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Phenotypic and Molecular Identification of Bacteria Involved in Decubitus Ulcers

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ABSTRACT

Background: Bacterial secondary infection of pressure ulcers (bedsores), so called as decubitus ulcers, leads to ulcer development and it interferes with the healing process. Thus, such infections can be lethal due to the sepsis if no constructive medicinal measures regarded. Drug resistance of bacteria in pressure ulcers leads to healing inhibition. Molecular identification of bacteria involved in such infections seem necessary as culture and phenotypic approaches may result in misidentification. . The purpose of this study was to isolate and identify aerobic bacteria detected in bedsores in three Hospitals: Rasool-e-Akram, Imam Hossein and Tajrish Shohada Hospitals, Tehran, Iran.

***Methods*:** To this end, decubitus ulcer samples of 49 patients were obtained using sterile swabs. After direct microscopic examination, the swabs were used to streak BHI agar plates supplemented with %5 defibrinated sheep blood for enrichment of probable aerobic cultures. Bacterial isolates diagnosed by biochemical tests. Antibiotic susceptibility of the isolates determined based on CLSI guideline. For molecular identification, PCR amplification of the 16S rRNA gene performed using Eubacterial universal primers. Then, the PCR products were sequenced and the nucleotide sequences of the PCR products were analyzed by BLASTN similarity search program available at NCBI.

***Results*:** Among the isolates, *Pseudomonas aeruginosa* (36%) had the highest frequency, followed by *Staphylococcus aureus* (32%) and *Escherichia coli* (30%). The frequencies of *Klebsiella pneumonia* and *Proteus* spp. were 10% and 8%, respectively. Most of the isolated bacteria showed a widespread antibiotic resistance. Molecular identification of the bacterial isolates resulted in 6 isolates of *Escherichia coli*, two isolates of each of *Proteus mirabilis* and *Shigella* spp., 4 isolates of *Enterobacter cloacae*, and 1 isolate of each of *Cronobacter sakazakii* and *Morganella morganii*.

***Conclusion*:** Results showed that *Pseudomonas aeruginosa* and *Staphylococcus aureus* as the most frequent bacterial species detected in pressure ulcers; however, bacterial prevalence may be different in different hospital wards.

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Introduction

A pressure ulcer is an injury to skin and surface tissues that usually occurs when a person remains in one position without moving or shifting his/her weight. Such chronic wounds, such as ulcers, have troubled the medical community for centuries. The first known decubitus ulcer was detected during the autopsy of an Egyptian mummy (1). Today, approximately 20% of long-term care patients and 9% of all hospitalized patients develop bedsore (2). The etiology of pressure sores is multifactorial and it is associated with factors such as stress, time, spastics, edema, enervation, moisture, poor nutrition and infection (REF). As reviewed elsewhere, high-risk people for decubitus ulcers are the aged, debilitated, paralyzed and unconscious patients or patients with incontinentia pigmenti (3-6).

There are four general accepted stages of pressure ulcers: Stage I: the ulcer is confined to the epidermis and superficial dermis, Stage II: the ulcer extends through the skin and subcutaneous tissue, Stage III: the ulcer extends to the muscle, and Stage IV: the ulcer invades bones or joints (7). The microbiota of chronic wounds and infection caused by them are known to play a significant role in hampers healing of pressure sore, even in the absence of inflammation (5, 8, 9).

Currently, drug resistance of microorganisms involved in pressure sores is going to be alarming. Antibiotic susceptibility pattern of bacteria isolated from pressure sores gives us a comprehensive data for prescribing antibiotics for pressure sores. Traditionally, identification of bedsore bacteria in clinical microbiology laboratories performed similar to other samples and it includes phenotypic tests: Gram-staining and biochemical tests, taking into account the culture tests and growth characteristics (10, 11, 12). However, these methods have major limitations. First, microorganisms with the characteristics of any known genus and species are occasionally encountered. Second, they cannot be used for un-culturable microorganisms. Third, identification of some particular groups such as anaerobes would require additional equipment. Thus, 16S rDNA sequence analysis is a helpful molecular approach which solve these problems and also facilitates the discovery of novel bacterial taxa (10, 13-15).

