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Improved protocols for isolation of *Mycobacterium ulcerans* from clinical samples

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Abstract

Background The isolation and culture of Mycobacterium ulcerans (Mu) as a primary diagnostic modality for Buruli ulcer (BU) disease are limiting due to their low sensitivity and slow-growing nature. *M. ulcerans* cultures can also be overgrown with other bacteria and fungi. Culture, however, remains an important tool for the study of persisting viable *M. ulcerans*, drug susceptibility tests, and other molecular assays to improve management of the disease. The challenge of contamination with other fast-growing bacteria necessitates decontamination of clinical samples prior to culturing, but current methods may be too harsh, resulting in low yields of *M. ulcerans*. We aimed to evaluate a Tika-Kic decontamination process for *M. ulcerans* that uses supplements to stimulate *M. ulcerans* growth to improve recovery.

Methods Swab and Fine Needle Aspirate (FNA) samples were collected from 21 individuals with confirmed BU at baseline (week 0) and weeks 2 and 4 after initiating antibiotic treatment. Samples were decontaminated with Tika-Kic decontamination medium and the modified Petroff (NaOH) methods then inoculated each into Mycobacterium Growth Indicator Tube (MGIT) or Löwenstein Jensen (LJ) medium. Time to growth detection and confirmation by qPCR as well as the proportion of positive cultures for all three methods and the proportion of positive cultures for all three time points were documented. Common contaminating bacteria were also isolated and identified.

Results The proportion of *M. ulcerans* positive cultures obtained was higher for Tika-MGIT samples [14/43 (32%)] compared to Petroff-MGIT samples [10/43 (23%)] and Petroff-LJ samples [8/43 (19%)]. Baseline samples had a higher isolate proportion [17 (53%)] compared to samples collected after treatment initiation [9 (28%) for week 2 and 6 (19%) for week 4]. Contaminating bacteria isolated include *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Pasteurella pneumotropica*, *Proteus mirabilis*, *Morganella morganii*, *Staphylococcus aureus* and *Enterococcus*.

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Conclusion Our study shows an advantage for culturing *Mycobacterium ulcerans* from clinical samples using the Tika-Kic decontamination and growth medium. Further research is needed to refine sample processing to improve *M. ulcerans* recovery.

Keywords Buruli ulcer, *Mycobacterium ulcerans*, Culture, Decontamination, TiKa, Contamination, Growth media

Background

Buruli ulcer disease is caused by *Mycobacterium ulcerans* which produces a toxin that causes necrotizing cutaneous infection. Buruli ulcer disease is endemic and focally distributed in regions of Africa and Australia with the highest burden in West Africa where over 1000 cases are reported annually although numbers have been declining since 2017 [1–3]. In West Africa, the disease affects mainly children below 20 years who live in remote communities, whereas in Australia, elderly persons living in retirement homes in coastal areas are most affected. BU presents as painless pre-ulcerative skin nodules, papules, plaques, or oedema, which go on to ulcerate with undermined edges after a median incubation period of 5 months [4, 5].

Treatment is mainly orally administered rifampicin (10 mg/kg) and clarithromycin (15 mg/kg) daily for 8 weeks [6, 7]. The World Health Organization (WHO) recommends that all clinically suspected cases of BU be laboratory confirmed before treatment initiation. The current gold standard for laboratory confirmation is by *M. ulcerans* IS2404 PCR which can be achieved within 48 h and has a sensitivity of 98–100% [8, 9]. Other confirmation methods are detecting acid-fast bacilli via microscopy, histology from skin biopsies, or culture of fine needle aspirates (FNA), wound swabs, or punch biopsies. Microscopy to detect acid-fast bacilli is simple and cheap, but the sensitivity is low (34-59%) [9–11]. The results of histopathology are often delayed due to a lack of technical expertise in areas where BU is most prevalent although it has a high sensitivity between 87 – 90% [9–11]. *M. ulcerans* culture has 20–60% sensitivity for disease confirmation and usually takes 6–8 weeks or months to achieve a positive culture [9–11].

Antibiotics have greatly improved the outcomes of Buruli ulcer but there is still high variability in time to healing among treated patients [7, 12]. Studies based on categorisation of lesions have reported a median healing time between 11 and 15 weeks [13], healing of oedematous lesions between 2 and 48 weeks [14], and some 14–25 weeks after the initiation of antibiotic treatment [6, 15–17]. Slow healing wounds have been attributed to persisting viable *M. ulcerans*, persisting mycolactone or presence of secondary bacterial infections [18, 19]. Improved tools for monitoring persisting and resistant organisms during and after treatment are needed. Improved *M. ulcerans* culture has the advantage of providing accurate estimates of viable counts following initiation of antibiotics but *M. ulcerans* is widely accepted as a difficult organism to culture. Primary isolation of *M. ulcerans* remains essential for studies on treatment efficacy, molecular epidemiology, and drug sensitivity testing. Thus, new methods for improving recovery of *M. ulcerans* are needed.

