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Bacterial diversity in Buruli ulcer lesions in Ghana

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ABSTRACT

Background: Previous studies have demonstrated secondary microbial infection of Buruli ulcer (BUD) lesions before, during and after treatment. However, there is limited data on the bacterial diversity across treatment and their influence on clinical outcome. The present study aimed to investigate the relationship between bacterial diversity within BUD lesions and clinical outcome in affected individuals.

Methods: We investigated the bacterial diversity within lesions of individuals with PCR confirmed BUD from 5 endemic districts within central Ghana. Samples were collected longitudinally from lesions over treatment period. Microbiological analyses including isolation of bacteria, and species identification were performed using the VITEK 2 compact.

Results: Out of 36 participants included, 80.5 % presented with ulcers on the lower limbs. Higher bacterial diversity was observed in ulcers compared to other clinical forms of BUD. There was a significant association between bacterial diversity and clinical outcome ($p = 0.002$). ESBL producing bacteria and MRSA were isolated in slow healing BUD lesions.

Conclusion: Higher diversity of secondary organisms colonizing BUD lesions may have an impact on clinical outcome in affected individuals. There is the need for the development of treatment guidelines for simultaneous management of *M. ulcerans* and other potential pathogens within lesions to improve clinical outcome.

1. Introduction

Buruli ulcer disease (BUD) is a chronic, necrotizing skin condition caused by the environmental pathogen, *Mycobacterium ulcerans.* Globally, the disease occurs in more than 30 countries with most cases from west Africa. Despite major advancements in understanding the disease mechanisms, the mode of transmission remains elusive despite its association with wetlands [1–3]. Clinically and epidemiologically, BUD varies across geographical regions. In Africa for instance, the rate of occurrence is higher in children aged 15 years and below while adults aged 60 years and above are commonly affected in Australia [4]. BUD lesions initially present as painless subcutaneous nodule, plaque or oedema which may enlarge with time into ulcers with necrotic bases and undermined edges. Before 2005, surgery was the mainstay of treatment. Currently, the standard treatment involves administration of oral antibiotics for eight weeks and wound dressing for ulcerative lesions. Surgery is now considered an add-on for large lesions [5–7].

Chronic wounds are known to have complicated and impaired wound healing due to superinfection by secondary pathogens. Previous studies have reported BUD lesions can be colonized by secondary pathogens debunking initial beliefs of "sterility" within lesions as a result of the macrolide exotoxin, mycolactone [8–11]. The work by Yeboah-Manu et al, demonstrated that BUD lesions can be colonized by potential pathogens before, during and after the 8-week course of

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treatment. In that study, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Proteus mirabilis* were organisms frequently isolated from BUD lesions [11]. The study found isolates within BUD lesions especially *S. aureus* to be resistant to first line antibiotics used in Ghana. Similarly in Nigeria, common bacterial isolates from BUD lesions included *Staphylococcus aureus, Aeromonas hydrophilia, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [12]. *S. aureus*was again found to have a high frequency of resistance to commonly prescribed antibiotics. Another study conducted in Ghana further assessed the impact of BUD antibiotics on the resistance profile of secondary organisms isolated from lesions [9] found resistance to common antibiotics especially streptomycin which was a drug of choice for BUD treatment [9].

Even though studies on microbiota have shown the presence of potential pathogens and their resistance profile within BUD lesions, there is no information on the interaction of these potential pathogens and their influence on clinical outcome in BUD. This study aimed at investigating the bacterial diversity within BUD lesions longitudinally and their influence on clinical outcome. Understanding how potential pathogens within lesions impact clinical outcome will guide treatment and improve overall management of the disease.

2. Methods

2.1. Study design and participant recruitment

This was a prospective observational cohort study to assess relationship between bacterial diversity and clinical outcome of BUD in clinically confirmed BUD cases. Participants were recruited from Agogo Presbyterian Hospital in the Asante Akim North District, Pakro Health Centre in Akwapim South District, Tepa Government Hospital in the Ahafo Ano North District, Dunkwa Government Hospital in the Upper Denkyira East District and Wassa Akropong Municipal Hospital in Wassa Amenfi East Municipal, all within central Ghana. These hospitals are established BUD treatment centres located within endemic districts for the management of cases within the district and other nearby communities. A convenience sampling technique was used in the selection of participants. The aims and study procedures were explained to participants and consent was sought before recruitment was done. Participants included confirmed BUD patients between the ages of 5 to 80 years willing to participate in the study as evidenced by consenting, persons not previously treated for Buruli ulcer and individuals with good general health not requiring long term medication. Pregnant or breastfeeding female patients confirmed BUD patients who had begun antibiotic treatment and individuals with chronic ulcers other than BUD were excluded. In all, 38 participants were recruited.

