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Major Article

Secondary bacterial isolates from previously untreated Buruli ulcer lesions and their antibiotic susceptibility patterns in Southern Nigeria

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Abstract

Introduction: Mycolactones, secreted by *Mycobacterium ulcerans*, were previously believed to prevent super infection in Buruli ulcer lesions. However, little is known about secondary bacterial infections in these lesions. This study evaluated contaminating bacterial flora and their antibiotic susceptibility patterns in cases of previously untreated Buruli ulcer disease from three states in Southern Nigeria. **Methods**: A prospective analysis was conducted between January and June of 2015 using wound swabs from eligible patients with Buruli ulcer disease, confirmed by quantitative-polymerase chain reaction, with active ulcers. Microbiological analyses including isolation of bacteria, species identification of isolates, and drug susceptibility tests were performed. **Results**: Of 51 patients, 27 (52.9%) were female. One or more bacterial species of clinical importance was isolated from each patient. A total of 17 different microbial species were isolated; 76.4% were Gram-negative and 23.6% were Gram-positive isolates. The most common bacterial species detected was *Staphylococcus aureus* (24%), followed by *Aeromonas hydrophila* (13%), *Pseudomonas aeruginosa* (13%), and *Klebsiella pneumoniae* (11%). Drug susceptibility tests showed a particularly high frequency of resistance to commonly used antimicrobials in Nigeria for *Staphylococcus aureus*. **Conclusions**: Super bacterial infections occur in Buruli ulcer lesions in Nigeria, and these infections are associated with high rates of resistance to commonly used antibiotics in the country.

Keyword: Bacterial contamination. Chemotherapy. *Mycobacterium ulcerans*. Mycolactones. Wounds.

INTRODUCTION

Buruli ulcer disease (BUD) is a necrotising skin and soft tissue infection caused by *Mycobacterium ulcerans*[1]. It is an emerging neglected tropical disease and the third most common mycobacterial infection, after tuberculosis and leprosy[1] [2] [3]. BUD has been reported in over 30 countries globally, including Australia, and those in Asia, the West Pacific, and South America; however, the highest disease burden occurs in West and Sub-Saharan Africa[1] [2]. Buruli ulcer (BU) lesions can occur as very large ulcers often with scarring, contractures, and limb deformities that can require surgical amputations[1] [2] [3] [4] [5].

Tissue destruction by most pathogenic bacteria is mediated by the release of toxins. Unlike *Mycobacterium tuberculosis* and *Mycobacterium leprae*, of which pathogenesis is not mediated by the release of toxins, skin and soft tissue damage with BUD is mediated by the release of two polyketide-derived macrolide toxins from *M. ulcerans*, designated as mycolactones A and B[6] [7]. In mouse and guinea pig models, studies have shown that mycolactones play a central role in the pathogenesis of *M. ulcerans*. For example, injection of 100µg of mycolactones was sufficient to cause characteristic ulcers in guinea

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pig skin[8]. In mice, mycolactones have been shown to be associated with vacuolar nerve tissue injury, which might explain the painlessness of BU lesions[9]. There has been substantial progress made in characterising the activity of mycolactones, especially their necrotising and immunosuppressive effects[6][7][10][11]. The cytotoxic effects of mycolactones released by clusters of *M. Ulcerans* include the destruction of the skin and surrounding soft tissues. This results in the formation of devitalized, avascular tissue and *necrotic slough* at the wound bed, which is characteristic of BU[6][7][8]. This devitalised tissue might provide an ideal medium for secondary bacterial infections, which consequently might interfere with wound healing[12]. However, as mycolactones are macrolides that can have antibiotic activity against a broad spectrum of bacteria, it has been suggested that secretion of these compounds by *M. ulcerans* during active disease might prevent superinfection in BUD wounds[10][12]. This speculation was strengthened by a study demonstrating that high levels of mycolactones in BU patients correlated with clinical and bacteriological response to therapy[13].