Clinical specimens from non-sterile sites require decontamination for optimal recovery of mycobacterial species which can be easily overgrown by colonizing bacteria during the long periods needed to obtain growth [20]. Commonly used decontamination methods in the isolation of *M. ulcerans* from clinical specimens include Petroff / Modified Petroff method (NaCl, -NaOH), reverse Petroff method (HCl), oxalic acid, N-acetyl-L-cysteine and sodium lauryl sulphate methods [21, 22]. These conventional decontamination methods, whilst often effective on many commensal organisms contain strong acid/alkali or detergents that can have detrimental effects on the recovery of *M. ulcerans* [22–24]. Studies have proposed the enrichment of growth medium with selective supplements to increase the recovery rate of mycobacteria [25, 26]. Current standard methods culture *M. ulcerans* on a Löwenstein–Jensen medium (LJ medium) slant or enriched broths comprising Middlebrook 7H9/7H10/7H11, supplemented with nutrients to optimize growth and antibiotics to reduce contamination. Commercial culture broths such as MGIT may have polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) added to reduce overgrowth [20]; similarly, PANTA can also be added to Löwenstein–Jensen media [27].

The Tika-Kic culture kit (Tika Diagnostics, London, UK) is a novel medium designed to decontaminate at neutral pH and stimulate mycobacterial growth from low sample loads. This approach has been successfully used with other slow-growing mycobacteria like *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *M. bovis*, and *M. tuberculosis* [28–32]. However, the Tika-Kic culture kit has not been evaluated in the culture of *Mycobacterium ulcerans.*

This study compares the efficacy of Modified Petroff and Tika-Kic methods to decontaminate and recover *M. ulcerans* from clinical samples. We further identified common bacterial contaminants present in these cultures.

Methods

Study setting

The study was conducted in three locations in central Ghana where BU is endemic. The study sites were the Agogo Presbyterian Hospital in the Asante Akim North District, Tepa Government Hospital in the Ahafo Ano North District (both in the Ashanti region), and Dunkwa Government Hospital in the Upper Denkyira East District of the Central region of Ghana. The skin Neglected Tropical Diseases (NTD) team at the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) provides oversight for clinics for individuals with skin NTDs including BU and yaws in these districts.

Study design and participant recruitment

This was a cross-sectional study. Individuals with IS*2404* PCR confirmed BU (or their legal guardians) attending the skin NTD clinics at the study sites were contacted in person for recruitment. PCR confirmation of BU using IS*2404* was performed using standard procedures in the laboratory as previously published [33, 34].

At initial contact, the study was explained to all potential participants. Only individuals who were willing to participate and provided written informed consent were included in the study.

Sample collection and transport

Cotton swabs were used to collect samples from the undermined edges of ulcerative lesions and Fine Needle Aspirates (FNA) were collected from the centre of pre-ulcerative lesions. Four samples were collected from each participant. One sample each was subjected to decontamination and culturing by three different methods; the fourth sample collected was used for the 16 S rRNA assay. If a participant had more than one lesion, the same lesion was sampled at baseline and during follow-up (weeks 2 and 4). Samples were transported to the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), at the Kwame Nkrumah University of Science and Technology (KNUST) for laboratory analysis. Samples for Petroff decontamination were transported in a liquid medium containing albumin in Dubos broth (Becton, Dickinson and Company, Difco, Erembodegem, Belgium) and supplemented with PANTA (Becton, Dickinson and Company, Erembodegem, Belgium). Samples designated for Tika-Kic decontamination procedure were placed in phosphate-buffered saline (PBS). Subsequently, samples were placed in biohazard bags, kept in cold boxes and transported by car at temperatures between (2℃ – 8℃) to the laboratory at KCCR for processing. Samples were transported from the field to the laboratory in an average time of 3 h. Samples were processed immediately after arrival at the laboratory.

Laboratory procedures

Tika-Kic decontamination procedure

Sample replicate swabs were decontaminated as per the manufacturer's instructions (Tika Diagnostics, UK). Briefly, samples in PBS were placed on a shaker for 5 min after which the swab sticks were removed; samples were then pelleted by centrifugation at 1700xg for 15 min at room temperature. Pellets were then resuspended in 10 ml of complete Tika-Kic base decontamination buffer and incubated overnight at 37 °C with mild agitation (150 rpm). Samples were then centrifuged at 1700xg for 15 min at 25 °C and the supernatants carefully discarded. Pellets were then resuspended in 0.5 ml of PBS (Gibco, Thermofisher Scientific, Germany) and inoculated into 7 ml MGIT tubes (Becton Dickinson, US) supplemented with 0.8 ml OADC/PANTA and 8.5 µl TiKa growth supplement B1 (Tika Diagnostics, UK). Cultures were incubated at 31 °C and read weekly for growth detection for up to 12 months.

