

Research Article,

Microbiological Analysis of Pseudomonas Aeruginosa and Staphylococcus Aureus Strains Isolated From Chronic Wounds

Ömer AKGÜL¹, Gülhan BORA²

^{1,2} Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Van Yüzüncü Yıl University, VAN, Turkey

Abstract:

Background: Wounds are classified as acute and chronic according to their clinical course. Although chronic wounds are rare, they can lead to death due to delays in healing. In both types of wounds, colonization with gram positive and gram negative bacteria can be observed. The diagnosis of microorganisms and antibiotic susceptibility profiles are known to be very important in treatment.

Material and Method: Swabs were taken from patients diagnosed with chronic wounds in our hospital. The samples were subjected to isolation and identification methods for microbiological analysis. Phenotypic antibiotic susceptibility analysis of each isolate was performed. Target gene analysis was performed for Staphylococcus aureus and Pseudomonas aeruginosa strains.

Discussion: Acute and chronic wound wounds can cause significant health problems and even death. Especially chronic wounds may cause sepsis due to their delayed healing. Wounds can be colonized with hospital and community-acquired bacteria. Wounds can also be colonized with both gram-positive and gram-negative bacteria. In our study, colonization with S. aureus and P. aeruginosa was more common. Males were more susceptible than females.

Conclusion: The most frequently isolated strains from patients with chronic wounds were S. aureus and P. aeruginosa. These strains were found to have multiple drug resistance. It was observed that strains with resistance genes posed a serious risk. A good understanding of the profiles of hospital and community-acquired wounds is very important in terms of establishing the correct treatment processes.

Introduction:

The primary role of intact skin is to control microbial populations colonizing at the skin surface and prevent deep tissue from being colonized and invaded by pathogens (1). The exposure of deep tissue following the breakdown of skin integrity (i.e. the wound) provides a moist, warm and nutritious environment conducive to microbial colonization and growth. Since many microbes are involved in wound colonization and contain a large number of potentially pathogenic microorganisms, any wound is at risk of becoming infected (2). Common microorganisms associated with wound infection include Staphylococcus aureus (S. aureus), which causes 20-40% of nosocomial infection according to different studies. Pseudomonas aeruginosa (P. aeruginosa), which mainly causes infections after surgery and

burns, has been reported to account for 5-15%. Other pathogens such as Enterobacteriaceae and Enterococci cause infection, especially in immunosuppressed patients and following abdominal surgery (3).

Chronic leg ulcers (CLUs) affect 1-2% of the population worldwide. They are a major cause of morbidity associated with delayed healing and high recurrence rates (4). CLUs particularly affect the elderly (over 65 years of age) and are associated with increased cost of diagnosis, treatment, care and rehabilitation (5). Chronic venous ulcers (CVUs) are the most common type of CLU (70% of cases). In addition, arterial ulcers account for 5-10%. Diabetic ulcers are mostly associated with a neuropathic cause and about 60% have an ischemic origin. Mixed venous and

arterial ulcers account for 15-30% of all CVUs (6; 7). Physiologically, the mechanism of wound healing is associated with connections between fibroblasts, Langerhans cells, keratinocytes and endothelial cells (8). Furthermore, this mechanism is characterized by hemostasis, inflammation, proliferation and remodeling (9). In the chronic wound, active leukocyte cytolytic enzymes, such as matrix metalloproteinase (MMPs) (10; 11), free oxygen radicals and inflammatory mediators are released, causing a mismatch between pathological local factors. This environment allows the colonization of both Gram-negative and Gram-positive microorganisms (12), especially Staphylococcus, Pseudomonas, Enterococcus, Enterobacter and Finegoldia (13; 14). It is observed that 93.5% of CVUs are caused by Staphylococcus aureus, 52.2% by Pseudomonas aeruginosa, 71.7% by Enterococcus faecalis, 45.7% by coagulase negative staphylococci, 41.3% by Proteus spp. and 39.1% by anaerobic bacterial infections (15). In diabetic and arterial foot lesions, Gram-positive aerobic cocci were found in 59% of cultures, of which 24% were S. aureus. In addition, the percentage of Gram-negative aerobic cultures, which are frequently colonized by Escherichia coli and Proteus mirabilis strains, is 35% (16; 17).

