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Bacteriology of Wound - Clinical Utility of Gram Stain Microscopy and the Correlation with Culture

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Abstract

Aim: To determine the most common bacteria isolated from wound specimens and to compare those culture results to Gram stain slides.

Material and methods: A total of 1970 specimens from 1788 patients, treated in the University Clinics in Skopje during a one year period were examined by standard microbiology techniques (inoculation onto standard agar media and direct Gram-stained smears). Automatized Vitek system was used for identification of all anaerobes.

Results: Out of a total of 1970 specimens, 1094 (55.5 %) were positive by culture. A total of 1462 strains were isolated: 753 Gram positive (Gram+), 661 Gram negative (Gram-) and 48 anaerobic bacteria. The number of specimens yielding one, two or more different strains was 788, 244 and 62, respectively. Gram + bacteria, in 44.7 % of positive samples were a single isolate. The most commonly isolated potential pathogen was *Staphylococcus*. In 23.7% samples, Gram negative bacteria were a single isolate (*E. coli* was the most common isolate). 1094 specimens were positive by culture, 419 (38.3%) were positive by both culture and Gram stain and 675 (61.7%) were negative by Gram stain (leukocytes were present in 276 specimens). 876 specimens were negative by culture, 789 (90%) were negative by both culture and Gram stain (leukocytes were present in 271 specimen) and 87 (9.9%) were positive only by Gram stain.

Conclusion: Our study demonstrated only a 38.3 % of microbiological correlation between Gram stain and culture. This data makes the clinical utility of Gram stain for the microbiological analysis of wounds questionable.

Introduction

From a microbiological perspective, the primary function of normal, intact skin is to control microbial population that live on the skin surface and to prevent underlying tissue from becoming colonized and invaded by potential pathogens. A loss of skin integrity (i.e., a wound) provides a moist, warm and nutritious environment that is conductive to microbial colonization and proliferation. The diversity of microorganisms in any wound will be influenced by factors such as wound type, depth, location, level of tissue perfusion and the antimicrobial efficacy of the host immune response [1]. Wounds can be broadly categorized as having either an acute or a chronic etiology. Acute wounds are caused by external damage to intact skin (surgical wounds, bites, burns, cuts and abrasions, lacerations and crush and gun shot injuries). Chronic wounds are most frequently caused by endogenous mechanisms associated with a predisposing condition that compromises the integrity of dermal and epidermal tissue (leg ulcer, foot ulcer and

pressure sores) [2-5].

The microbial flora in wounds appears to change over time. In early acute wound, normal skin flora predominate (Gram positive cocci are common organisms). After about four weeks, facultative aerobic gram negative rods colonize the wound (*Proteus, E. coli* and *Klebsiella* are most common once). As the wound deteriorates deeper structures are infected. Oftentimes infections are polymicrobial. Long-term chronic wounds usually contain more anaerobes than aerobes. Sometimes can be very difficult to detect when the wound is infected.

When typical features of wound infections appear (increased exudate, swelling, erythema, pain, local temperature, periwound cellulites etc) wound swab is commonly taken [2, 6, 7]. Wound swabbing involves the use of a cotton-tipped swab to sample superficial wound fluid and tissue debris and enables a semiquantitative and qualitative analysis of the wound microflora. Although the value of acquiring superficial swab samples has been questioned, the procedure is simple, inexpensive, non-invasive and convenient for the majority of wounds. The gold standard collection method is a tissue biopsy. If the biopsy is not possible, wound swab is a potentional alternative. However, taking a sample of pus or liquid is preferable [2, 3, 8].

A Gram stain should always be performed on a wound specimen. If numerous leukocytes are observed, together with a single bacterial morphology, an early pathogen can be provided. A specimen characterized by a foul odor usually indicates the presence of anaerobic bacteria. Wound specimens should be cultured for both aerobic and anaerobic microorganisms. An antibiograms should be performed if one microorganism is clearly prevalent, as well as, on prevalent microorganisms in mixed cultures, particularly if large numbers of leukocytes are observed in the Gram stain [9].