Recent studies using synthetic mycolactones from *M. ulcerans* showed that these compounds had no antimicrobial activity against any of the microorganisms tested[11]. Another study recently demonstrated that *M. ulcerans* infection and mycolactone secretion do not prevent secondary bacterial infections in BU lesions as previously speculated, and indeed that these super infections might delay wound healing[12]. Thus, to optimise BUD treatment in endemic settings, there is a need to characterise these infections and their antibiotic susceptibility patterns, especially in under-resourced populations. This will aid in the identification of common secondary bacterial infections in BU, and suggest possible empirical regimens for treating these infections according to geographical regions/settings. Nigeria was previously thought to not be endemic for BUD due to very few cases being reported over four decades since it was first detected in the country[14]. However, in 2012, we piloted a case-search strategy in three rural districts of Southern Nigeria and found 36 BUD cases that were confirmed by polymerase chain reaction (PCR)[14]. As a result, a scale-up has been underway since 2014. The aim of this study was to evaluate contaminating bacterial flora and their antibiotic susceptibility patterns from previously untreated BUD lesions from three states in Southern Nigeria.

METHODS

Study participants

This prospective analysis was conducted between January and June of 2015. The study population comprised pre-treatment patients with quantitative-PCR (q-PCR)-confirmed BUD with active ulcers (**Figure 1**). Fifty-one study participants were recruited from three States in Southern Nigeria. The states included Cross River State (South-South zone), Ogun State (South-West zone), and Anambra State (South-East zone). Information about the gender and age of the patients was also provided. The ulcers were sampled for microbiological analysis before administration of any chemotherapy for BUD.

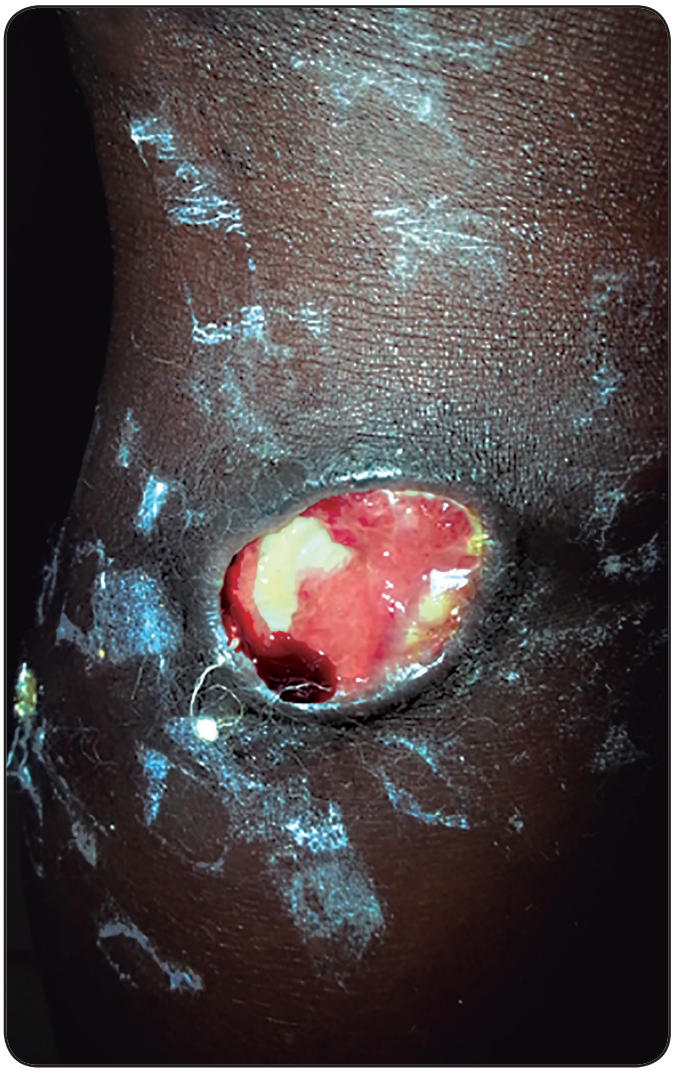

FIGURE 1. A Buruli ulcer lesion of the right lower limb showing undermined edges.