2.2. Study procedures

Clinical and demographic data were collected prospectively using standard skinNTD 01 forms. Lesion sizes, appearance and characteristics were reviewed by an experienced clinician every two weeks till 8 weeks and thereafter monthly until one year after treatment completion. The time to complete healing of study participants was documented. All participants received a combination of oral rifampicin and clarithromycin for 56 days as recommended by the World Health Organization [13]. Clinical outcome was defined by complete healing following the 8-week antibiotic treatment course. Participants whose lesions healed within or by 8 weeks were categorized as fast healers whereas those whose lesions healed after 8 weeks were grouped as slow healers.

Laboratory confirmation of clinically suspected BUD cases by IS*2404* qPCR was performed per standard protocol as described elsewhere [14]. Following confirmation of BUD, samples were collected at baseline, week 4, week 8 and week 16 for unhealed lesions. At each sampling time, a pre-moistened sterile swab was used to collect samples from the undermined edges and surface of ulcers. For pre- ulcerative lesions, fine needle aspirate (FNA) was collected from the centre of the lesion into a sterile 2 ml cryogenic vial. Clinical samples were preserved at 4°–8 °C and transported to the Kumasi Centre for Collaborative Research (KCCR). The samples were processed immediately upon arrival at KCCR. Samples were cultured on MacConkey agar, Blood agar, Chocolate agar and Sabouraud Dextrose agar at 37 °C for 18–24 h. Colonies were consecutively sub cultured until pure isolates were obtained for identification by the Vitek 2 compact (Biomérieux, France). Briefly, bacteria suspensions were prepared by emulsifying 2–3 single colonies of pure isolates in 3 ml 0.45 % sterile saline. The turbidity of the suspensions was examined using the Densicheck Plus (Biomérieux, France) device to ensure they were within the range of 0.5–0.63 (McFarland standard). Vitek cards (Biomérieux, France) for identifying Gram-positive and negative bacteria were placed in the appropriate bacteria suspension. For the Antibiotic Susceptibility Test (AST) of the isolates, a fixed volume of the initial suspensions made were pipetted into new polysterene tubes containing 3 ml 0.45 % sterile saline. The Vitek cards, AST-N214 and AST-GP67 were placed in the second set of bacteria suspensions made according to Gram result of isolate in suspension. The two sets of bacteria suspensions were loaded onto the Vitek 2 compact following the manufacturer's protocol for simultaneous identification and AST analysis. Susceptibility of isolates to the antimicrobials were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline [15].

2.3. Statistical analysis

Data generated from the study was entered into Microsoft excel (Microsoft Corporation, Redmond, WA) and analysed using GraphPad Prism version 9.0 (GraphPad Software, Inc., La Jolla, CA). Study data was described using frequencies and percentages. Pearson's chi test was used to determine associations between sociodemographic characteristics and rate of healing in BUD lesions. Mann-Whitney test was used test for differences in bacterial diversity between fast and slow healers. Any result with a $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Study population

Of 38 participants recruited at baseline, one patient was lost to follow up and another withdrew from the study. The reason given for withdrawal was that lesion was healing at a slower rate than anticipated, hence opted for an alternative treatment. Thus, 36 participants were included in the analysis. The median age of the study participants was 17 (5–63) years. Significant associations were found between the lesion categories ($p < 0.001$), clinical forms ($p = 0.011$) and the rate of healing. Table 1 shows the demographic and clinical characteristics of the study participants.

3.2. Bacterial diversity between clinical forms at baseline

Table 2 shows a comparison of isolates between the clinical forms of study participants at baseline. The clinical form with the most diverse group of organisms was the ulcer group. The least diverse group of bacteria was observed among patients who presented with nodules at baseline (2, 3 %).

3.3. Variation in bacterial diversity across study time points

To assess the influence of the 8-week antimycobacterial treatment duration on bacterial within Buruli ulcer lesions, we assessed the longitudinal distribution of bacterial over the study time points. Fig. 1a and 1b are heat maps showing the variation in the diversity of Gram-negative and Gram-positive organisms across the study time points respectively. In general, there was a decreasing frequency in bacterial

Table 1
Demographic and clinical characteristics of study participants.