Gramstain was a part of a routine microbiological examination in the Institute of Microbiology in Skopje ten years ago. After a period of few years, Gram stain was implemented again. The clinical utility of adding a Gram-stained slide and its correlation with culture results has not been evaluated previously in the Institute of Microbiology in Skopje.

The aim of this study was to determine the most common bacteria isolated from wound specimens and to evaluate the use of Gram stain compared to microbiological culture.

Material and Methods

A total of 1970 specimens (swab, pus, aspirates, punctuates, necrotic tissue) from 1788 patients, treated in the University Clinics in Skopje during a one year period were examined.

On receipt by the laboratory, all specimens were inoculated onto standard agar media (Columbia agar with 5% sheep blood for the isolation of aerobes, Schaedler agar for the isolation of anaerobes and glycose broth). The aerobic plates were read within 24–48 hours and the anaerobic plates at 48 and 72 hours. Any growth was subsequently identified by standard microbiological methods, i.e the appearance of the colonies, biochemical identification and automated Vitek 2 system. Automatized Vitek system (bioMerieux, Marcy l'Etoile, France) was used for identification of all anaerobes, confirming the identification of 13 strains of Gram negative aerobes. The turbidity of the bacterial suspension was adjusted with Vitek 2 Densichek (bioMerieux) to match the McFarland 0.5 standard in 0.45% sodium chloride. The Vitek 2 ID cards were used. The VITEK 2 system reported the results automatically with software release 2.01 according manufacturer's recommendations.

Quantitative bacteriology was not performed.

Gram-stained smears from wound specimens were also performed at the time of culturing, in which the presence of bacteria and/or leukocytes (Le) was detected.

Gram-stained slides were analyzed under oil immersion (x1000) magnification. For each morphologically distinct microorganism seen, the Gram reaction (Gram-positive or Gram-negative), morphology (e.g., coccus, rod, yeast), other distinguishing features (e.g., formation of chains or clusters) were determined.

The Gram stain results were defined as follows: a positive Gram stain requires that organisms with or without appreciable numbers of white blood cells were seen under oil immersion light microscopy; a negative Gram stain requires that no organisms were seen under oil immersion light microscopy. The Gram stain results were studied in comparison with isolation of viable organisms in cultures. A true positive was defined as a positive Gram smear from a wound from which an organism was subsequently cultured within 48 hours. A false positive had a positive Gram smear but a negative culture within 48 hours. A false negative had a negative smear but a positive culture within 48 hours of incubation.

Student t-test was used for the statistical analysis.

Results

Out of a total of 1970 specimens, 1094 (55.5 %) were positive by culture. A total of 1462 strains were isolated: 753 Gram positive (Gram+), 661 Gram negative (Gram-) and 48 anaerobic bacteria. The number of specimens yielding one, two, or more different strains was 788, 244 and 62, respectively. The total number of specimens negative by culture was 876 (44.5%).

Table 1: Gram positive (Gram+) and Gram negative (Gram-) bacteria isolated from wound specimens.

Bacteria	A single isolate	With other Gram+ bacteria	With other Gram- bacteria	With >2 Gram+ and Gram- bacteria	Total
Staphylococcus aureus	262	13	13	2	290
Staphylococcus aureus-MR	52	1	7	7	67
Staphylococcus-coagulasa negativ	81	1	1	/	83
Enterococcus	58	5	6	4	73
Streptococcus β-haemolyticus	31	3	1	1	36
Corynebacterium spp (JK and D2)	5	/	/	/	5
	489	23	28	14	554
Escherichia coli	75	31	23	11	140
Klebsiella spp	43	12	10	14	79
Pseudomonas aeruginosa	43	13	17	5	78
Proteus mirabilis	37	17	7	10	71
Acinetobacter spp	28	26	7	5	66
Enterobacter/Serratia	20	11	7	3	41
Stenotrophomonas maltophilia	12	/	1	/	13
Citrobacter spp	1	/	3	/	4
	259	110	75	48	492
Anaerobic bacteria	40	4	4	/	48
Total	788	244		62	1094

Gram positive bacteria in 489 (44.7%) samples included only a single isolate, and in 227 (20.7%) samples were together with other Gram positive and Gram negative bacteria.