Sample collection

After superficial pre-cleansing of the ulcers with physiologic saline, specimens were collected by rotating a sterile, pre-moistened swab (Nuova Aptaca SRL, Canelli, Italy) across the ulcers. Two swabs were collected from the undermined edges of the BU lesions using the Levine method of specimen collection. The third swab was rotated over a small area with slight pressure to collect fluid from the wound tissue[15]. To preserve secondary bacteria, swabs were placed in Ames agar gel with charcoal and sent to the laboratory for analysis. The analysis was performed at the microbiology laboratory of the National Orthopaedic Hospital, Enugu, Nigeria.

Microbiological analysis

***Isolation of bacteria*:** swabs were inoculated on appropriate media including blood agar, chocolate agar, and MacConkey agar and incubated under aerobic conditions for 18 to 24hours. Pure cultures of the isolates were obtained for identification.

***Bacterial species identification*:** bacterial isolates was identified through morphological characteristics, gram staining, and biochemical reactions. Bacterial colonies were gram-stained[16] and identified by biochemical tests. Gram-negative rods were characterized using substrates on the Microbact 24E Gram-negative identification system (Oxoid Limited, UK), according to manufacturer's instructions. Gram-positive cocci were first identified by Gram staining and then analysed using the catalase test to differentiate between *Staphylococcus* spp and *Streptococcus* spp. *Staphylococcus* spp were further differentiated into coagulase-positive and coagulase-negative strains.

***Antibiotic susceptibility testing*:** susceptibility of the isolates to specific drugs was tested using the Kirby-Bauer disc diffusion method on Mueller Hinton agar[17]. Antibiotics used for sensitivity testing included ampicillin, amoxicillin/clavulanic acid, ceftazidime, cefuroxime, gentamicin, ciprofloxacin, and ofloxacin. Gram-positive cocci were tested against ceftriaxone, erythromycin, and cloxacillin.

Ethics considerations

This study was approved by the Ethics and Research Advisory Board of the German Leprosy & TB Relief Association, Nigeria. All patients or legal guardians (for minors) gave written informed consent for all diagnostic processes and for publication of clinical photographs derived from the management of patients. All identified BU cases were investigated and treated at no financial cost to the patients.

Data analysis

The data collected were entered into a Microsoft Excel spreadsheet and analysed using Epi Info. Results are presented as absolute and relative frequencies.

RESULTS

Samples were collected from 51 patients; 27 (52.9%) were female and their ages ranged from 4 to 75 years. One or more bacterial species of clinical importance was isolated from a specimen from each patient (**Table 1**). A total of 17 different microbial species were isolated; 76.4% were Gram-negative and 23.6% were Gram-positive isolates. The most common bacterial species detected were *Staphylococcus aureus* (24%), followed by *Aeromonas hydrophila* (13%) *Pseudomonas aeruginosa* (13%), *Klebsiella pneumoniae* (11%), *Enterobacter cloacae*(6%), *Pseudomonas pseudomallei* (6%), and *Burkholderia cepacia* (6%) (**Figure 2**).

The presence of only one bacterial species isolated from each sample was the most frequent occurrence (92%). Polymicrobial infections were found in four (8%) of the lesions and consisted of two species per sample. The predominant species found in polymicrobial infections was *S. aureus*, occurring with either of *P. aeruginosa*, *Pseudomonas pseudomallei*, *A. hydrophila*, or *Escherichia coli*.

The antimicrobial susceptibility patterns of both Gram-positive and Gram-negative isolates from BU wounds are shown in **Table 2**. *S. aureus* was the only Gram-positive species isolated from the samples. *S. aureus* showed a high frequency of resistance to different classes of antimicrobial agents including amoxicillin/clavulanic acid, cloxacillin, ciprofloxacin, erythromycin, and cefuroxime (each with resistance rates exceeding 80%). The most active agents against *S. aureus* were ofloxacin, gentamicin, ceftazidime, and ceftriaxone with sensitivity rates ranging between 53.8 and 76.9%. *S. aureus* did not show 100% sensitivity to any of the antimicrobials tested.