In this study, the prevalence of pressure ulcers in patients hospitalized at Rasool-e-Akram, Imam Hossein and Tajrish Shohada Hospitals, Tehran, and the etiological factors that affected ulcer development were evaluated. Identification of aerobic bacteria involved in pressure ulcers performed using conventional culture methods. Moreover, their antibiotic resistance profiles were determined. Also, the bacterial isolates remained unidentified by phenotype-based methods, were correctly identified using the 16S rDNA sequence analysis.

Materials and Methods

Patients

A total of 49 patients (33 men and 16 women) admitted to the internal and other departments of the above mentioned Hospitals, (Emam Hossein, Rassol-e-Akram and Tajrish Shohada), Tehran, were randomly selected. Among the overall population, 19 patients were from Emam Hossein Hospital (11 patients from the internal ward and 8 from other wards), 21 were from Rassol-e-Akram Hospital (14 patients from the internal ward and 7 from other wards) and 9 were from Tajrish Shohada Hospital. The patients were classified into two age groups of < 40 and ≥ 40 years.

Pressure ulcer sampling

Patients enrolled in the study protocol after being educated and signing the informed consent after their pressure ulcers diagnosed. The pressure sores were included by purposive sampling, based on some inclusion and exclusion criteria. The patients with pressure sores were evaluated by relevant history and clinical examination. The pressure sore

was sampled with a cotton swab. Deep tissue specimens for quantitative culture were taken from the sore site, where clinical signs of infection were evident. The specimens were then put into a pre-weighted saline (2 ml) containing container and delivered to laboratory for further assessments.

Bacterial identification

After direct microscopic examination by Gram staining, the swabs were spread on the surface of heart infusion agar supplemented with %5 defibrinated sheep blood for aerobic cultures and incubated for 24-48 h. After incubation, bacterial isolates diagnosed by phenotypic tests based on their Gram staining result. Tests such as catalase, oxidase, coagulase, Dnase and mannitol salt agar were used for Gram positive isolates and tests such as TSI, SIM, Citrate, MRVP and citramide agar were used for characterization of Gram negative isolates (facultative fermentative bacteria like enterobacteriaceae as well as non-fermentative bacteria like *Pseudomonas*).

Antibiotic susceptibility of bacterial isolates

Antibiotic susceptibility of all the bacterial isolates was determined by disc diffusion method according to the CLSI 2014 guidelines and Kerbeybauer method (REF). The antibiotic discs (Padtanteb) and concentrations (µg) used in the study are as follows; Ciprofloxacin (V;30), Amoxycilin, Penicillin (P;10), Gentamicin (CN;10), Vancomycin (CP;5), Nitrofurantoin (FM;300), Azithromycin (AZM;15), Ampicillin (Am;10), Kanamycin (K; 30), Cefalotine (CF;30), Tetracyclin (TE;30). Results were interpreted as susceptible, resistant and intermediate according to criteria recommended by the CLSI 2014.

Molecular identification

The genomic DNA of all the isolates was extracted and purified using a Bioflux kit (Japan) (Kit No.). For molecular diagnosis, 12 specimens, undetected by the culture methods, were selected. PCR amplification of a partial fragment of 16S rRNA gene performed using a gradient thermal cycle (SinnaGen Co., red master mix 2X). The universal primers (forward primer AGAGTTTGATCCTGGCTC CAG and reverse primer GCTCGTTGCGGGACT TAACC) were used for the amplification of 16S rDNA fragment (REF). The reaction mixture of 50 µL consisted of 1 µL of genomic DNA, 20 µL of Taq DNA polymerase, 250 µL of 10X PCR buffer, 50 µL of dNTP, 1 pmol of each of primers and 75 mM of MgCl2. Amplification reactions performed in 35 cycles as follows: 1 min at 94 °C, 40 sec of annealing at 62 °C, 2 min at 72 °C and a final extension for 10 min at 72 °C. The PCR products were gel agarose electrophoresed and visualized by UV transilluminator (GenDirex). The PCR amplified fragments were purified by gel extraction kit and sequenced from both directions using BigDye 1A technology on an AB13700XL DNA sequencer (Applied Biosystems). The nucleotide sequence of the PCR products were compared with those of known 16S rDNA gene sequences in the nucleotide database of GenBank (http://www.ncbi.nlm.nih.gov) at NCBI by multiple sequence alignment using the ClustalW program. Neighbor-joining tree of the selected 16S rDNA gene sequences of the bacterial genus obtained from BLASTN search was drawn. All of this gene sequences have been submitted in gen bank (www.ncbi.nlm.nih.gov) (13, 14).