S. aureus and P. aeruginosa represent the most common agents isolated from CLUs and form a type of biofilm that is often resistant to antimicrobial therapy (18; 19). S. aureus usually colonizes at the top layer of wounds, while P. aeruginosa colonizes at the deepest part of the wound bed. S. aureus is a common opportunistic pathogen that is highly susceptible to many antibiotics such as methicillin (20), although treatment has been complicated in recent years by methicillin-resistant S. aureus (MRSA). P. aeruginosa has high chromosomal and acquired antibiotic resistance (21), which makes it difficult to treat (22). A wound determined to be infected with P. aeruginosa is characterized by a significantly larger area and a delayed or inhibited healing process (4). De Leon et al. (23) reviewed literature data and found that the ability of P. aeruginosa and S. aureus to survive the antibiotic treatment was enhanced when they were grown together in co-cultures. Similarly, Pastar et al. (24) interpreted the interactive effects of MRSA and P. aeruginosa on wound ulcers in an experimental study of a porcine wound healing model, reporting

that bacterial associations can alter virulence, prolong the healing process and alter response to antimicrobial therapy. Accurate diagnosis and treatment of leg ulcer infections requires detailed knowledge of skin bacterial epidemiology and risk factors for infection.

The aim of this study was to isolate S. aureus and P. aeruginosa from patients who applied to Van Regional Training and Research Hospital with chronic wound ulcers and to evaluate the antibiotic resistance status of these strains.

Material and method:

Bacterial isolation and identification analysis

Patients diagnosed with chronic wound ulcers were followed up between January 2020 and December 2021 at Van Training and Research Hospital. A swab sample was taken after the wound was washed with soap. The samples were taken to the laboratory for microbiological analysis in cold chain. For isolation, 5% sheep blood agar (Acumedia, USA), Mc Conkey agar (Oxoid, UK), Eosin Methylene Blue Agar (EMB, Oxoid, UK), Mannitol Salt agar, chromID® P. aeruginosa (bioMerieux) and chromID® MRSA SMART (bioMerieux) media were used. Catalase, oxidase and coagulase tests were performed. VITEK 2 device was used for identification and antibiotic resistance analysis of the isolated bacteria. Isolates were stored at -20°C.

Genomic DNA extraction and gene amplification

Bacteria were brought to the molecular unit of Van Yüzüncü Yıl University, Faculty of Pharmacy, Pharmaceutical Microbiology Laboratory for DNA extraction. The bacteria were cultured on Trypton Soy Agar (Acumedia, USA) and incubated at 37°C for 24 hours. Then, DNAs of S. aureus and P. aeruginosa strains showing multidrug resistance were extracted using the EcoSpin Bacterial Genomic DNA kit (Echotech Biotechnology, TURKEY) protocol. Bacterial DNA samples were stored at -20°C.

May Taq™ DNA Polymerase (Bioline, Bio-21105) protocol was used for DNA amplification of bacteria. For Polymerase Chain Reaction (mPCR), 10µL 5x MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl₂), 5µL template DNA, 1µL of each primer (20µM), 1µL MyTaq DNA polymerase and 8µL PCR water (ddH₂O) were calculated as 25µL final solution. PCR conditions