Strains from genus *Staphylococcus* (the most commonly isolated potential pathogen) and *Streptococcus* were a single isolate in 395 (36%) and 89 (8.1%) of positive specimens, respectively.

Gram negative strains were isolated from 538 samples. The most common isolate was *E. coli* -140 (12.8% of positive specimens), followed by *Klebsiella spp*. - 79 (7.2%), *Pseudomonas aeruginosa* - 78 (7.1%) and *Proteus mirabilis* - 71 (6.5%). In 259 (23.7%) samples, Gram negative bacteria were a single isolate and in 279 (25.5%) they were isolated together with other bacteria. Gram negative bacteria were more commonly isolated with other strains compared to Gram positive strains. This difference is statistically significant (p=0.00001) (Table 1; Figure 1).

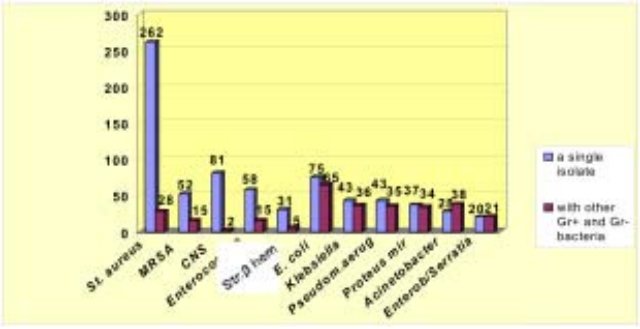

Figure 1: The common isolates, present in wound specimens as a single bacteria or together with other Gram positive or/and Gramnegativebacteria.

Stenotrophomonas, S. aureus, MRSA, *Enterococcus, Streptococcus, Staphylococcus coagulasa negative* and anaerobes appeared frequently as a single isolate, than together with other Gram positive and Gram negative bacteria. This difference was statistically significant.

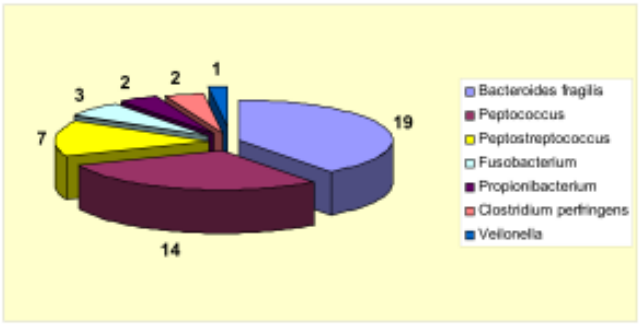

Figure 2: Anaerobes isolated from wound specimens.

Out of a total of 1094 specimens positive by culture, in 48 (4.4%) of them, anaerobes were isolated and confirmed by Vitek. *Bacteroides fragilis*-19 and *Peptococcus*-14 were the most frequently isolated anaerobes. Forty anaerobic bacteria (3.7%) appeared more as a single isolateand the rest 8 (0.7%) were combined with other Gram positive and Gram negative aerobic bacteria. This difference was statistically significant (p=0.00001) (Figure 2).

One thousend ninety four (1094) specimens were positive by culture; 419 (38.3%) of them were positive by both culture and Gram stain and the rest of 675 (61.7%) were positive only by culture and negative by Gram stain (leukocytes were present in 276 specimens). 876 specimens were negative by culture,

Table 2: Analysis of Gram stained slides from specimens with isolated bacteria.