Statistical analysis

Statistical analysis performed using the SPSS statistical package, windows version 16. A $\chi 2$ test used to study the correlation between sex, age and complication factors of the patients with pressure ulcers. A value of $P<0.05$ was considered statistically significant.

Results

Classification of patients according to sex, age and complication

Of the 49 studied patients, men comprised 66.35% and women 33.65%. A greater percentage was ≥40 years of age (34/49, 69.4 %) than <40 years of age (15/49, 30.6 %). Among patients with different diseases or complications, the elderly and then, those with diabetes were most frequent (18.4% and 14.3%) (Fig. 1).

Classification of patients according to site and stage of pressure ulcer

Among the 49 samples taken from the patients, 83.6% (41/49) were from sacrum, 10.3% (5/49) from ischium and 6.1% (3/49) from heel (Fig. 2). Regarding the frequency of the pressure ulcer stages, the frequency was 4% in stage 2, 20.5% in stage 3 and 71.5% in stage 4. There was no stage 1 among the pressure ulcers (Fig. 3).

Distribution of bacterial isolates in the samples

The frequently identified bacteria detected by aerobic culturing from the pressure ulcers of all studied patients who admitted to the three hospitals showed that *Pseudomonas* species (18/9, 36%), *Staphylococcus aureus* (16/49, 32%) and *Escherichia coli* (15/9, 30%) were the most abundant microorganisms isolated. However, *Proteus* spp. (4/49, 8%) and *Klebsiella* spp. (5/49, 10%) were less abundant (Fig. 4).

By comparison of the identified bacteria from the pressure ulcers of the patients in the three hospitals, *Staphylococcus aureus* isolates were observed with the highest frequency (8/19,42%), followed by *Escherichia coli* (6/19,31%) and *Pseudomonas* spp. (5/19,26%), at Imam Hussein Hospital (Table 1); however, at Rasool-e-Akram Hospital, *Pseudomonas* spp. (9/21, 42%) followed by *Staphylococcus aureus* isolates (7/21, 33%) had the highest frequency (Table 2). The results also showed that multiple isolates may be obtained from the same sample. The frequency of Pseudomonas species and Escherichia coli (4/9, 44%) was equal and at highest rate. *Staphylococcus aureus* frequency was (1/9, 11%) (Table 3).

Antibiotic susceptibility patterns of bacterial isolates

All the microorganisms isolated from pressure sores were mostly resistant to Ampicillin, Cefalotin and Amoxicillin. Most of the isolates showed 100% resistance to Azithromycin, but the drug resistance in Proteus isolates was 25%. The E. coli isolates were 80% sensitive to Nitrofurantoin. All the Staphylococcus aureus isolates were sensitive to Vancomycin, but the other bacteria were 100% resistant. There was a relative resistance to other antibiotics such as Kanamicin, Gentamicin and Ciprofloxacin. Tetracyclin had 60-100% resistance among *Pseudomonas*, *E. coli* and *Proteus* isolates (Table 4).

Molecular identification

In this study, the 16S rRNA sequence analysis was used for identification of bacterial isolates from some pressure sore specimens, which remained undetected by the culturing method. With molecular methods, *Enterobacter cloacae* and then, *Escherichia coli* were the most frequently identified bacterial species. *Morganella morganii* was isolated from the patients in infectious wards (Table 5).

