagnostic tests, in comparison with traditional culture methods, enable more patient care improvement. However, it is also necessary to continue to include all these introduced newer methods. In addition, it has been proven that culture-based methodologies in bacterial detection are markedly less sensitive to DNA detection methodologies (Han *et al.*, 2011). Moreover, the results obtained using these traditional culture-based methods are usually associated with misidentification reflecting overestimation of the relative presence of easily cultured and identifiable microbes (Davies *et al.*, 2004).
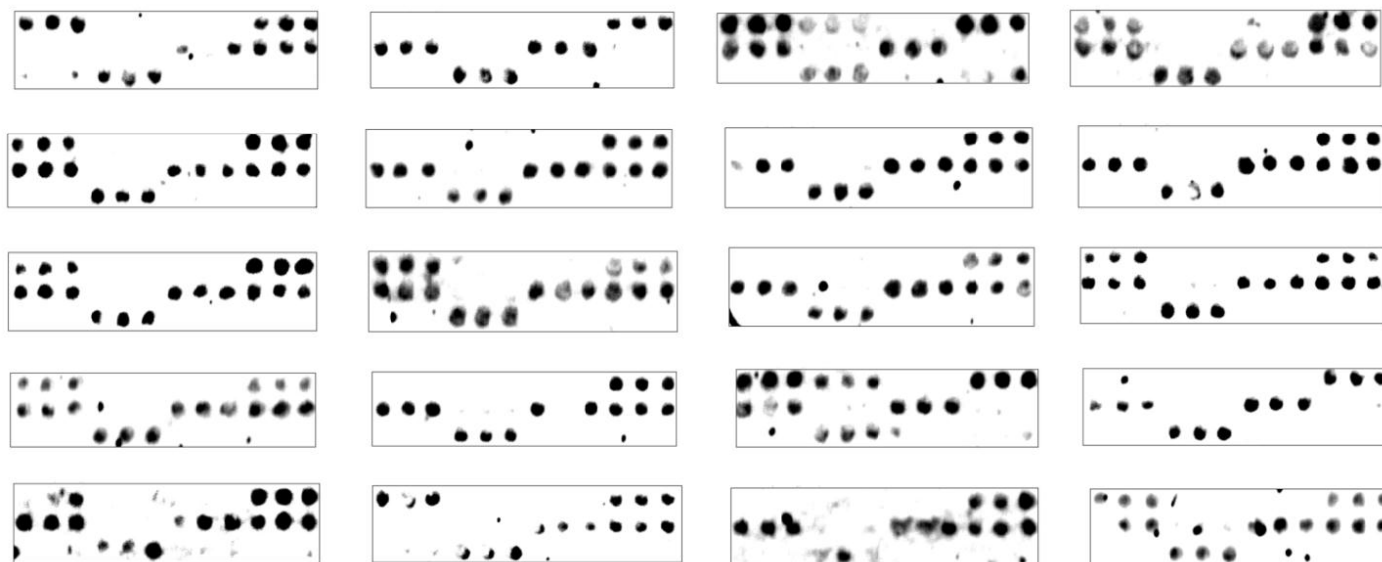
Figure 2. Results of hybridization with the target wound sample DNAs from Mansoura Hospital.

According to the recent reports, the results obtained using PCR and 16S sequencing commonly used have demonstrated the close correlation between most of these molecular methods. As previously documented, different molecular methods such as real-time, uniplex, and multiplex PCR, especially in mixed samples, have enhanced both the speed and the sensitivity of microbial detection (Gebert *et al.*, 2008; Lehmann *et al.*, 2008). However, multiplex PCR, in each reaction, can only detect a limited number of pathogens (Edin *et al.*, 2015; Gadsby *et al.*, 2015). For these reasons, when dealing with samples containing a mixed culture or mostly environmental samples, a comprehensive view of microbial communities is required. In the same respect, a better solution is provided by DNA microarrays, especially in mixed microbial infections.

Microarray testing is usually based on the panel of taxa selection, detection capacity, and probe coverage, which may differ between sequence targets. Microarray-detecting oligonucleotides are mainly designed based on the available sequence data and the variation between target organisms exhibiting reduced power for identifying other different organisms (Cannon *et al.*, 2010; Shen *et al.*, 2015).

As previously reported (Costello *et al.*, 2009; Grice and Segre, 2011; Grice *et al.*, 2009; Oh *et al.*, 2014), in sequencing surveys of healthy adults, dry, moist, and sebaceous microenvironmental changes usually affect the relative abundance of bacterial taxa. However, a lower bacterial diversity is usually detected (Scharschmidt and Fischbach, 2013), as the normal skin is usually dominated by only a few taxa, mainly lipophilic *Propionibacterium* species of the phylum Actinobacteria thriving sebaceous sites, while *Staphylococcus* and *Corynebacterium* species of the phyla Firmicutes and Actinobacteria are preferentially abundant in moist areas (Costello *et al.*, 2009; Grice *et al.*, 2009). Interestingly, some members of the skin microbiota such as *Staphylococcus epidermidis* are nonpathogenic; however, when reaching the blood circulation, serious systemic diseases in some individuals can be caused (Blum and Rodvold, 1987). In this study,

to distinguish some major bacterial species, frequently causing wound-associated infections including *E. faecalis*, *Enterobacter* spp., *K. pneumoniae*, *H. influenzae*, *E. coli*, *S. pyogenes*, *Clostridium* spp., *S. aureus*, *Mycobacterium* spp., *Citrobacter* spp., *P. aeruginosa*, and *Proteus* spp., 12 oligonucleotide probes, complementary to 16S PCR products, were selected for detection based on several species-specific regions of each pathogen.