Characteristic	Frequency, n (%)			
	Slow healers, n = 30	Fast healers, n = 6	All, n = 36	p-value
Sex				
Male	18 (90)	2 (10)	**20 (100)**	0.230
Female	12 (75)	4 (25)	**16 (100)**	
Age (years)				
≤15	14 (78)	4 (22)	**18 (100)**	0.729
16–29	7 (88)	1 (12)	**8 (100)**	
30–49	5 (83)	1 (17)	**6 (100)**	
≥50	4 (50)	0	**4 (50)**	
Occupation				
Farmer	7 (100)	0	**7 (100)**	0.364
Student	16 (80)	4 (20)	**20 (100)**	
Artisans and traders	3 (60)	2 (40)	**5 (100)**	
Miner	3 (10)	0	**3 (100)**	
Unemployed	1 (100)	0	**1 (100)**	
WHO Lesion category				
Category I (≤5 cm)	4 (40)	6 (60)	**10 (100)**	**<0.001**
Category II (5–15 cm)	12 (100)	0	**12 (100)**	
Category III (>15 cm)	14 (100)	0	**14 (100)**	
Clinical form				
Ulcer	25 (83)	4 (67)	**29 (100)**	**0.011**
Plaque	3 (100)	0	**3 (100)**	
Nodule	0	2 (100)	**2 (100)**	
Oedema	2 (100)	0	**2 (100)**	
Lesion location				
Upper Limb	11 (92)	1 (8)	**12 (100)**	0.373
Lower Limb	16 (76)	5 (24)	**21 (100)**	
Others	3 (100)	0	**3 (100)**	

Categorization of study participants into fast and slow healers was done based on standard 8-week treatment for BUD. If healed by 8 weeks = fast healer, healing after 8 weeks = slow healer.

Table 2
Bacterial diversity between Buruli ulcer clinical forms at baseline.

Organism	Ulcer, n (%)	Nodule, n (%)	Plaque, n (%)	Oedema, n (%)
Staphylococcus spp.	11 (69)	2 (12)	3 (19)	0
Enterococcus spp.	10 (91)	0	0	1 (9)
Bacillus spp.	8 (100)	0	0	0
Proteus mirabilis	7 (88)	0	0	1 (12)
Escherichia coli	5 (100)	0	0	0
Providencia stuartii	5 (100)	0	0	0
Enterobacter spp.	4 (66)	0	1 (17)	1 (17)
Pseudomonas spp.	2 (67)	0	0	1 (33)
Klebsiella spp.	1 (50)	0	0	1 (50)
Citrobacter spp.	1 (100)	0	0	0
Acinetobacter spp	1 (100)	0	0	0
Vibrio mimicus	1 (100)	0	0	0
Serratia spp	1 (100)	0	0	0
Morganella morganii	1 (100)	0	0	0
Alcaligens faecalis	1 (100)	0	0	0
Stenotrophomonas maltophilia	1 (100)	0	0	0
Micrococcus luteus	0	0	1 (100)	0
Kocuria kristinae	1(100)	0	0	0
Total	**61 (83)**	**2 (3)**	**5 (7)**	**5 (7)**

*In addition, two fungi (*Candida spp*) were isolated in two ulcer lesions.

diversity, although most of the organisms persisted throughout the study. However, *Pseudomonas* spp., *Klebsiella* spp., and *Providencia stuartii* were observed to increase in frequency during treatment period but decreased during the follow up period.

3.4. Bacterial diversity at baseline and clinical outcome of study participants

To assess if bacterial diversity is associated with the rate of healing, we compared the diversity between the fast and slow healing Buruli ulcer lesions. Higher diversity was associated with the rate of healing in Buruli ulcer lesions ($p < 0.002$). There was a preponderance of Gram negative isolates in the slow healing group. Gram negative organisms including *Pseudomonas* spp., *Klebsiella* spp. and *Escherichia coli* were present in the slow healing lesions but not in the fast healing lesions. Organisms including *Staphylococcus* spp., *Bacillus* spp., *Enterococcus* spp. and *Proteus mirabilis* which were isolated in both fast and slow healers were relatively higher in the slow healing lesions (Table 3).