Bacteria	Le+	Le+++	Le, bacteria	Bacteria	Negative direct slide	Total
Klebsiella spp.	13	14	30	7	15	79
Acinetobacter spp	6	5	13	11	31	66
Pseudomonas	8	13	23	8	26	78
Proteus	6	10	33	10	12	71
E. coli	12	20	51	16	41	140
Enterobacter / Serrat.	7	8	7	3	16	41
Citrobacter	/	/	3	/	1	4
Stenotrophomonas	1	5	3	4	/	13
St. aureus	45	33	56	21	135	290
MRSA	4	11	13	11	28	67
Enterococcus	12	8	26	10	17	73
Streptococcus	2	3	12	8	11	36
Staphylococcus coag. 0	9	5	14	5	50	83
Corynebacterium spp.	2	/	/	1	2	5
Anaerobes	2	12	16	4	14	48
Total	**129**	**147**	**300**	**119**	**399**	**1094**
			419 (38.3%)			

789 (90%) were negative by both culture and Gram stain (leukocytes were present in 271 specimen) and 87 (9.9%) were positive only by Gram stain.

Table 3: Analysis of direct Gram stained slides from specimens negative by culture.

Negative direct slide	Le+	Le+++	Le, bacteria	Bacteria	Total
518	133	138	48	39	876
	789		87		

Organisms examined by Gram stain yielded a sensitivity of 38%, specificity of 90%, positive predictive value of 83% and negative predictive value of 54% when used to predict positive culture results for bacterial wound infection.

Table 4: Results of Gram stain and culture of the 1970 specimens.

Gram stain	Positive culture	Negative culture	Total
Positive Gram stain	419	87	506
Negative Gram stain	675	789	1464
Total	1094	876	1970

Sensitivity, 419/1094 = 38%; Specificity, 789/876 = 90%; Positive predictive value, 419/506 = 83%; Negative predictive value, 789/1464 = 54%.

Discussion

The microorganisms most frequently involved in wound infections change from time to time and also vary with hospital settings. The majority of open and chronic wounds are polymicrobial. Superficial wounds and surgical incision are usually monomicrobial [2, 6, 7, 10]. Giacometti et al. (2000) reported monomicrobial and polymicrobial wound infections [10]. For most surgical site infections, the source of pathogens is the endogenous flora of the patient's skin, mucous membranes, or hollow viscera. These organisms are usually aerobic gram-positive cocci (e.g., *Staphylococci*), but may include fecal flora (e.g., anaerobic bacteria and Gram negative aerobes) when incisions are made near the perineum. When a gastrointestinal organ is opened during an operation and is the source of pathogens, Gram negative bacilli (e.g., *E. coli*), Gram positive organisms (e.g., *Enterococci*), and sometimes anaerobes (e.g., *Bacillus fragilis*) are the typical isolates. Exogenous sources of pathogens include surgical personnel, the operating room environment (including air), and all tools, instruments, and materials brought to the sterile field during an operation [10].

In present study, 53% of contaminating bacteria were Gram positive and 47% were Gram negative bacteria. Most of the specimens showed monobacterial growth. Out of a total number of Gram positive bacteria, the most commonly isolated potential pathogen was *Staphylococcus* (47.5%). Gram positive bacteria, in 44.7% samples, were a single isolate. Out of a total number of Gram negative bacteria, the most common isolate was *E. coli* -12.8%, followed by *Klebsiella spp*.- 7.2%, *Pseudomonas aeruginosa*- 7.1% and *Proteus mirabilis* – 6.5%. In 23.7% samples, Gram negative bacteria were a single isolate. Gram negative bacteria were more commonly isolated with other Gram positive and Gram negative bacteria compared to Gram positive ones. This difference is statistically significant (p=0.00001).