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for MecA were set as 15 min at 94°C, 30 s at 94°C, 1 min at 59°C, 1 min at 72°C, 10 min at 72°C, for 30 cycles. MecA gene amplification for isolated and identified S aureus was performed using F: 5'-TCCAGATTACAACCTTCACCAGG-3'; R: 5'-CCACTTCATATATCTTGTAACG -3' primer (25). PCR conditions for VIM and IMP were set as 5 min at 94° C, 1 min at 94°C, 1 min at 54°C, 2 min at 72°C, for 30 cycles. VIM and IMP gene amplifications for isolated and identified P. aeruginosa were performed by using F: 5'-GTTTGGTCGCATATCGCAAC-3'; R: 5'-AATGCGCGCAGCACCAGGATAG-3' and F: 5'- GAAGGCCGTTTATG- TTCATAC-3'; R: 5'-GTATGTTTTCAAGA- GTGATGC-3' primers (26). HyperLadder™ marker (50 Base Pair, Bioline, USA) was used to evaluate amplicon sizes. Bacterial amplicon products were run on a 1.5% agarose gel in a Thermo EC300XL2 electrophoresis device at 100 volts for 1 hour. Amplicons were visualized using Bio-Print- ST4 (Vilber Lourmant, France). P. aeruginosa ATCC27853 and S. aureus ATCC297213 strains were used as controls.

Ethics Committee Approval

The ethics committee approval of our study was approved by the clinical research ethics committee of Van Training and Research Hospital with the decision dated 11/09/2020 and numbered 2020/04.

Results:

Two hundred sixty-nine patients with chronic ulcerative wounds were microbiologically analyzed. 185 (69%) of the patients were male and 84 (31%) were female. After isolation and identification from these patients, all of the wounds were found to be contaminated with different microorganisms. It was determined that 47 (17.5%) of the wounds were mono-microbial and the others were poly-microbial. Of these wounds, S. aureus and P. aeruginosa were the most frequently isolated microorganisms in 81 (34%) and 55 (23%) wounds, respectively.

The analysis of S. aureus by Vitek II showed that the highest antibiotic resistance was found to Tetracycline, Erythromycin and Gentamicin. In addition, the highest susceptibility was found to Vancomycin. 17 isolates were determined to have MRSA phenotype. As a result of the analysis of P. aeruginosa strains with Vitek II, it was determined that the highest resistance was to Cefoxitin, Cefazolin and Cefepime. In addition, the highest susceptibility was found to Colistin. Furthermore, carbapenem resistance was observed among P. aeruginosa strains, although not very high. Information about the antibiotic resistance phenotype of both S. aureus and P. aeruginosa strains is given in Table I.

Table I. Phenotypic antibiotic susceptibility results of S. aureus and P. aeruginosa strains

Antibiotics	S. aureus (n=81)			Antibiotics	P. aeruginosa (n=55)		
	R	I	S		R	I	S
Cefoxitin screening	17	-	64	Ampicillin	37		18
Benzyl penicillin	56	-	25	Piperacillin/Tazobactam	33		22
Oxacillin	17	-	64	Cefazolin	44		11
Gentamicin	60	4	17	Cefuroxime	42		13
Ciprofloxacin	27	-	54	Cefuroxime Acetyl	39		16
Levofloxacin	25	-	56	Cefoxitin	45		10
Moxifloxacin	35	-	46	Ceftazidime	36		19
Inducible Clindamycin resistance	21	-	60	Ceftriaxone	37		18
Erythromycin	63	-	18	Cefepime	43		12
Clindamycin	50	-	31	Imipenem	16		39
Quinupristin/Dalfopristin	43	-	38	Meropenem	13		42
Linezolid	29	-	52	Amikacin	37		18
Vancomycin	2	-	79	Gentamicin	35		20
Tetracycline	67	-	14	Ciprofloxacin	22		33
Tigecycline	21	-	60	Tigecycline	19		36
Nitrofurantoin	32	-	49	Cholistin	2		53
Rifampin	44	-	37	SXT	34		21
SXT	21	-	60				

Genotypic analysis of *S. aureus* strains phenotypically identified as MRSA was performed. All MRSA strains were found to carry *MecA* gene. Among the 16 *P. aeruginosa* strains phenotypically showing carbapenem resistance, 7 of them were positive for VIM and 3 of them were positive for IMP genes.