3.5. Prevalence of Extended Beta lactamase enzymes (ESBL), Methicillin resistant Staphylococcus aureus (MRSA) and Methicillin resistant Staphylococcus hominis (MRSHo) in BUD lesions

We screened bacterial isolates for the presence of ESBL, MRSA and MRSHo in Buruli ulcer lesions. Out of 37 Gram-negative bacteria isolated at baseline, two (2) ESBL organisms; *Klebsiella pneumoniae* and *Escherichia coli* were isolated in slow healing lesions. No ESBL producing organism was isolated in fast healing lesions. There was a higher frequency of *Staphylococcus* spp. (including Methicillin Resistant *Staphylococcus hominis* and Methicillin Resistant *Staphylococcus aureus*) in slow healing wounds compared to fast healing lesions (Table 4).

4. Discussion

BUD lesions may harbour secondary organisms which could be associated with worsening treatment outcomes such as delayed wound healing. Notwithstanding, the extent to which these organisms interact during the course of treatment, their influence on clinical outcome has not been extensively studied. This study aimed to investigate the bacterial diversity variation within BUD lesions over treatment period and their influence on clinical outcome. The study showed that higher bacterial diversity within Buruli ulcer lesions may be associated with slow healing in affected individuals. Multi drug resistant organisms including *Klebsiella pneumoniae* and *Escherichia coli* ESBL positive organisms, MRSA and MRSHo were more prevalent in slow than fast healing BUD lesions.

In this BUD cohort, most of the participants presented with category II or III ulcers on the lower limbs in keeping with the known epidemiology of the disease in West Africa [16]. Our study isolated secondary bacterial and fungal species of clinical importance affirming previous reports of BUD lesions being colonized by secondary organisms [8–12]. Bacteria isolates included *Staphylococcus* spp, *Klebsiella* spp, *Pseudomonas* spp., *Escherichia coli*, *Enterococcus* spp., *Enterobacter* spp., *Bacillus* spp., *Proteus mirabilis*, *Alcaligens* spp., *Providenica stuartii* among others. These organisms have been frequently reported in BUD lesions and are known to delay wound healing, particularly in chronic wounds. Fungal species isolated in this cohort were mainly *Candida* spp. A key characteristic of this group of fungi is their ability to form biofilms in chronic wounds which can delay healing in the lesions [17]. *Bacillus* spp. including *B. cereus*, *B.thuringiensis* and *B.firmus* were isolated. The most dominant species among the isolated Bacillus group was *B. cereus*. These organisms are commonly associated with soil and plants [18]. Finding *B. cereus* within BUD lesions is not surprising, given that a number of participants in this study were farmers. Although *B.cereus* infections have mainly been associated with food poisoning, there have been reports of infection from wounds and insect bites [19–21].