The evolutionary relationships was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.10072983 is shown. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 925 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

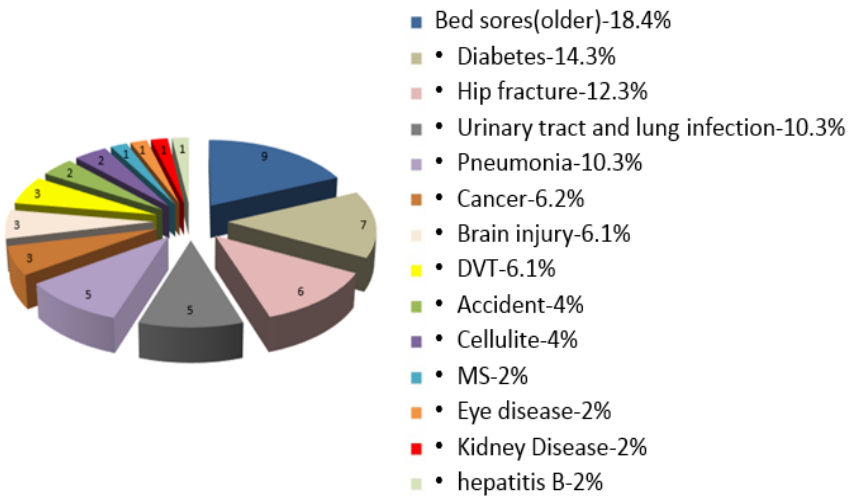

Figure 1. Patient's complications.

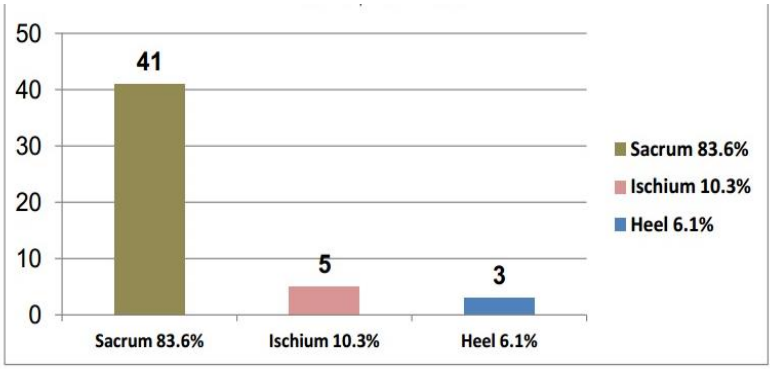

Figure 2. The site of pressure ulcers.

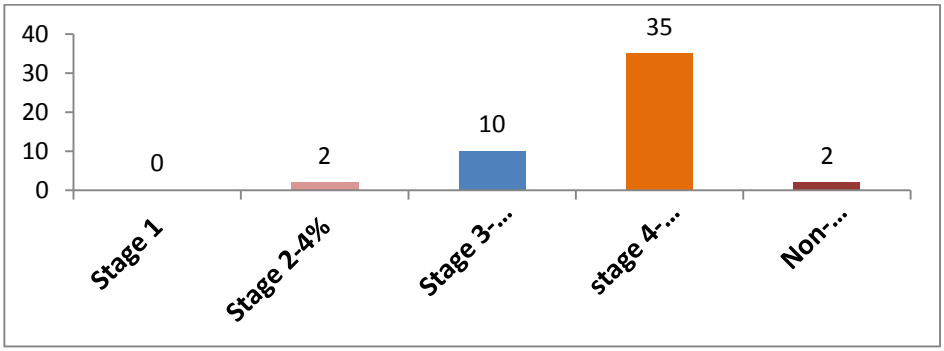

Figure 3. The frequency of pressure ulcers stages.

