The utility of Gram stains and culture in the management of limb ulcers in persons with diabetes

Zulfiqarali G Abbas, Janet K Lutale, Mapoko M Ilondo, Lennox K Archibald

ABSTRACT

In Tanzania, limited laboratory services often preclude routine identification of microorganisms that cause infections in persons with diabetes. Thus, we carried out this study to determine the utility of a Gram stain alone versus culture in guiding appropriate antimicrobial therapy. During February 2006 to December 2007 (study period), deep tissue biopsies were obtained from persons with diabetes presenting to the Muhimbili National Hospital (MNH) with infected limb ulcers. Specimens were Gram-stained then cultured for bacteria and fungi. Biopsies were obtained from 128 patients. Of 128 cultures, 118 (92%) yielded bacterial or fungal growth; 59 (50%) of these 118 cultures yielded mixed growth (80% included Gram-negative organisms); 38 (32%) and 20 (17%) yielded Gram-negative and Gram-positive organisms alone, respectively. The predictive value positive of a Gram stain for bacterial growth was 93% (110/118); a Gram-positive stain was 75% (15/20) predictive of growth of Gram-positive organisms whereas a Gram-negative stain was 82% (31/38) predictive of growth of Gram-negative organisms. In regions with limited resources, a Gram stain of an ulcer biopsy that is carefully procured is largely predictive of the type of microorganism causing infection. Gram staining of deep tissue biopsies might have a potential role to play in the management of infected diabetic limb ulcers.

Key words: Bacterial culture ● Deep tissue biopsy ● Diabetic foot ● Gram stains ● Ulcer biopsy

BACKGROUND

In Sub-Saharan Africa, foot complications are the main cause of prolonged hospital stays for persons with diabetes, and are associated with substantial morbidity and mortality (1). In Tanzania, high mortality rates are observed in persons with diabetes who have developed ulcers that have progressed to gangrene (2).Concomitant local infections in the foot play a critical role in the pathogenesis of foot ulcer disease, which can progress to systemic infection, loss of limb or death (3,4). In addition, lower limb amputation is a common sequelae for persons with diabetes who have been hospitalised for progressive infection of their ulcers (2). Infection itself may be the primary cause of foot ulcers in persons with diabetes who have neither neuropathy nor vascular disease (2). Prompt laboratory-directed therapy with appropriate antimicrobials rather than blind empiricism would be expected to improve the outcomes among patients for whom the pathogenesis of foot ulcers was associated with bacterial infection. Unfortunately, limited laboratory services in Tanzania often

Authors: ZG Abbas, MMed, DTM&H, Department of Medicine, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania, Abbas Medical Centre, Dar es Salaam, Tanzania; JK Lutale, MMed, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania; MM Ilondo, PhD, World Partner Project, Copenhagen, Denmark; LK Archibald, MD, FRCP, Division of Infectious Diseases, College of Medicine, University of Florida, Gainesville, FL, USA
Address for correspondence: ZG Abbas, MMed, DTM&H, P. O. Box 21361, Dar es Salaam, Tanzania
E-mail: zabbas@cats-net.com

Key Points

- in Tanzania, high mortality rates are observed in persons with diabetes who have developed ulcers that have progressed to gangrene
- infection itself may be the primary cause of foot ulcers in persons with diabetes who have neither neuropathy nor vascular disease

preclude routine microbiological identification of microorganisms involved in the pathogenesis of various bacterial infections occurring in persons with diabetes. Because of the simplicity of a Gram stain and the relatively low cost of Gram stain reagents, we carried out this study to determine the utility of Gram staining alone versus culture in guiding appropriate antimicrobial therapy.

PATIENTS AND METHODS

Muhimbili National Hospital (MNH) is located in Dar es Salaam, Tanzania and is the largest hospital and main referral medical facility in the country. MNH serves a catchment area population of about 6·6 million people of diverse ethnic backgrounds. The Department of Medicine at MNH runs a busy diabetes out-patient service with >5000 patients attending each year. The MNH Department of Medicine has been aggregating epidemiological, clinical and microbiological data on upper and lower limb ulcers in persons with diabetes since 2000 with patients' consent. All data are recorded without patient identifiers. We studied surveillance data for the period February 2006 through December 2007 (study period) for all diabetes patients presenting to the MNH diabetes clinic with upper or lower limb ulcers and clinical signs of infection.