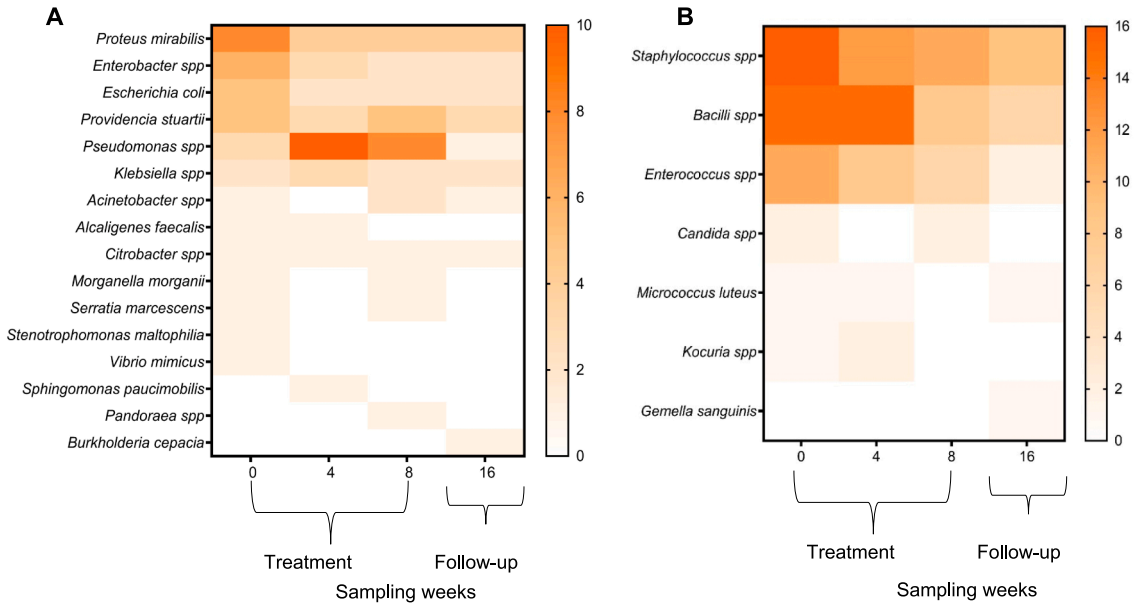

Fig. 1. Heat map representation of bacteria isolates over study period. A Distribution of Gram-negative bacteria over study period. **B** Distribution of Gram-positive bacteria over study period. The colour intensity shows the frequency of isolates obtained for each organism at specific time points. The red colour indicates highest frequency of isolates while the white colour indicates no isolates obtained. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Distribution of lesion isolates between Buruli ulcer healing groups at baseline.

Organism	Slow healers, n (%)	Fast healers, n (%)	p-value
Staphylococcus spp.	12 (75)	4 (25)	**<0.002**
Bacillus spp.	5 (63)	3 (37)	
Enterococcus spp.	9 (82)	2 (18)	
Proteus mirabilis	7 (88)	1 (12)	
Enterobacter spp.	5 (83)	1 (17)	
Providencia stuartii	5 (100)	0	
Escherichia coli	5 (100)	0	
Pseudomonas spp.	3 (100)	0	
Klebsiella spp.	2 (100)	0	
Acinetobacter spp.	1 (100)	0	
Alcaligens spp.	1 (100)	0	
Citrobacter spp.	1 (100)	0	
Morganella morganii	1 (100)	0	
Micrococcus spp.	1 (100)	0	
Serratia spp.	1 (100)	0	
Stenotrophomonas maltophilia	1 (100)	0	
Vibrio mimicus	1 (100)	0	
Kocuria spp.	0	1 (100)	
Total	**63 (100)**	**12 (100)**	

*p-value connotes the statistical significance between the summation of the bacterial diversity between the fast and slow healers using the Mann Whitney test.

To the best of our knowledge, this is the first study systematically comparing bacterial diversity between different clinical forms of BUD. The highest bacterial diversity was found in ulcers with *Staphylococcus* spp. being dominant. Given that the cytotoxic effects of mycolactone leads to the breakdown of skin and surrounding tissues [22], it is plausible a favourable environment was thus provided for the colonization and proliferation of normal skin flora and other potential pathogens within this clinical form [11]. The microbiota diversity within pre-ulcerative lesions on the other hand was relatively lower. The reason for our finding here is unclear though we believe the Gram-positive organisms may likely have been contaminants on the skin which came in contact with the needles used for FNA collection. In addition, a few of the pre-ulcerative lesions especially oedemas that were presented at baseline had begun ulcerating which possibly allowed for colonization by these organisms.

In the present study, we show bacterial diversity within BUD lesions varied across treatment period; this is consistent with findings from previous studies [8,10,11]. Generally, there was a decrease in the frequency of isolates during antibiotic treatment and this agrees with findings from other studies [10,18]. The frequency of *Pseudomonas* spp. and *Klebsiella* spp. increased within the treatment period but declined before treatment completion. The increase in frequency of *Pseudomonas* spp. and *Klebsiella* spp. is not surprising given their high resistance to most antibiotics and they are a major source of nosocomial infections [19–22]. Also, these organisms have been frequently associated with wound infection and delayed wound healing [20,23,24].

Studies assessing healing in Buruli ulcer have reported factors such

Table 4
Prevalence of ESBL and Methicillin resistant isolates in Buruli lesions.

MDR isolates	Slow healers				Fast healers			
	Week 0	Week 4	Week 8	Week 16	Week 0	Week 4	Week 8	Week 16
ESBL	2	1	0	0	0	0	0	0
MRSA	2	1	1	4	2	0	0	0
MRSHo	0	0	1	1	0	0	0	0
Total	**4**	**2**	**2**	**5**	**2**	**0**	**0**	**0**

*MRSA=Methicillin Resistant *Staphyococcus aureus*; MRSHo = Methicillin Resistant *Staphylococcus hominis*; ESBL=Extended Spectrum Beta lactamase producing organisms; MDR=Multi drug resistant. The numbers represent the actual frequency of organisms isolated at the different time points.