Modified Petroff decontamination procedure

Two replicate samples were subjected to the Modified Petroff decontamination procedures (NaOH, NaCl). Briefly, samples were transferred into 50 ml Falcon tubes and shaken for 5 min at 300 rpm after which swab sticks were removed from the swab samples. Decontamination was performed with 4 ml of 4% NaOH added to the samples and shaken for 15 min at 100 rpm. Samples were then centrifuged at 1700xg for 15 min at room temperature, and pellets were washed with 15 ml of 0.9% NaCl. Samples were further centrifuged at 1700xg for 15 min at 25 °C then pellets resuspended in 500 µl of 0.9% NaCl until complete dissolution. Resuspended pellets from one replicate decontaminated sample were inoculated onto Löwenstein–Jensen (LJ) medium (Becton Dickinson, Erembodegem, Belgium) slopes and the other inoculated in 7 ml MGIT supplemented with 0.8 ml OADC/PANTA (Becton Dickinson, USA). Cultures were incubated at 31 °C and read weekly for growth detection for up to 12 months.

Confirmation of positive cultures

Time to actual growth detection and confirmation was recorded. Positive primary bacterial growth from all media were confirmed as *M. ulcerans* by Acid-fast Bacilli (AFB) -Fluorescent Microscopy (Auramine stain) [35] and qPCR targeting the IS*2404* [33, 36] as described elsewhere.

Cultures with no visible growth were reported as negative after 12 months of incubation. Cultures were reported as contaminated when there was a change in colour of liquid medium, liquifying of LJ medium or when observed growths were different from typical mycobacterial growth, confirmed negative with AFB

microscopy and qPCR for *M. ulcerans.* Contaminating bacteria were identified based on colony morphology, microscopic examination by Gram staining and culture in blood agar to identify specific organisms.

Isolation and identification of contaminating bacteria

Samples from contaminated *M. ulcerans* cultures (both MGIT and LJ) were pre-enriched in Brain Heart Infusion (BHI) for 24 h at 37 °C. For LJ cultures, one loop full of the growth was transferred to 0.5 ml of BHI and mixed thoroughly. For growths in MGIT, 0.5 ml was taken from the tube and transferred to 0.5 ml of BHI and mixed thoroughly. The pre-enriched samples were plated on Blood and MacConkey agars. Plates were incubated at 37 °C for 24 h. Initial identification of bacteria was based on their morphology, colour and reaction to media. Sub-cultures were prepared from primary cultures until pure isolates were obtained. Pure isolates were stained to confirm their Gram status and were further identified using biochemical tests, including catalase, coagulase, and oxidase tests. *Staphylococcus aureus* and Gram-negative bacteria were identified using the Analytical Profile Index (API) tests; ID 32 STAPH V3.0, API 20NE and API 20E (Biomerieux, Marcy-l'Étoile, France).

16S rRNA assay

The 16S rRNA assay for the detection of viable *M. ulcerans* was performed using standard procedures as previously described [33]. Briefly, swab or FNA samples were transported in RNA Protect bacterial solution, and DNA and RNA were extracted simultaneously using the Qiagen AllPrep DNA/RNA kit (Qiagen, Hilden Germany). The extracts were then subjected to 16S rRNA and IS*2404* qPCR for quantification and detection of viable organisms.

Data analysis

Data was entered into Microsoft Excel version 2013 (Microsoft Corp., Redmond, Washington, USA). Data entered comprised participant demographics and outcomes of microbiological cultures. Data analysis was performed using Microsoft Excel and GraphPad Prism version 9 (GraphPad Prism, San Diego, California, USA).

Pearson chi-square test was used to compare proportions, and the Mann-Whitney non-parametric test was applied to compare the differences between groups. A p-value < 0.5 was considered statistically significant.

Results

Number of samples processed

Clinical samples were collected from 21 newly confirmed BU patients recruited into the study to evaluate the decontamination procedures for *M. ulcerans* culture. A total of 43 samples were processed for each of Tika-Kic decontaminated-MGIT culture (Tika MGIT), modified Petroff decontaminated-MGIT cultures (Petroff-MGIT), and modified Petroff decontaminated-Löwenstein Jensen cultures (Petroff-LJ) for all three time points. The number of samples processed for each method at the different time points is detailed in Fig. 1.

Characteristics of study participants

Table 1 shows the clinical characteristics of the study participants, who comprised 10 (47.6%) males and 11 (52.4%) females. The participants' median age (IQR) was 13 (5–63) years.

At baseline, there were 3 (14.3%) nodules, 5 (23.8%) plaques, 1 (4.8%) of oedema and 12 (57.1%) ulcers. Lesions were category III in 7 (33.3%), category II in 8 (38.1%) and 6 (28.6%) had category I. Eleven (52.4%) of the lesions were located on the lower limb, while 7(33.3%) were on the upper limb, and 3 (14.3%) had the lesions located on the trunk.

Comparison of *M. ulcerans* cultures at the three time points for all three methods

A total of 32/129 (25%) positive cultures for *M. ulcerans* were recovered across all decontamination methods, comprising 14/43 (33%) for Tika-Kic samples and 18/86 (21%) for Petroff samples. Of the 18 positive samples processed using the Petroff decontamination method, 10/43 (23%) were for Petroff-MGIT and 8/43 (19%) for Petroff-LJ, as shown in Table 2. Baseline samples had a higher positivity rate of 17/63 (27.0%). The rate declined to 9/39 (23.1%) by week 2 and 6/27 (22.2%) by week 4 after the start of antibiotic treatment (Fig. 2). There was no statistically significant difference in positivity rates at the different time points ($p = 0.97$).