TABLE 1

Characteristics of 51 Buruli ulcer patients and associated bacterial isolates.

Variable	Number	Percentage
Age (years)		
≤10	13	25.6
11–20	7	13.7
21–30	6	11.8
31–40	10	19.6
41–50	7	13.7
51–60	4	7.8
≥61	4	7.8
Sex		
female	27	52.9
male	24	47.1
Gram staining		
Gram-negative	42	76.4
Gram-positive	13	23.6
Isolates per patient (number)		
1	47	92.2
2	4	7.8

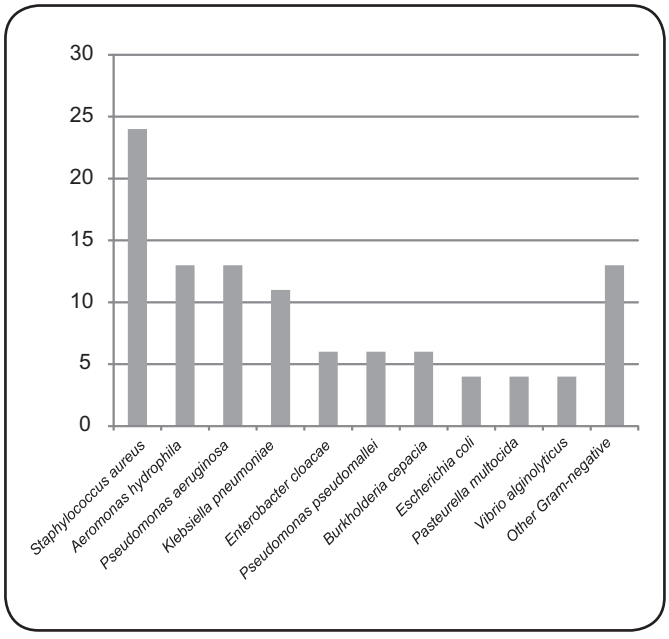

FIGURE 2.Percentage of bacterial species isolated from wound swabsamples collected from 51 patients with Buruli ulcer disease. The *Other Gram-negative* group includes *Citrobacter koseri, Enterobacter agglomerans, Enterobacter sakazakii, Kluyvera ascorbata, Moraxella* spp, *Proteus mirabilis,* and *Providencia rettgeri*.

TABLE 2

Antibiotic susceptibility pattern of bacterial isolates from Buruli ulcer wounds in Nigeria.

Organism	Antibiotic tested	Number tested	Susceptible		Resistant	
			n	%	n	%
Staphylococcus aureus	Amoxi-Clav	13	1	7.7	12	92.3
	Ceftazidime	12	7	58.3	5	41.7
	Ciprofloxacin	5	0	0.0	5	100.0
	Cefuroxime	13	2	15.4	11	84.6
	Ceftriaxone	12	8	66.7	4	33.3
	Erythromycin	13	2	15.4	11	84.6
	Gentamicin	13	10	76.9	3	23.1
	Ofloxacin	13	7	53.8	6	46.2
	Cloxacillin	11	1	9.1	10	90.9
Aeromonas hydrophila	Ampicillin	7	1	14.3	6	85..7
	Amoxi-Clav	7	1	14.3	6	85.7
	Ceftazidime	7	3	42.7	4	57.1
	Ciprofloxacin	7	6	85.7	1	14.3
	Cefuroxime	7	2	28.6	5	71.4
	Gentamicin	7	3	42.9	4	57.1
	Ofloxacin	7	5	71.4	2	28.6
Pseudomonas aeruginosa	Ampicillin	7	0	0.0	7	100.0
	Amoxi-Clav	6	0	0.0	6	100.0
	Ceftazidime	7	6	85.7	1	14.3
	Ciprofloxacin	7	7	100.0	0	0
	Cefuroxime	7	0	0.0	7	100.0
	Gentamicin	7	2	28.6	5	71.4
	Ofloxacin	7	3	42.9	4	57.1
Klebsiella pneumoniae	Ampicillin	5	0	0.0	5	100.0
	Amoxi-Clav	6	2	33.3	4	66.7
	Ceftazidime	6	4	66.7	2	33.3
	Ciprofloxacin	6	6	100.0	0	0
	Cefuroxime	6	0	0.0	6	100.0
	Gentamicin	6	5	83.3	1	16.7
	Ofloxacin	6	5	83.3	1	16.7
Other Gram-negatives	Ampicillin	22	0	0.0	22	100.0
	Amoxi-Clav	22	3	13.6	19	86.4
	Ceftazidime	22	12	54.5	10	45.5
	Ciprofloxacin	22	21	95.5	1	4.5
	Cefuroxime	22	7	31.8	15	68.2
	Gentamicin	22	13	59.1	9	40.9
	Ofloxacin	20	20	90.9	2	9.1