In other studies on Gram stain examination, 31.2% pathogens were Gram positive and 68,8% were Gram negative, as against 70% for Gram positive and 30% for Gram negative organisms in other setups. This difference may be due to variation in common nosocomial pathogens inhabitant in different hospital set up. Though percentage of Gram negative bacilli from the wounds was more, *Staphylococcus aureus* was the predominant organism isolated followed by *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Escherichia coli* [10]. Similar findings were observed in the studies done previously [10-13]. In the other study of surgical wound infections, the most common bacterium was *Escherichia coli* (50%) followed by *Staphylococcus aureus* (20%), *Pseudomonas aeruginosa* (20%), *Enterococcus faecalis* (10%), *Proteus mirabilis* (10%), and Coagulase-negative staphylococci (10 %) [2, 14].

Surface swab cultures, by virtue of their ease of collection and reduced laboratory processing cost, have

attracted much attention as a potential alternative to the gold standard histology and quantitative culture method for microbiological wound monitoring. Gram stain proved to be simple, rapid, cheap, and of acceptable predictive value. A rapid Gram stain technique was shown to reliably predict a microbial load of $< 10^5$ CFU/g of tissue if a single microorganism was seen on the slide preparation [2,15-17]. Breidenbach and Trager demonstrated that a critical level of bacteria of $< 10^4$ CFU/g of tissue must be reached to cause infection, and that quantitative tissue cultures predict the likelihood of wound infection more effectively than swab cultures do [18]. In contrast, Pruitt et al. reported that quantitative culture are incapable of differentiating between wound colonization and infection and in burn wound infection histological analysis was the most effective method [19].

Careful examination of Gram-stained slides is required to determine Gram-stain affinity, morphology and arrangement of the organisms,however it has to be emphasized that Gram stain should not be a substitute for culture. The Gram stain information would not have been sufficient to guide possible choice of antimicrobial chemotherapy, although the impact of these results on antibiotic choice was not the focus of our study.

There were few reports on the clinical utility of Gram stain microscopy and the correlation of the results with culture [9, 20-22]. Our study demonstrated that Gram stain compared to culture showed lower sensitivity (38%), but fair specificity (90%), and a positive predictive value (82.8%). Although the organisms seen on Gram stain were commonly isolated in culture, many specimens yielding a potential pathogen in culture had no organisms seen on the Gram stain. This situation mainly occurred when growth in culture was poor. Inoculation of agar media was done prior to Gram staining and we suppose that the inoculum's size may have been reduced, although this remains to be determined. It is hypothetically possible that surface wound Gram stain might be a better predictor of infection than wound biopsy or surface culture, because culture-positive, Gram stain-negative wounds might have a very low bacterial concentration, and the wound is unlikely to become infected, although this requires further investigations.

A total of 876 wound cultures were negative and did not grow viable organisms. This might be attributed to difficult-to-grow fastidious organisms, inappropriate processing of specimens in the laboratory, or the administration of antibiotics prior to specimen collection.

False positive Gram stain results could be due to either stained cotton swab fibres or stain deposits or crystals [21]. We suppose that the presence of anaerobes in the wound that die from oxygen exposure due to delayed processing or bacterial contaminants that are not multiplying in the wounds or the presence of non-viable microorganisms could be the reason for false-positive Gram stain, although this has yet to be proven.

In conclusion, the most commonly isolated potentional pathogen from wound specimens were bacteria from the genus *Staphylococcus (Staphylococcus aureus,* followed by *Staphylococcus coagulasa-negative*, and *Staphylococcus aureus*-methicillin resistant-*MRSA).* The most common Gram negative isolate was *E. coli,* followed by *Klebsiella spp*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. Gram positive bacteria and anaerobes more often appeared as a single isolate compared to Gram negative bacteria. Our study demonstrated only a 38.3% microbiological correlation between Gram stained slide and culture. This data makes the clinical utility of Gram stain for the microbiological analysis of wounds questionable. In most cases, the Gram stain information would not have been sufficient to guide possible choice of antimicrobial chemotherapy or implementation of appropriate infection control measures. Therefore, for precise microbiological diagnosis of wound infections, cultivation of wound specimens should be implemented.

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