Discussion:

Healthcare professionals are responsible for wound management and care to ensure that wound infections are prevented or reduced. However, in cases where infection develops, wound management practices should be addressed in detail. In other words, wound treatment requires a systematic study from the analysis of the patient profile to the treatment process. Antibiotic treatment protocol is prepared, but antibiotic susceptibility testing should be performed first. The study by Mama et al (27), showed that the incidence of wound infection was higher in males (89.7%) than females (81.4%). This is in line with studies conducted in different parts of Ethiopia (28) and other countries (29). This may be explained by the fact that traditionally, men are predominantly involved in occupations such as farming, construction work, transportation and industrial work where trauma exposure is common. In our study, as in the studies mentioned above, wound infections were more common in men than in women.

In a study by Bessa et al. 28 microbial species were isolated from wounds with signs of infection. Most of the wounds were colonized by a single bacterial species. The most common isolate was *S. aureus*, which has been reported as the dominant microorganism isolated from different types of wounds in other studies (30; 31). In a study by Mama et al. (27) 91.6% of culture positive wounds showed mono-microbial growth, 8.4% showed poly-microbial growth and 12.7% showed no bacterial growth. Similarly, high rates of mono-microbial growth have been reported in India (86-100%) and Pakistan (98%) (32; 33). In our study, 47 (17.5%) of the wounds were mono-microbial and the rest were poly-microbial. Based on this, unlike the above studies, poly-microbial culture growths were found to be higher in our study. It is seen that *P. aeruginosa* is the most frequently detected Gram-negative bacterium

according to the studies (4; 34). *S. aureus* and *P. aeruginosa* are known to produce highly destructive virulence factors responsible for maintaining infection and prolonging healing in chronic wounds. *S. aureus* causes clinically significant infections, mostly through virulence factors such as coagulase, catalase, clumping-factor A and leukocidins (35). Furthermore, elastase released by *P. aeruginosa* has been associated with pathogenicity in the wound environment (36). Therefore, our results confirm the usual most common microorganisms found in infected wounds. However, the role that each specific pathogen plays in both non-healing and infected chronic wounds is not yet fully defined; it is mostly based on hypotheses. Apart from the presence of pathogens, the presence of specific bacterial combinations and interactions is thought to be crucial in both acute and chronic wounds (37). In a separate study, *S. aureus* (32.4%) and *E. coli* (20%) were the main organisms isolated from wound infections (27). A number of previous reports on wound infection from Ethiopia and different parts of the world showed that *S. aureus* and *E. coli* were the most common isolates (38; 39). The high prevalence of *S. aureus* may be because it is an endogenous source of infection. Infection with this organism may also be due to contamination from the environment, e.g. contamination of surgical instruments. *S. aureus*, a common bacteria found on surfaces, easily colonizes into wounds when the natural skin barrier is disrupted. In our study, *S. aureus* and *P. aeruginosa* were the most common bacteria found in 81 (34%) and 55 (23%) chronic wounds, respectively. Compared to other studies, we have identified a possible bacterial profile in our patients.

Bacterial isolates were examined for patterns of susceptibility to the antibiotics most commonly used in treatment. Despite growing concerns about antibiotic-resistant bacteria, the appropriate use of systemic antibiotics is still recommended where there is clear evidence of infection (40). Resistance to oxacillin is particularly important because it can give us the percentage of methicillin-resistant *Staphylococcus aureus* (MRSA). In the study by Bessa et al., it was found that 21.8% of *S. aureus* was resistant to oxacillin. *S. aureus* has always been the main source of