During the study period, two deep tissue biopsy specimens were procured by one of the authors (ZGA) using aseptic surgical technique and probing deep into the tissue at the base of the ulcer with a sterile scalpel and forceps. The biopsies were placed in a sterile container and taken immediately to the microbiology laboratory for processing. A smear of one tissue specimen was stained using Gram reagents followed by plating of the tissue on sheep blood agar media for bacterial and fungal culture. The second tissue specimen was stained with Ziehl-Neelsen reagents for mycobacteria. Both smears were analysed under light microscopy. Available resources did not enable routine mycobacterial culture of this second tissue specimen. All microbiological processing and isolate identification were carried out by a trained laboratory technician in the microbiology department using validated microbiological procedures and standard biochemical testing methods. Because patients resided in a region of East Africa

endemic for human immunodeficiency virus (HIV) infection, HIV testing had been offered to all patients with appropriate pre- and post-test counselling.

Data were analysed using SAS statistical software package (SAS Institute, Cary, NC). We compared the results of Gram stains showing Gram-positive and Gram-negative microorganisms on microscopy with culture results (i.e. growth of Gram-positive and Gram-negative microorganisms on solid agar) in 2×2 tables and calculated sensitivities and positive predictive values. The utility of Gram stains specifically as a detection mechanism was compared with bacterial cultures on solid agar media using the McNemar modification of the chi-square test and, where appropriate, the Yates correction for small numbers of observations.

RESULTS

One hundred and twenty-eight persons with diabetes were enrolled during the study period. Of these, 95 (74·2%) were male; median age was 56 (range: 14–80) years; 90 (70%) were indigenous Africans, 19 (15%) were Asian Indian, and 19 (15%) were Arab; 116 (91%) controlled their diabetes with oral agents or diet only. One hundred and eight (84%) ulcers were located in the lower limb of study participants while 20 (15·6%) were located on the upper limb.

Of the 128 Gram stains performed on prepared smears of the deep tissue specimens, 118 (92·2%) showed microorganisms on light microscopy. Of these 118 positively stained smears, 64 (54·2%) showed mixed organisms (i.e. various combinations of Gram-positive and Gram-negative organisms, and yeasts). Thirty-eight (32·2%) smears stained for Gram-negative bacteria only and 17 (14·4%) stained for Gram-positive organisms only. Just one (0·9%) smear showed yeasts alone on Gram stain.

Of the 128 individual cultures performed, 126 (98·4%) yielded growth of microorganisms: 58 (46·8%) of these positive cultures yielded a single microorganism (Table 1); 68 (54·0%) yielded mixed growth involving a total of 138 Gram-positive and Gram-negative bacteria and yeasts (Table 2). Forty-four (75·9%) of the 58 single isolates were Gram-negative bacteria; 13 (22·4%) were Gram-positive bacteria and

Table 1 Cultures that yielded a single organism (Gram-positive or Gram-negative)

Organism	Numbers isolated (n = 58)	%
Escherichia coli	14	24·1
Klebsiella pneumoniae	13	22·4
Staphylococcus aureus	8	13·8
Pseudomonas aeruginosa	7	12·1
Coliforms	6	10·3
Proteus mirabilis	4	6·9
Staphylococcus epidermidis	3	5·2
Yeast	2	3·5
Enterococcus sp.	1	1·7