As shown in Fig. 2, Tika-MGIT had the highest *M. ulcerans* recovery rate of 38.1% (8/21) at baseline compared to 23.8% (5/21) for Petroff-MGIT and 19% (4/21) for Petroff-LJ. At week two, growth rates were 30.8% (4/13) for Tika-MGIT, 23.1% (3/13) for Petroff-MGIT and 15.4% (2/13) for Petroff-LJ. All three methods had an equal growth rate of 22.2% (2/9) at week 4. There were no statistically significant differences between the proportions of positive cultures in the three groups ($p = 0.98$).

Comparison of the decontamination methods and culture media

The median time (IQR) to *M. ulcerans* growth detection of 5 (3–9) weeks for Petroff-LJ was shorter compared to 8 weeks (4–11) for Tika-MGIT samples and 8.5 weeks (8–13) for Petroff-MGIT samples (Table 2).

Twenty two (51.2%) samples from the Tika-MGIT, 27 (62.8%) from the Petroff-MGIT and 24 (55.8%) from the Petroff-LJ did not grow and were reported as negative cultures.

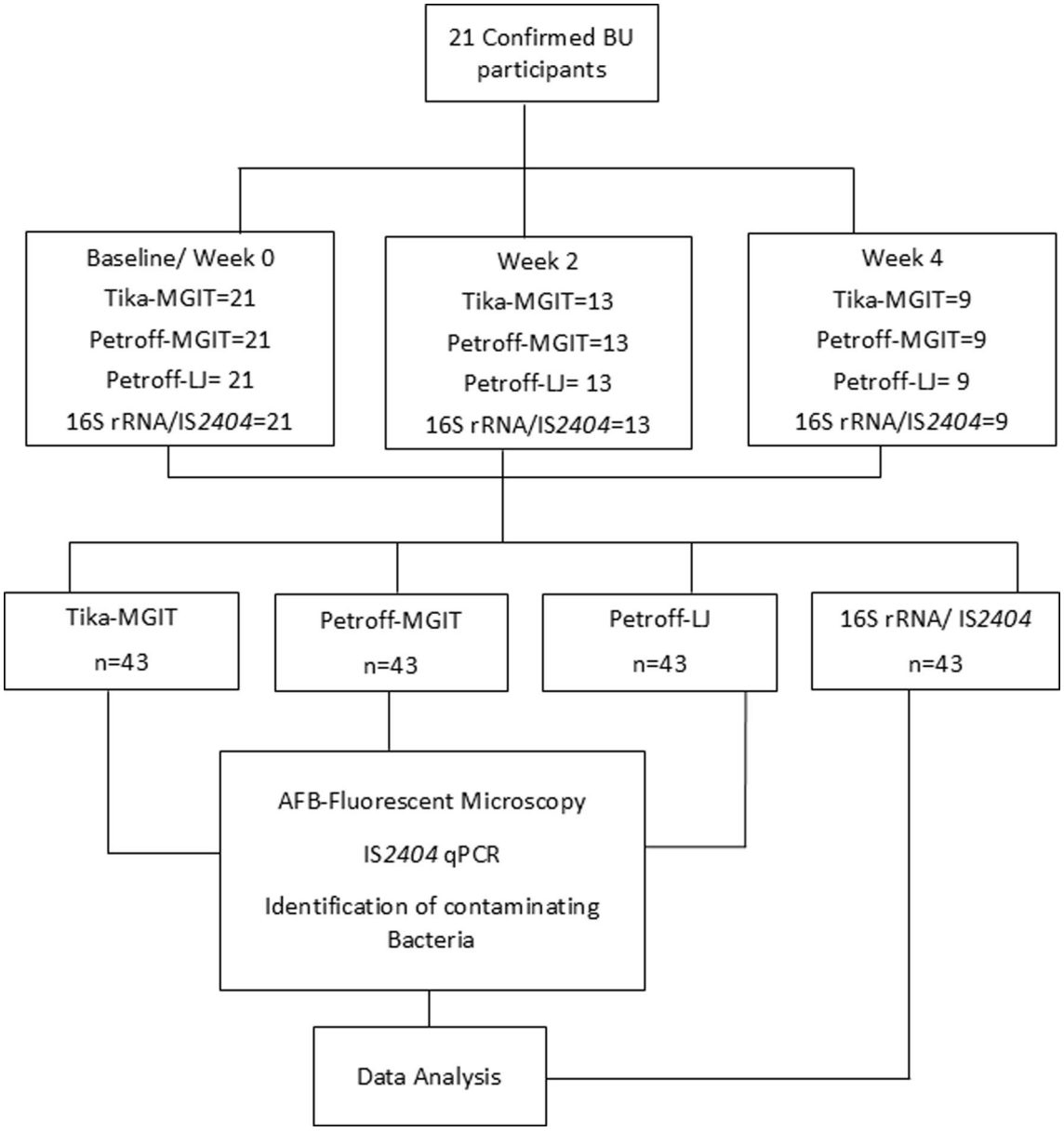

Fig. 1 Workflow for Mycobacterium ulcerans decontamination and culture assessment. *Details of the workflow for samples collected from 21 BU confirmed participants. Four samples were collected from each lesion (patient) at time points 0 (baseline; before initiation of antibiotic treatment), Week 2–(two weeks after treatment initiation), and week 4 (4 weeks after treatment initiation).The four samples at each time point were processed as follows: (a) One sample was for Tika-Kic decontamination and culture in Mycobacterium Growth Indicator Tube (MGIT); (b) one sample was for modified Petroff decontamination and culture in MGIT; and (c) one sample was for modified Petroff decontamination and culture on Löwenstein Jensen (LJ) medium; (d) one sample was used for the 16S rRNA assay. Confirmation of M. ulcerans growth was by fluorescent microscopy for Acid Fast Bacilli (AFB) and Polymerase Chain Reaction (PCR) for Insertion Sequence (IS) 2404. Week 2: 13 individuals were sampled (5 participants had lesions that were healed or almost healed, and 3 did not attend a visit on the scheduled date). Week 4: 9 individuals were sampled (2 more lesions had healed and 2 individuals did not attend the scheduled visit)

The minimum time to process the Tika samples was 15 h, including an overnight incubation (between 14 and 18 h). Comparatively, it took approximately one hour to process the Petroff samples. However, technical processing time was similar for all procedures.

Clinical characteristics and corresponding *M. ulcerans* culture recovery