as lesion size or category at baseline, development of paradoxical reactions, *Mycobacterium ulcerans* bacterial load at baseline [23–25] as being associated with delayed healing. In this study, we noted a significantly higher bacterial diversity in patients whose wounds healed slowly compared to those who healed fast. Given this observation, it is probable bacterial diversity within lesions may be contributing to delayed healing in Buruli ulcer disease. Our observation here is consistent with the findings by Xu et al who suggested an overall decrease in bacteria numbers and diversity were beneficial for wound healing [25]. Delayed wound healing arising from higher wound bacterial diversity may be attributable to increased virulence of wound bacteria as a result of the dynamic interactions between the different species within the microbial environment capable of modifying bacterial behaviour, hence impacting healing [26].

It was evident the presence of some specific organisms in lesions were associated with delayed wound healing in the study cohort. Extended Spectrum Beta Lactamase (ESBL) positive *Escherichia coli* and *Klebsiella pneumoniae* were isolated only in slow healing lesions at baseline. Our findings here are consistent with previous reports of ESBL producing bacteria in BUD wounds [8,11]. There is increasing concern on ESBL producing bacteria in infections as they have been implicated in numerous outbreaks of nosocomial infections and are known to significantly negatively impact therapeutic decisions [26–29]. Also, there was a higher diversity of Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus hominis* (MRSHo) in slow healing lesions across the study time points. Infections caused by *Staphylococcus* spp. carrying the *mec*A gene are a major cause of nosocomial infections (especially in immunocompromised individuals) and are difficult to treat as most are resistant to methicillin and penicillin antibiotics [30]. Recently, methicillin-resistant *S. hominis* was found to be prevalent among filarial lymphoedema patients in the western region of Ghana [31]. Our finding of MRSHo colonizing lesions during and after treatment is worrying given that they had not been previously reported in Buruli ulcer lesions; this warrants further investigation to ascertain their impact on healing outcomes. Highly resistant organisms with antimicrobial resistance (AMR) in Buruli ulcer lesions can lead to delayed healing and increase treatment costs; this is particularly worrying within the low resource settings where BUD is endemic. Even though BUD treatment is free, the presence of these secondary organisms may lead to hospitalization and the need for additional antibiotics. This could impose a financial burden on affected individuals and caregivers who may have to pay for these additional expenses themselves [32].

4.1. Strengths and limitations of the study

We were unable to perform cultures to isolate anaerobic bacteria within BUD lesions using the Vitek 2 Compact. Some secondary organisms may have been missed as we were unable to perform high throughput sequencing. Further, we did not document clinical signs of superinfection. However, our research has demonstrated that the diversity of bacteria and the presence of multi-drug resistant organisms in BUD lesions may have an impact on the clinical outcomes of Buruli ulcer. This highlights the need for treatment guidelines for managing secondary organisms.

5. Conclusion

BUD lesions can be colonized by secondary bacterial organisms which may have an impact on clinical outcome of affected individuals. There is an urgent need for the development of treatment guidelines for the effective concurrent management of both *M. ulcerans* and other potential pathogens in order to improve healing outcomes.

6. Ethics statement

This study was approved by the Committee on Human Research, Publication and Ethics (CHRPE) of the Kwame Nkrumah University of Science and Technology (KNUST) with approval number CHRPE/AP/472/17. All participants provided written informed consent. All study procedures conformed with the principles guiding research in human subjects as set out in the Declaration of Helsinki [33].

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CRediT authorship contribution statement

Nancy Ackam: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Abigail Opoku-Boadi:** Data curation, Investigation, Writing – review & editing. **Bernadette Agbavor:** Data curation, Investigation, Writing – review & editing. **Jonathan Kofi Adjei:** Data curation, Investigation, Writing – review & editing. **Abigail Agbanyo:** Data curation, Investigation, Writing – review & editing. **Michael Ntiamoah Oppong:** Data curation, Investigation, Writing – review & editing. **Charity Wiafe-Akenten:** Formal analysis, Methodology, Supervision, Writing – review & editing. **Augustina Sylverken:** Formal analysis, Methodology, Supervision, Writing – review & editing. **Kwasi Obiri-Danso:** Formal analysis, Methodology, Supervision, Validation, Writing – review & editing. **Mark Wansbrough-Jones:** Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. **Yaw Ampem Amoako:** Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Richard Odame Phillips:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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