Table 2 Results of cultures that yielded polymicrobial growth of microorganisms

Organisms isolated	Number of cultures (n = 68)
Staphylococcus aureus Escherichia coli	13
Staphylococcus aureus Klebsiella pneumoniae	11
Staphylococcus aureus Pseudomonas aeruginosa	6
Staphylococcus aureus Yeast	6
Staphylococcus aureus Proteus mirabilis	5
Staphylococcus aureus Coliforms	5
Staphylococcus aureus Staphylococcus epidermidis	1
Staphylococcus aureus Enterococcus sp.	1
Staphylococcus epidermidis Escherichia coli	4
Staphylococcus epidermidis Coliforms	1
Staphylococcus epidermidis Klebsiella pneumonia	2
Klebsiella pneumoniae Yeast	6
Pseudomonas aeruginosa Yeast	2
Proteus mirabilis Yeast	1
Coliforms Yeast	1
Enterococcus sp. Escherichia coli	1
Staphylococcus aureus Staphylococcus epidermidis Klebsiella pneumonia	1
Staphylococcus aureus Coliforms Yeast	1

2 (3·4%) were yeasts (Table 1). Of the 68 cultures that yielded mixed growth, 60 (88·2%) included Gram-negative pathogens, 58 (85·2%) included Gram-positive organisms and 17 (27%) included yeasts (Table 2).

The overall sensitivity of Gram stains for growth of any type of bacteria or fungi was 94% (118/126). When we correlated the Gram stain appearance of biopsy smears by matching each with their respective culture results (Table 3) and applying the McNemar test, there were just two discordant stain/culture pairs, with 96·4% congruency between Gram stain appearance and culture results; there was no statistically significant difference ($P = 0·25$) between the predictive value of Gram stains and cultures in the ascertainment of the identity of the microorganism responsible for the infection. Of the 40 cultures that yielded Gram-negative organisms, 38 (95%) had a corresponding Gram stain for the presence of Gram-negative organisms only: that is, the sensitivity of a Gram stain for growth of Gram-negative organisms was 95%. Similarly, of the 15 cultures that yielded Gram-positive organisms only, all 15 (100%) had a prior Gram stain showing the presence of Gram-positive organisms only, that is, the sensitivity of a Gram stain for growth of Gram-positive organisms was 100%. The probability that a biopsy with a Gram stain showing the presence of Gram-negative microorganisms only would yield growth of a Gram-negative microorganism on culture was 100% (38/38), that is, the predictive value positive was 100%. Similarly, the probability that a biopsy with a Gram stain showing Gram-positive organisms only would yield Gram-positive growth on culture was 88·2% (15/17) – the predictive value positive (Table 3).

For any culture that yielded a Gram-negative microorganism, singly or mixed with Gram-positive organisms or yeasts, the sensitivity of Gram stains in predicting growth of Gram-negative microorganisms was 86·6% (84/97). Similarly, the sensitivity of Gram stains in predicting the presence of Gram-positive organisms singly or mixed with Gram-negative organisms or yeasts was 68·4% (54/79). Of yeast cells observed on Gram stain, only 53%

Table 3 Results of Grams stains with the corresponding matched culture result*

Gram stain result on light microscopy	Growth of Gram-negative microorganisms (single species)	Growth of Gram-positive microorganism (single species)
Gram-negative bacilli	38	0
Gram-positive cocci	2	15

*Discordancy not significant by McNemar test ($P = 0.25$). The matched data array indicates the complementarity of Gram stains and culture.

Key Points

- our study showed significant correlation between the Gram stain appearance of deep tissue biopsy specimens and the identity of organisms grown from microbiological culture of the respective tissue specimens obtained from patients with chronic diabetic upper and lower limb ulcers in Tanzania
- these results suggest that Gram stains, although not an absolute alternative for bacterial culture, are highly representative and reliable in the characterisation of pathogens linked with infected ulcers in the upper and lower limb of persons with diabetes
- we contend that in regions with limited microbiology resources, routine Gram stains are more cost effective and easier to conduct than microbiological cultures for the characterisation of infections, and therefore more likely sustainable

had matched growth of yeasts on culture. All 128 participants were HIV-negative.