A total of 13/21 participants had positive cultures at different time points in at least one method. One participant's sample had concordant positive (same outcome) results across all three culture methods, and 7 had

Table 1 Clinical characteristics of study participants

Characteristic	Frequency *n* = 21
Sex, N (%)	
Male	10 (47.6)
Female	11 (52.4)
Age	
*Median (IQR), years	13 (5–63)
Lesion Form, N (%)	
Nodule	3 (14.3)
Plaque	5 (23.8)
Oedema	1 (4.8)
Ulcer	12 (57.1)
Lesion Site, N (%)	
Lower Limb	11 (52.4)
Upper Limb	7 (33.3)
Trunk	3 (14.3)
Lesion Category, N (%)	
I	6 (28.6)
II	8 (38.1)
III	7 (33.3)

* IQR-Interquartile range

Table 2 Comparison of the performance of the decontamination methods and growth media for *M. ulcerans* culture

Variable	Tika-MGIT *n* = 43	Petroff-MGIT *n* = 43	Petroff-LJ *n* = 43
Time to growth detection			
Median (IQR), weeks	8 (4–11)	8.5 (8–13)	5 (3–9)
Positivity Rate			
Number of positives (%)	14 (33)	10 (23)	8 (19)
Contamination rate			
Number of contaminated samples (%)	7 (16.3)	6 (14.0)	11 (25.6)
Growth comparisons			
M. ulcerans growth vs. Contamination	14/7	10/6	8/11
M. ulcerans growth vs. No growth	14/22	10/27	8/24
Minimum time for processing (hours)	15	1	1

*Comparison of M. ulcerans cultures for Tika-Kic medium/ Petroff decontamination procedures and the Mycobacterium Growth Indicator Tube (MGIT)/ Löwenstein Jensen (LJ) media. Median time to growth was measured in weeks with interquartile range (IQR). Proportions of M. ulcerans growth were compared with (versus-vs) proportion of contaminated cultures and those with no growth after 12 months

concordant positive results by at least two of the three methods (Table 3).

Culture results were compared with results from *M. ulcerans* 16S rRNA assay used for the detection of viable organism in BU wounds. 16S rRNA assay was performed at all time point (Weeks 0, 2 and 4) samples. There was concordance between the final culture result and the final 16S rRNA assay result in 19/21 (90.5%) of participants' samples. For Tika-MGIT, concordance with 16S rRNA assay was 18/21 (85.7%) compared to 16/21 (76.2%) for the Petroff-MGIT and 13/21 (61.9%) for the Petroff-LJ method (Supplementary Tables 1–7).

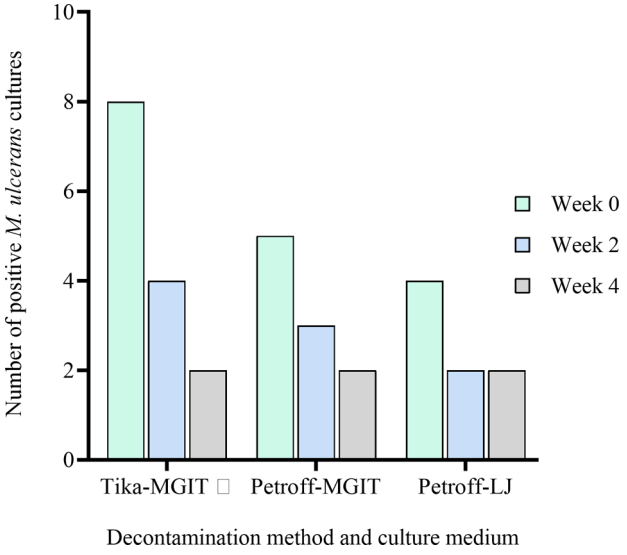

Fig. 2 Yield of positive cultures at different time points. *Positive M. ulcerans cultures were compared among the decontamination methods and growth media. Cultures were done at week 0 (before the start of antibiotic treatment), and weeks 2 and 4 (after the start of antibiotic treatment)