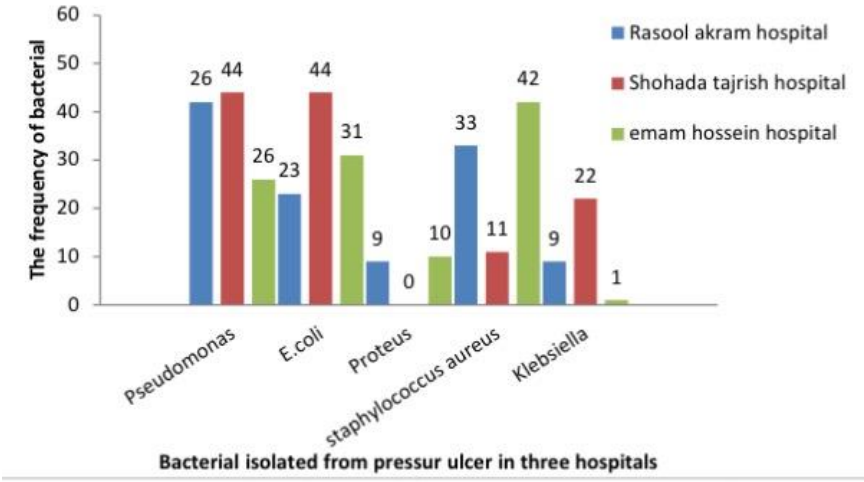

Figure 4. The prevalence of bacteria in the three hospitals.

Table 1. The frequency of bacterial isolated from pressure ulcers at Imam Hussein Hospital.

Bacterial isolates	Total number of pressure ulcers n =19	Internal department n=11	Other Departments n=8
Pseudomonas species	5 (26%)	3 (27%)	2 (25%)
E. coli	6 (31 %)	3 (27%)	3 (37%)
Proteus spp.	2 (10%)	2 (18%)	0
Staphylococcus aureus	8 (42%)	6 (54%)	2 (25%)
Klebsiella spp.	1 (5%)	0	1 (12%)
No growth	2 (10%)	2	-

Table 2. The frequency of bacterial isolated from pressure ulcers at Rasool-e-Akram Hospital.

Bacterial isolates	Total number of pressure ulcers n=21	Internal department n=14	Other Departments n=7
Pseudomonas species	9 (42%)	5 (35%)	4 (57%)
E. coli	5 (23%)	3 (21%)	2 (28%)
Proteus spp.	2 (9%)	0	2 (28%)
Staphylococcus aureus	7 (33%)	5 (35%)	2 (28%)
Klebsiella spp.	2 (9%)	0	2 (28%)
Not detected	5 (23%)	3	2

Table 3. The frequency of bacterial isolated from pressure ulcers at Rasool-e-Akram Hospital.

Bacterial isolates	Total number of pressure ulcers in Departments (n=9)
Pseudomonas species	4 (44%)
E. coli	4 (44%)
Proteus spp.	0
Staphylococcus aureus	1 (11%)
Klebsiella spp.	2 (22%)
No growth	1 (11%)

Table 4. Antibiotic susceptibility patterns of bacterial isolated from pressure ulcers.

Antibiotics	Bacteria isolated from bedsore ulcers of patients														
	***Staphylococcus aureus* (*n= 16*)**			***Pseudomonas* (n= 18)**			***E. coli* (n=15)**			***Klebsiella* (n=5)**			***Proteus* (n= 4)**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Ampicillin	16%	0	84%	0	0	100%	0	0	100%	0	0	100%	0	0	100%
Cefalotine	0	0	100%	0	0	100%	0	0	100%	0	0	100%	0	0	100%
Azithromycin	0	0	100%	0	5%	95%	0	0	100%	0	0	100%	50%	25%	25%
Nitrofurantoin	17%	0	83%	16%	11%	73%	80%	0	20%	25%	0	75%	0	0	100%
Vancomycin	100%	0	0%	0	0	100%	0	0	100%	0	0	100%	0	0	100%
Kanamycin	0	0	100%	0	0	100%	33%	0	67%	0	0	100%	0	0	100%
Gentamicin	33%	0	67%	5%	22%	73%	23%	0	77%	0	0	100%	0	0	100%
Ciprofloxacin	50%	0	50%	5%	22%	73%	33%	0	67%	0	0	100%	0	50%	50%
Tetracyclin	75%	0	25%	22%	11%	67%	30%	0	70%	80%	0	20%	0	0	100%
Amoxycillin	0	0	100%	0	0	100%	0	0	100%	0	0	100%	0	0	100%

Table 5. Distribution of the bacterial isolates in the sampled Hospitals.