Amoxi-Clav: Amoxicillin-Clavulanic acid.

Furthermore, the antimicrobial sensitivity pattern of the three most common Gram-negative isolates from BU wounds is shown in **Table 2**. The most ineffective antibiotic against all Gram-negative isolates was ampicillin; *P. aeruginosa* and *K. Pneumonia* were not susceptible to any antibiotics with a resistance rate of 100%. *A. hydrophila* had a high resistance rate (85.7%) to ampicillin and amoxicillin/clavulanic acid. *P. aeruginosa* showed 100% resistance to ampicillin, amoxicillin/clavulanic acid, and cefuroxime. *K. pneumoniae* showed 100% resistance to ampicillin and cefuroxime. The three most common Gram-negative isolates (*P. aeruginosa*, *A. hydrophila*, and *K. pneumoniae*) showed high susceptibility to ciprofloxacin (rates ranged from 85.7-100%). In addition, *K. pneumoniae* and *P. aeruginosa* had susceptibility rates of 66.7% and 85.7%, respectively, to ceftazidime. Additionally, among *other Gram-negative bacteria* isolates, resistance rates were highest to ampicillin (100%), amoxicillin/clavulanic acid (86.4%), and cefuroxime (68.2%). However, antimicrobial susceptibility rates were highest for ofloxacin (90.9%) and ciprofloxacin (95.5%).

DISCUSSION

The pathogenesis of *M. ulcerans* is mediated by macrolide toxins such as mycolactone, which exhibits both cytotoxic and immunosuppressive activities[10] [11] [12] [13]. Macrolides exhibit

antimicrobial activity against a wide spectrum of bacteria including Gram-positive, Gram-negative, and atypical bacteria like *Rickettsia* and *Chlamydia*[18][19][20]. Therefore, mycolactones, like other macrolides, were suggested to have antimicrobial properties that could prevent secondary bacterial infections in BU lesions. This speculation was recently debunked by a pilot study[12]. To confirm this observation, we evaluated bacterial contamination of BU lesions as well as associated antimicrobial susceptibility patterns.

In this study, 17 bacterial species were isolated from BU lesions. The majority of the ulcers were colonised by a single bacterial specie, whereas fewer than 10% had polymicrobial infections. The most common and only Gram-positive isolate was *S. aureus*. This is consistent with studies that surveyed different types of wounds and chronic ulcers including BU lesions and found *S. aureus* as the most common isolate[12][18][19][20]. Virulence in *S. aureus* is mediated by the release of several virulence factors like invasins, hyaluronidase, catalase, coagulase, hemolysins, leukotoxin, and leukocidin[19][20]. Furthermore, several Gram-negative bacteria were also isolated from BU wounds with the most common being *A. hydrophila, P. aeruginosa*, and *K. pneumoniae*. Our study agrees with a recent study suggesting that *M. ulcerans* infection and mycolactone secretion does not prevent secondary bacterial infections in BU lesions, and that most contaminants are *S. aureus* and *P. aeruginosa*[12]. The relative high rates of infection with *A. hydrophila* suggest environmental transmission of *M. ulcerans* as this organism has been found in a variety of aquatic environments, including lakes, rivers, streams, swamps, rain water, and swimming pools, and it has also been isolated from tap water and soil[21][22].This organism has also been isolated from other water- or soil-associated traumatic wound infections[21][22].