Sensitivity of different culture methods for *M. ulcerans* recovery

The final culture result was deemed positive if the *M. ulcerans* organism was recovered from any of the three culture methods employed in this study at any of the three time points. To determine the sensitivity of the culture methods, the *M. ulcerans* culture results were compared with the gold standard confirmatory qPCR for all 21 patient samples (Table 4). The Tika-MGIT method had a sensitivity of 47.6% (95% CI 28.3–67.6) while the Petroff-MGIT method had a sensitivity of 28.6% (95% CI 13.8–49.9). The Petroff-LJ method had a sensitivity of 23.8% (95% CI 16.6–45.1) (Table 4). The overall sensitivity of culture compared to the qPCR was 61.9% (95% CI 40.9–79.3). The sensitivity of Tika-MGIT compared to Mu 16S rRNA assay was 82% (95% CI 52–97) (Table 5).

Clinical characteristics and *M. ulcerans* recovery from culture

The clinical characteristics were compared to determine the proportions with a positive culture, which was defined as a positive *M. ulcerans* culture for at least one method at any time point. *M. ulcerans* was recovered from 8 out of 12 ulcers compared to 4 out of 5 plaque lesions. No nodules produced a positive *M. ulcerans* culture. More positive cultures were also obtained from category II lesions (6/8), and lesions on the lower limbs (7/11). Clinical characteristics did not show any

Table 3 Demographic details and culture outcomes for study participants

						Microbiological Confirmation				Molecular Confirmation
Participant Code	**Sex**	**Age**	**Lesion form**	**Lesion site**	**Lesion Category**	**Tika-MGIT**	**Petroff-MGIT**	**Petroff-LJ**	**Final Mu Culture Positivity**	**Mu 16S rRNA Positivity**
BUCP1	F	13	Ulcer	LL	III	+	+	-	+	+
BUCP2	M	6	Ulcer	LL	II	+	-	+	+	+
BUCP3	F	7	Ulcer	LL	II	-	+	-	+	+
BUCP4	M	7	Ulcer	GN	III	-	-	-	-	-
BUCP5	F	27	Ulcer	AB	I	+	-	-	+	+
BUCP6	M	47	Ulcer	UL	III	-	-	-	-	-
BUCP9	M	6	Ulcer	LL	I	-	+	-	+	+
BUCP10	F	18	Ulcer	LL	II	+	-	-	+	+
BUCP12	F	63	Ulcer	BP	III	-	-	-	-	-
BUCP14	F	19	Ulcer	UL	II	+	+	+	+	+
BUCP19	F	10	Ulcer	LL	I	+	+	-	+	+
BUCP20	F	53	Ulcer	LL	II	-	-	-	-	-
BUCP8	M	46	Plaque	UL	III	+	+	-	+	+
BUCP13	M	10	Plaque	UL	II	+	-	+	+	+
BUCP15	M	10	Plaque	LL	III	+	-	+	+	+
BUCP17	F	33	Plaque	LL	II	-	-	-	-	-
BUCP21	M	12	Plaque	UL	II	+	-	-	+	-
BUCP18	M	45	Oedema	UL	III	-	-	+	+	-
BUCP7	F	48	Nodule	UL	I	-	-	-	-	-
BUCP11	M	5	Nodule	LL	I	-	-	-	-	-
BUCP16	F	7	Nodule	LL	I	-	-	-	-	-

*Details of culture outcomes (defined as a positive M. ulcerans recovered for at least one of the different methods at any time point) on 21 BU confirmed participants evaluated by Tika-MGIT (Mycobacterium Growth Indicator Tube), Petroff-MGIT and Petroff-LJ (Löwenstein Jenssen) and compared with 16 S rRNA assay for viable M. ulcerans. (+) indicates a positive outcome, (-) indicates a negative outcome); (C) indicates all time point samples contaminated (Mu) indicates Mycobacterium ulcerans; LL- lower limb; UL- upper limb; GN- genitalia; AB- Abdominal region; BP- back

Table 4 Comparison of *M. ulcerans* culture recovery, using the Tika-MGIT, Petroff-MGIT and Petroff-LJ methods

Decontamination and Culture Method	**Confirmatory qPCR result**		**Sensitivity % (95% CI)**
	Positive	**Negative**	
Tika-MGIT			
Positive	10	0	47.6 (28.3–67.6)
Negative	11	0	
Petroff-MGIT			
Positive	6	0	28.6 (13.8–49.9)
Negative	15	0	
Petroff-LJ			
Positive	5	0	23.8 (16.6–45.1)
Negative	16	0	
Final Culture Results			
Positive	13	0	61.9 (40.9–79.3)
Negative	8	0	