NO of bacterial strains	Bacteria	Hospital	Hospital sector
Sample 3	*Enterobacter cloacae*	Imam Hussein Hospital	Orthopedics
Sample 6	*Escherichia coli*	Imam Hussein Hospital	Internal
Sample 11	*Proteus mirabilis*	Rasoul Akram Hospital	Internal
Sample 19	*Enterobacter cloacae*	Imam Hussein Hospital	Orthopedics
Sample 20	*Morganella morganii*	Imam Hussein Hospital	Infectious
Sample 23	*Enterobacter cloacae*	Shohada Tajrish Hospital	Oncology
Sample 24	*Escherichia coli*	Shohada Tajrish Hospital	Surgery
Sample 26	*Enterobacter cloacae*	Imam Hussein Hospital	Internal
Sample 34	*Escherichia coli*	Imam Hussein Hospital	Internal
Sample 37	*Escherichia coli*	Rasoul Akram Hospital	Internal
Sample 46	*Escherichia coli*	Rasoul Akram Hospital	Neurosurgery
Sample 48	*Cronobacter sakazakii*	Imam Hussein Hospital	Internal

Discussion

The etiology of chronic wounds such as pressure ulcers commonly relates to underlying pathologies; initiation is often associated with primary tissue damage, which creates a portal of entry for microorganisms, Hence, there is first an infection and then the microbial community develops (16-18).

The prevalence of pressure ulcers in the three hospitals was reported 14.8% in this study. In a study by Ahmadinejad, the prevalence was obtained as 5.34% (19). Cho et al. in South Korea reported the prevalence of these injuries as 9.5% (20). Also, in a study by Shahin et al. (2009), the incidence of these wounds was reported to be 3.8% to 12.4%, worldwide (21). In a study by Hossein and Khundkar (2012) on 50 patients with bedsores, 48.65% of the patients had grade 3 lesions and 39.19% had grade 4 ulcers (22). Also, the results showed that the prevalence of pressure ulcers in patients with >40 years ages was higher than in lower ages. Hossein and Khundkar also showed that the 35-45 age group had the highest frequency (35%) and the 45-55 age group had 30% frequency (22). The pressure ulcers usually occur as a second complication after hospitalization due to other illnesses. In the present study, majority of the patients with bedsores had an underlying condition such as diabetes (14.3 %). However, a high percent of these people (18.4%) suffered from bedsores because of aging. Also, Keller (2002) suggested the impact of diabetes and impaired blood flow on the pressure ulcer development. Diabetic patients, due to the failure of vascular function, are exposed to bedsores more than other patients (23). Wound care providers treat wounds as if the bioburden is one of the major barriers to wound healing. Debridement, antibiotics, and topical antiseptics are routinely used in managing the microbes that are universally found on chronic wounds.

To study the bedsore and its progress, various factors such as degree of injury, age, complications and length of hospitalization of patients are important. In this study, a total of 71.5% of ulcers were at stage 4, 20.5% at stage 3 and 4% at stage 2. The occurrence of pressure ulcers with grade 3 or 4 in patients, in addition to the unsuccessful wounds treatment due to infectious agents and bacterial antibiotic resistance in biofilm infections,

as well as the costs for maintenance of patients and antibiotics prescribed for the treatment have created several problems. In 2007, the Centers for Medicare and Medicaid Services (CMS) reported 257/412 patients with bedsores grade 3-4 in health centers, with a treatment cost of 43,180 USD for each patient (24).