infection in acute soft tissue wounds. MRSA, however, has been an organism causing infection in only a small portion of the total. However, MRSA is recently becoming a more common wound pathogen (41). The emergence of MRSA presents two problems: first, it is associated with the chronic wound, which is the source of other MRSA nosocomial infections. Second, with the impact of MRSA on the chronic wound itself, there is an increased risk of bacteremia (41). Among Gram-positive bacteria, all isolates were susceptible to vancomycin and linezolid; no resistance was detected despite the massive use of these two antibiotics. According to the study by Mama et al. (27), *S. aureus* tended to be resistant to a wider range of antibiotics in the determination of the susceptibility of *S. aureus* to fifteen antibiotics selected by disk diffusion technique. In this study, *S. aureus* showed high resistance to ampicillin (95.7%), penicillin (91.5%) and tetracycline (51%). This was consistent with studies conducted in Ethiopia (42) and elsewhere (29). The same isolate was highly susceptible to amikacin (100%), vancomycin (100%), ciprofloxacin (96%), norfloxacin (96%) and gentamicin (96%). In addition, clinical *Staphylococci* were found to be 100% susceptible to vancomycin and (43) amikacin. In the study by Mama et al. coagulase negative *Staphylococci* were found to be 100% susceptible to amikacin and vancomycin, sulfamethoxazole trimethoprim (86%), gentamicin (83%) and ciprofloxacin (76.2%). The same organism was highly resistant to ampicillin (90.5%), penicillin (76%), cephalothin (71%) and tetracycline (52%). This finding was comparable to studies conducted in the same country (44) and elsewhere in the world (45; 46). The remarkable susceptibility of Gram positive bacteria to vancomycin, amikacin and aminoglycosides (gentamicin) may be due to the less frequent use of these antibiotics due to less availability, cost and toxic effect respectively. In our study, seventeen isolates were found to be MRSA, indicating a low risk situation for total chronic wound patients. However, it is known that the risk is high in MRSA colonized patients. As a result of the antibiotic susceptibility analysis of *S. aureus*, the highest antibiotic resistance was found to Tetracycline, Erythromycin and Gentamicin. In addition, the highest susceptibility was found for Vancomycin. When evaluated with the above antibiotic resistance rates, it was seen that there

were differences and it was important to perform antibiogram testing.

Regarding antibiotic resistance of Gram-negative, the most common isolates, and especially *P. aeruginosa*, showed a relatively high resistance to most of the antibiotics. Multi-drug resistant isolates of *P. aeruginosa*, which are simultaneously completely resistant to ampicillin, amoxicillin/clavulanic acid, ertapenem and trimethoprim/sulfamethoxazole, are of great concern. In addition, the results show that *P. aeruginosa* tends to show a high level of resistance to carbapenems and third-generation cephalosporines. However, *P. mirabilis* and *E. coli* showed a low resistance profile compared to *P. aeruginosa*. In the study by Mama et al. (27), *P. aeruginosa* showed decreased susceptibility to commonly used antibiotics such as ampicillin, doxycycline, naldixic acid and tetracycline, except ciprofloxacin, norfloxacin (100%) and gentamicin (82%). Ciprofloxacin and norfloxacin were reported to be the most potent oral drugs available for the treatment of *P. aeruginosa* infections. This report is in agreement with the result of another study in which ciprofloxacin recorded the least resistance (6.2-24%) to *P. aeruginosa* isolates from wound infection (47). Currently, there is no doubt that the oral drug ciprofloxacin and injection gentamicin are the most effective antibiotics for *P. aeruginosa* involved in wound infection compared to many other commonly used drugs. Third generation cephalosporines (ceftriaxone 63.6%) are a real cure for resistant *Pseudomonas*. In fact, irrational and inappropriate use of antibiotics is responsible for the development of resistance of *Pseudomonas* to antibiotic monotherapy. The incidence of *P. aeruginosa* in wound infection in hospitalized patients is becoming more serious in developing countries due to the lack of general hygiene conditions, production of low-quality antiseptics and medical solutions for treatment (29). In our study, antibiotic susceptibility analysis of *P. aeruginosa* strains showed the highest resistance to cefoxitin, cefazolin and cefepime. In addition, resistance to colistin was found to be at the lowest rate. Again, carbapenem resistance was determined among *P. aeruginosa* strains, although it was not high. *P. aeruginosa* isolated from patients with chronic wounds showed multidrug resistance and this posed a risk.

Conclusion:

In our hospital, *S. aureus* and *P. aeruginosa* were found to be the most common bacterial agents in patients with chronic wounds. Since these bacteria may have multidrug resistance, it was determined that it is very important to perform antibiogram tests. Vancomycin and colistin can be used effectively in wound infections. It was revealed that improving the part of hospital surveillance related to wound infections would contribute to treatment.

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