*M ulcerans culture recovery was compared for Tika-MGIT, Petroff-MGIT and Petroff-LJ with the standard confirmatory qPCR results for all 21 participants. This was used to compute the sensitivity with a 95% CI (Confidence interval)

Table 5 Comparison of mu culture recovery, using the Tika-MGIT, Petroff-MGIT and Petroff-LJ methods with mu 16S rRNA results

Decontamination and Culture Method	**Number of samples with Mu 16S rRNA result**		**Sensitivity % (95% CI)**
	Positive	**Negative**	
Tika-MGIT			
Positive	9	1	82 (52–97)
Negative	2	9	
Petroff-MGIT			
Positive	6	0	55 (28–79)
Negative	5	10	
Petroff-LJ			
Positive	4	1	36 (15–65)
Negative	7	9	
Comparison for Final culture result and Mu 16S rRNA results			
Final culture results			
Positive	11	2	100 (74–100)
Negative	0	8	

*Mu (M. ulcerans) culture recovery was compared for Tika-MGIT, Petroff-MGIT and Petroff-LJ with a Mu 16S rRNA result (defined as a positive for any of the three methods at any of the three time points (Week 0, 2 and 4)). This was used to compute the sensitivity with a 95% CI (Confidence interval). A comparison was also made between the final Culture result and Mu 16SrRNA results

statistically significant association with *M. ulcerans* recovery from cultures (Table 6).

Contamination rate and bacteria identification

From all time points and decontamination methods, a total of 24 out of 129 samples (18.6%) were found to be contaminated. Specifically, 7 out of 43 samples (16.3%) from the Tika group, 6 out of 43 samples (14.0%) from the Petroff MGIT group, and 11 out of 43 samples (25.6%) from the Petroff-LJ group were contaminated (Table 2 and Supplementary Table 8).

Bacterial cultures and identification tests were performed to identify contaminating bacteria in *M. ulcerans* cultures. Commonly isolated Gram-negative bacteria included *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Pasteurella pneumotropica*, *Proteus mirabilis*, *Achromobacter xyloxidans*, and *Morganella morganii.* Isolated Gram-positive bacteria included *Staphylococcus aureus*, *Enterococcus (Streptococcus group D)*, Coagulase-negative *Staphylococcus*, and Gram-positive rods. In all, 11 different bacteria were isolated from the contaminated samples.

The Tika-MGIT had the highest proportion of isolated bacterial strains 7/11 (64%) which includes both Gram-positive and gram-negative bacteria. Petroff-MGIT and Petroff-LJ both had 6/11 (55%) of bacterial strains isolated though there were variations in the specific bacteria isolated. Petroff-MGIT cultures had the lowest isolated organisms including both Gram-positive and Gram-negative bacteria (Fig. 3).

Organisms were further compared between time points. Week 2 and 4 cultures were the most contaminated with about 64% of the total bacteria being isolated from week 2 and 4 samples. There were 4 (36%) contaminating bacterial strains at Week 0; no Gram positives were isolated from Week 0 cultures. However, there was no difference in the type of organisms across the decontamination method and growth medium (Fig. 4).

Discussion

The study compares the various efficacies of three decontamination techniques to enhance the yield and recovery of *M. ulcerans* in culture from clinical samples of Buruli ulcer lesions. We utilized the Tika-Kic decontamination kit for the first time in processing *M. ulcerans* cultures in a resource-limited setting and compared with the standard modified Petroff decontamination procedure. We show that the Tika-MGIT system, previously shown to be effective for the isolation of various mycobacteria from both human and animal specimens [28–31] demonstrated a 19% increase in sensitivity over a modified Petroff-MGIT and 23.8% higher than the Petroff-LJ method. Compared to the Mu 16S rRNA, the Tika-MGIT method has a sensitivity of 82%.

Studies investigating decontamination procedures and growth media for *M. ulcerans* isolation report varying recovery rates. A study by Yeboah-Manu et al. [27] evaluated four decontamination methods (including Petroff) and three growth media for isolating *M. ulcerans* from surgically excised tissues. Their findings suggested that 5% oxalic acid decontamination followed by culturing on Löwenstein-Jensen (LJ) medium supplemented with 0.75% glycerol and 2% PANTA yielded superior results. This method achieved a high recovery rate (75.6%) with minimal contamination (2.4%). It is important to note that the study also employed LJ medium with 0.5%

Table 6 Clinical characteristics of participants with positive *M. ulcerans* culture

Characteristic	Proportion with positive culture result			Proportion with positive final Mu culture result	
	Tika-MGIT	Petroff-MGIT	Petroff-LJ		*P*-value
Sex					
Male	5/10	2/10	4/10	7/10	0.66
Female	5/11	4/11	1/11	6/11	
Lesion form					
Nodule	0/3	0/3	0/3	0/3	0.09
Plaque	4/5	1/5	2/5	4/5	
Oedema	0/1	0/1	1/1	1/1	
Ulcer	6/12	5/12	2/12	8/12	
Lesion site					
Lower limb	5/11	4/11	2/11	7/11	0.52
Upper limb	4/7	2/7	3/7	5/7	
Trunk	1/3	0/3	0/3	1/3	
Lesion category					
I	2/6	2/6	0/6	3/6	0.25
II	5/8	2/8	3/8	6/8	
III	3/7	2/7	2/7	4/7	