The role of bacteria in chronic wounds and the appropriateness of using antimicrobial agents in the routine management of chronic wounds are a source of continual debate in the wound care community (25-27). The complex community of aerobic and anaerobic bacteria in chronic wounds slows the process of healing and increases the severity of wounds. The most common method for bacterial identification is based on culturing and biochemical tests. Using such methods, many culturable aerobic and anaerobic bacteria are diagnosed.

In this study, the most frequent bacteria isolated by the aerobic culturing method from the pressure ulcers of all the patients were members of *Pseudomonas* spp. (36%), *Staphylococcus aureus* (32%) and *E. coli* (30%). *Proteus* spp. (8%) and *Klebsiella* spp. (10%) had the lowest frequency among the isolates. In a study by Klaus Kirketerp-Møller using the culturing method, it has been demonstrated that *Staphylococcus aureus* strains are among the common bacteria in most chronic ulcers (26, 27). In 2013, Hossein and Khundkar showed that among bacteria isolated from pressure ulcers, *Pseudomonas* spp., followed by *Staphylococcus aureus* and *Proteus* spp. were the most frequent bacteria (22). In this study, 17 (34.6%) patients were detected to have more than two species of bacteria in their pressure ulcers with grades 3 and 4. Our results showed that ulcers with stage 3 or 4 are often infected by multiple bacteria. The study of bacterial antibiotic susceptibility patterns demonstrated that most bacteria have up to 70% resistance to antibiotics used in this study. In similar studies, the highest sensitivity was reported to Ceftazidime, Amikacin, Ciprofloxacin, and Gentamycin. This causes lack of timely recovery in the treatment of bedsore wounds with common antibiotics. Molecular methods are among the mostly used bacterial identification methods, which are not based on culturing. The accuracy of such methods is highly promising and allows identifying a wide range of bacteria, even those do not grow in culture methods or have a minority in bedsore ulcers microbial community. Thus, such bacteria can be simply identified using a single method; 16S rRNA gene sequence analysis (22).

In this study, we identified 12 bacterial species including 5 *Eshcerichia coli* strains, 4 *Enterobacter cloacae*, 1 *Proteus mirabilis*, 1 *Morganella morganii* and 1 *Cronobacter sakazakii*. In a study by Rhoads et al. (2012), 5 *Morganella morganii* and *Enterobacter cloacae*, 3 *Escherichia coli* and 10 *Proteus mirabilis* were identified by the 16S rRNA sequencing method (28). Another study by the same authors on 168 pressure ulcers resulted in the identification of 17 and 338 different genus using the culture and molecular method, respectively (28). Further, Price LB (2004) identified bacteria from wounds by aerobic and anaerobic culturing methods and 16S rRNA pyrosequencing. Their results showed that, by the molecular pyrosequencing method, 10 bacterial families were identified, which were 4 times greater in number than the number of bacterial diversity detected by the culture methods (29). Davies CE et al. (2014), by comparison of denaturing gradient gel electrophoresis (DGGE) molecular method based on the PCR-amplified 16S rRNA gene products and culturing method showed that *Staphylococcus* and *Pseudomonas* spp., are the common bacteria in chronic wounds that were identified by the culture method. However, the remaining 40% of bacteria that were not culturable were identified by this molecular method (30). In the present study, the microbes that were commonly predominant in wounds according to molecular testing and by using aerobic culture methods were often aerobic bacteria. This finding showed that aerobic bacteria alone (as in this study) fail to resolve the complexity of the polymicrobial nature of chronic wounds. The results in this study demonstrated that bedsores infections are most common in different hospital

wards due to prolonged hospitalization and different circumstances, and cause a lot of damage in terms of health expenditure and psychological problems in patients.

Conclusion

The progression and chronicity of wounds can be correlated with infection, which, from a microbiological perspective, is dependent upon the types of bacteria present and their relationship with the host immune responses. Using molecular techniques and sequencing combined with microbial cultivation techniques is expected to be more effective to decipher the bacterial diversity in bedsores. However, further appropriate actions must be carried out for antibiotic treatment and rapid recovery of patients and thus, for control of wound infections.

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Conflict of interest

None declared conflicts of interest.

Financial disclosure

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