The bacterial isolates were evaluated for susceptibility to commonly used antibiotics in Nigeria. All tested *S. aureus* strains were resistant to ciprofloxacin, 92% were amoxicillin-clavulanic acid-resistant, and 91% were cloxacillin-resistant. The level of resistance of *S. aureus* isolates to cloxacillin is crucial; beyond being one of the most commonly prescribed antibiotics for treating skin and soft tissue infections in Nigeria, it can also indicate the proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) present. However, in Nigeria and Ghana, relatively low rates of MRSA have been reported compared to those in South Africa[12][23][24]. *S. aureus* strains showed mild to moderate susceptibility to third-generation cephalosporins (ceftazidime/ceftriaxone) and gentamicin. This finding indicates that these agents are likely to be the most effective in managing BU lesions contaminated with *S. aureus*. This is worrisome because despite being administered intravenously, the results indicate that *S. aureus* has become more resistant to third-generation cephalosporins[20].

Furthermore, the antibiotic resistance of Gram-negative isolates is also of great concern; the most common isolates, especially *P. aeruginosa*, showed relatively high resistance to the majority of the antibiotics tested, especially, ampicillin, amoxicillin/clavulanic acid, and cefuroxime. Multidrug-resistant isolates of *P. aeruginosa*, those fully resistant to all of these agents, are of major concern[20]. Additionally, the results indicate that *P. Aeruginosa* is developing a high level of resistance to aminoglycosides and third-generation cephalosporins. Similar evidence was reported by Nicoletti et al.[25]in a study on severe infections from different human sources and Yeboah-Manu et al.[12] in a study of secondary infections of BU wounds[12][25]. In addition, our results showed that common Gram-negative bacterial isolates from the lesions (*A. hydrophila, P. aeruginosa*, and *K. pneumoniae*) demonstrated a high level of susceptibility to ciprofloxacin ,indicating that this antibiotic might be useful in managing secondary infections. Moreover, other less common Gram-negative isolates from the BU lesions also showed high susceptibility to quinolones [ciprofloxacin (96%) and ofloxacin (91%)].

This study had some strengths and limitations. An important strength is that it confirms a recent observation that BU lesions might be associated with secondary bacterial infections that could interfere with wound healing, therefore requiring additional treatment with antibiotics. Furthermore, the choice of drugs for treatment of secondary infections of BU lesions with locally available antimicrobial agents requires a better understanding of the infecting flora and its drug susceptibility patterns. Therefore, this study provides this information for Nigeria. One potential limitation of this study is that several samples were not collected longitudinally before and during BU treatment to determine the causes, sources, and consequences of secondary BU wound infections[12]. Another limitation is that we utilised wound swabs for specimen collection. Wound swab specimen collection for microbiological analysis has recently been questioned. The current gold standard for microbiological analysis of wounds requires the use of biopsy specimens[26][27]. However, as previously highlighted, limitations include determining which health workers should collect biopsies, a lack of laboratories with expertise on microbiological culture testing of biopsies, higher costs involved with the performance of these tests, and the potential for further tissue injury and delays in wound healing when biopsies are taken. These factors precluded the use of this method in the present study[12]. Moreover, wound swabs have been shown to be associated with the highest recovery rates[28]; in addition, it was shown that microbiological analysis of biopsies does not offer additional prognostic information when compared with analysis of the surface microflora[29].

In conclusion, secondary bacterial infections occur in BU lesions in Nigeria and are associated with a wide variation in the rates of susceptibility and resistance to commonly used antibiotics in the country. The findings of this study might be of interest to clinicians, investigators, and public health authorities, and offer suggestions to guide empirical therapy. In addition, the findings of this study might also guide policymakers to implement specific intervention strategies to eliminate antibiotic-resistant bacterial infections and their transmission in BU endemic settings. However, further studies are required to characterise the evolution and sources of secondary bacterial infections in BU lesions, and to assess their impact on the treatment duration of BUD to create guidelines for wound care during BU management.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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