DISCUSSION

Our study showed significant correlation between the Gram stain appearance of deep tissue biopsy specimens and the identity of organisms grown from microbiological culture of the respective tissue specimens obtained from patients with chronic diabetic upper and lower limb ulcers in Tanzania. These findings have important implications for the management of diabetic limb ulcers in Dar es Salaam, where the incidence of upper and lower limb infections remains relatively high and is associated with substantial morbidity and mortality. The relatively high (86–100%) positive predictive values of Gram stains for growth of organisms with the corresponding Gram profiles suggest that Gram stains can potentially provide the clinician with useful information about when to add agents with a broader or different spectrum than those that might have been initiated empirically. We found this was true for bacterial cultures that yielded growth of a single isolate as well as polymicrobial growth that included organisms with the opposite Gram stain profile. These results suggest that Gram stains, although not an absolute alternative for bacterial culture, are highly representative and reliable in the characterisation of pathogens linked with infected ulcers in the upper and lower limb of persons with diabetes.

In less-developed countries like Tanzania, factors that preclude the optimal management of infections include limited diagnostic microbiology services, shortages or lack of reagents, unavailability of antimicrobials or lack of sufficiently trained laboratory personnel. We contend that in regions with limited microbiology resources, routine Gram stains are more cost effective and easier to conduct than microbiological cultures for the characterisation of infections, and therefore more likely sustainable. In the current era of advanced technology, it is very easy for one to overlook the utility of basic light microscopy and Gram stains in the rapid diagnosis of infections. A Gram stain can confirm within minutes, the presence of Gram-positive or Gram-negative organisms in pus, smears of sputum or soft tissue, spun specimens of urine or cerebrospinal fluid, and urethral or cervical smears (e.g. presence of Gram-negative diplococci), allowing the clinician to choose and target antibiotic therapy at the likely pathogens while the patient is still seeing the clinician. In contrast, bacterial culture reports may take 3–5 days to reach those who need to know.

Additional advantages of Gram stains include their potential value in being able to show (i) organisms that do not grow on culture in a patient who is on antimicrobial therapy; (ii) the true infectious disease situation of an infected wound (e.g. the number of polymorphonuclear leucocytes or the degree of bacterial bioburden in the wound or ulcer) or (iii) the morphology of microorganisms observed under the microscope (microorganisms dying under the influence of antimicrobial therapy often acquire distorted shapes). It is important to emphasise that one cannot ascertain the species or antimicrobial susceptibility profile of microorganisms by merely visualising an organism on a Gram stain. We would like to point out, also, that the specimens obtained from our study-patients were deep tissue biopsies, not specimens of pus, fluid or a wound swab. The only way to assess inflammation and quantification of polymorphonuclear leucocytes in a tissue specimen is by histological examination and analysis of an appropriately stained specimen. We did not carry out histological analysis of the tissue specimens, only Gram and Ziehl-Neelsen stains and culture on sheep blood agar.

The reliable predictive performance of Gram stains highlighted by our study suggests an adjunct role for this diagnostic procedure in the management of infections in low-income countries. Besides, it is reasonable to

expect that reliable, microbiological characterisation of infection as part of the management strategy will almost certainly increase the effectiveness of antimicrobial therapy beyond mere empiricism, resulting in reductions in antimicrobial waste and unnecessary antimicrobial prescribing, especially in countries with limited resources or with increasing occurrence of infections caused by antimicrobial-resistant pathogens. Although none of the patients in our study had prior antimicrobial therapy before presenting to MNH with limb infections, published data suggest that recent antimicrobial treatment generally has no effect on the recovery of bacterial species in general (5).

There is a myriad of published literature showing that cultures of deep tissue biopsies are better representative of the aetiology of underlying infections for all degrees of severity of infections when compared with cultures of superficial swabs of the base of ulcers (6,7). The diagnosis of deep infection in a diabetic ulcer is generally based on clinical criteria rather than microbiological results (8). Thus, in countries with limited resources, a microbiological diagnosis should be reserved for cases in which the aetiology of infection is required, for example, when the infection is particularly severe, when less common microorganisms are suspected as the causative agent, when response to antimicrobial treatment is poor, or when a longstanding infection does not heal within a reasonable period of time (9). Gram stains can be used to assess and interpret of the pathogenic involvement of the different microorganisms isolated from the respective culture (9). Occasionally, swab cultures might yield results compatible with deep tissue biopsy cultures in certain circumstances, such as when the infection remains superficial and has not progressed to deeper tissue layers (10). However, in practice, superficial swab cultures generally yield polymicrobial growth and are often a reflection of colonisation rather than infection. When obtaining a deep tissue biopsy is not feasible, curettage or pus aspiration rather than superficial swabbing of the ulcer is more effective (11).