In samples obtained in the current study, by DNA microarray testing, all samples (100%) were found to be positive and identified mainly as Gram-negative bacteria, which is consistent with some previous studies in Saudi Arabia (Alkaaki *et al.*, 2019; Al-Mulhim *et al.*, 2014) and other countries (Allegranzi *et al.*, 2011). The percentages of identified bacteria were *Citrobacter* spp. (100%), *Enterobacter* spp., (100%), and *K. pneumoniae* (100%), followed by *P. aeruginosa* (90%), *Proteus* (80%), and *E. coli* (60%). Similarly, in a previous study, the same bacterial taxa were detected, but at lower percentages, 56.7%, 6.7%, and 3.3% for *E. coli, Klebsiella* spp., and *Enterobacter* spp., respectively (Adwan *et al.*, 2016). In addition, an increased level of *Pseudomonas* species (43%) was detected in a previous study (Gupta *et al.*, 2019), compared to *K. pneumoniae* and *Acinetobacter baumannii*, which were detected at the lower prevalence of 28% and 14.83%, respectively. Moreover, other studies in a tertiary care hospital in India reported the predominance of *P. aeruginosa*, followed by *K. pneumoniae* and *A. baumannii* (Dash *et al.*, 2013; Singh *et al.*, 2003). Interestingly, the wound microbiome, in which *Pseudomonas* dominates, exhibited increased temporal stability against multiantibiotic therapy in comparison to staphylococcal infections (Tipton *et al.*, 2017). However, other different results were obtained in another study on surgical site infections (Negi *et al.*, 2015), as the commonest organism identified was *S. aureus* (50.4%), followed by *E. coli* (23.02%), *P. aeruginosa* (7.9%), and *Citrobacter* spp. (7.9%). In addition, in another study (Be *et al.*, 2014), in wound samples, *A. baumannii* was mainly detected (23%) in 28 samples. In this current study, *S. aureus* was rarely identified in 10% of total isolates. A similar observation was

Table 3. Schematic representation of hybridization results obtained with the target DNAs from wound samples obtained from Mansoura Hospital.

	Ecoli	S.aureus	Clostridium spp.	K.pneumoniae	P.aeruginosa	Mycobacterium spp.	Enterobacter spp.	Proteus spp.	E. faecalis	Citrobacter spp.	H.influenzae	S.pyogenes
1	■			■	■		■	■		■		
2				■	■		■			■		
3	■	■	■	■			■			■		
4	■			■			■	■		■		
5	■	■		■			■			■		
6				■	■		■			■		
7				■	■		■			■		
8				■	■		■			■		
9	■			■	■		■			■		
10	■	■		■	■		■			■		
11				■	■		■			■		
12	■			■	■		■			■		
13	■			■	■		■			■		
14				■	■		■			■		
15	■	■	■	■			■	■		■		
16		■		■	■		■	■		■		
17	■	■		■	■		■			■		
18	■			■			■			■		
19				■	■		■			■		
20	■	■		■	■		■			■		

previously reported, as *S. aureus* was rarely detected (two wound samples) in one study using a microarray (Be *et al.*, 2014) and in another study on combat wound colonization (Brown *et al.*, 2011). On the contrary, a higher prevalence of *S. aureus* (30%) could be detected in one previous study (Adwan *et al.*, 2016). In addition, in another study, the most commonly isolated organism was *S. aureus*, followed by coagulase-negative *Staphylococci* (CNS), *Enterococcus* spp., and *E. coli*, while the pathogen detection was mainly dependent on the surgical procedure (Owens and Stoessel, 2008). Moreover, in another study, *S. aureus* was the most frequent pathogen detected (22.8%), while other pathogens, namely, *P. aeruginosa* (20.1%), *E. coli* (12.2%), *Klebsiella* spp., (12.2%),

Enterobacter spp., (7.7%), and *Enterococcus* spp. (5.9%), were identified at lower percentages (El-Saed *et al.*, 2020).

Interestingly, in previous studies, not so much data were found concerning the detection of mixed wound infection. In this current study, four, five, or six different bacteria were identified in each sample, representing mixed microbial infections. *K. pneumoniae*, *P. aeruginosa*, *Enterobacter* spp., *Proteus* spp., and *Citrobacter* spp. were the most identified mixed infections.

CONCLUSION

In the present study, by PCR detection, *Citrobacter* spp., *Enterobacter* spp., *K. pneumoniae*, *P. aeruginosa*, *Proteus* spp., and *E. coli* were mainly detected. In addition, pathogen detection with both PCR and the DNA chip indicated 100% matching. Moreover, the use of a DNA chip in a mixed culture facilitates the fast identification of wound-infecting pathogens and the proper treatments strategies.

CONFLICTS OF INTEREST

The author declares that there are no conflicts of interest regarding the publication of this paper.

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