For cultures that yielded mixed bacterial growth in our study, Gram-negative and Gram-positive organisms were present 94 and 89% of the time, respectively. For cultures that yielded growth of a single isolate,

76% of isolates were Gram-negative whereas 21% were Gram-positive organisms only. The presence of Gram-negative organism on staining of a deep tissue biopsy that goes on to yield polymicrobial growth is likely to be a manifestation of chronic, complex or previously treated wounds, as Gram-negative bacilli and anaerobes may join in a polymicrobial infection (12).

A search of the medical literature for studies of the utility of Gram stains in the management of limb infections in diabetes populations in less-developed countries showed a paucity of published data. We believe that the main reason for this scarcity of evidence is the fact the microbiology services have not been an integral part of the management algorithm for infection in the diabetes population in less-developed countries. Limited laboratory services in countries with limited resources often preclude routine microbiological identification of microorganisms involved in the pathogenesis of infections in persons with diabetes. There may be lack of skilled laboratory personnel or existing services are often unsustainable. These limitations preclude routine characterisation of infections using Gram stain and cultures.

Our study had few limitations. First, culture for anaerobic organisms was not feasible because of lack of laboratory facilities for processing the culture of this class of organisms. Although routine anaerobic cultures and bone radiology would have been desirable, especially in patients with compromised vascular perfusion and deep infection, such routine workup of diabetic patients was not feasible in Dar es Salaam. That said, we believe that deep underlying or concomitant anaerobic infection would have been discernible clinically through the manifestation of symptoms and signs such as smell, pain, or the presence of deep lying abscesses or gas in the tissues (13). Second, although we stained and examined tissue smears for tubercle bacilli, we did not routinely obtain mycobacterial cultures of the respective tissue specimens for reasons similar those given above for not culturing anaerobes. However, we do not believe this was a major limitation for two reasons: (i) mycobacterial skin infections are relatively uncommon in our patient population – none had symptoms to suggest underlying pulmonary or cutaneous mycobacterial infection;

and (ii) the major risk factor for deep mycobacterial infection in Tanzania is HIV infection, whereas our entire study population was HIV-seronegative (14). Neither mycobacteria nor HIV appear to be playing any role in the pathogenesis of infected ulcers in this population.

The results of our study provide evidence for policy and decision makers involved in the funding of laboratory services in Tanzania on which to base enhancement of basic microbiology services. Under the constraints of the limitation of sparse microbiology resources in developing countries, a Gram stain of a tissue biopsy specimen obtained deep within an infected ulcer is largely predictive of the microorganism causing underlying infections and is a relevant example of the use of Gram stain as a useful tool to 'do more with less' in managing diabetic infections in Africa (3). To ensure the most representative result to guide antimicrobial therapy, management of infected diabetic limb ulcers could potentially be enhanced by obtaining a Gram stain of deep tissue biopsy rather than a superficial swab of the ulcer. Gram stains are relatively less expensive than culture, are sustainable for routine management of diabetic infections, and promote initiation of guided antimicrobial therapy rather than blind empiricism.

Limited laboratory services often preclude routine microbiological identification of microorganisms involved in the pathogenesis of infections acquired by persons with diabetes. There may be lack of skilled laboratory personnel or existing services are often unsustainable. These limitations preclude routine characterisation of infections associated with cultures. For persons with diabetes in an HIV endemic region of sub-Saharan Africa, where microbiology resources are limited, a Gram stain alone of a deep biopsy of an ulcer is predictive of the microorganism causing underlying infection. Management of infected diabetic limb ulcers should include at least a Gram stain of deep tissue, especially when culture facilities are not available.

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