*Numerators represent the absolute number within a group with positive culture result; the denominators represent the total number of individuals within each group

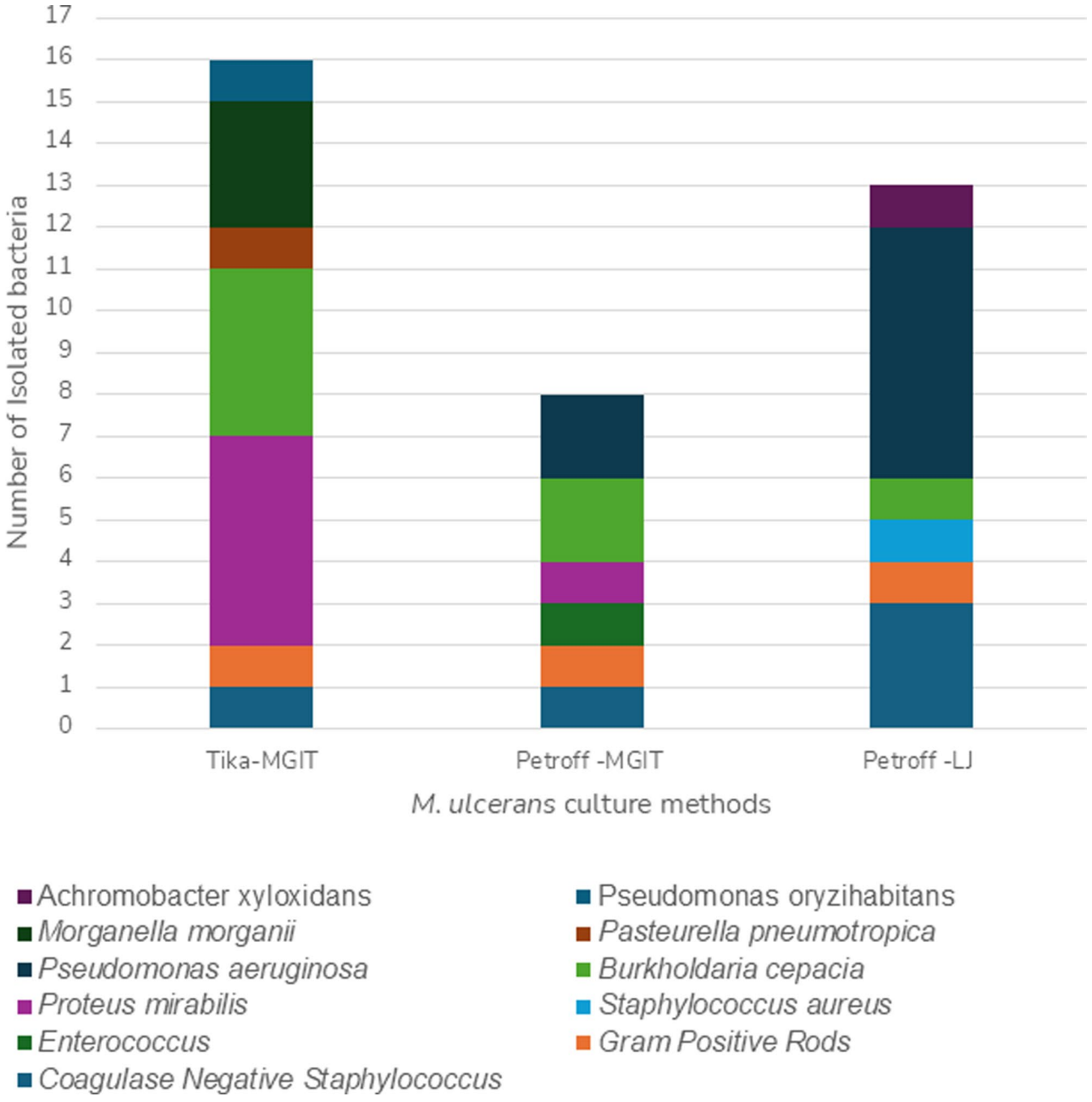

Fig. 3 Isolated bacteria strains from contaminated *M. ulcerans* culture from the three culture methods

pyruvate (LJP) alongside LJ supplemented with glycerol and PANTA. While their results highlighted the superiority of LJ supplemented with glycerol and PANTA, LJP may also a viable option [27].

In another research study, eight decontamination protocols were evaluated using various chemicals on different types of samples. The study found that using 0.5% and 1% povidone-iodine resulted in a low contamination rate (0%), but also led to the lowest recovery rate (0%). On the other hand, using 2% cetylpyridinium chloride (CPC) with 4% sodium chloride solution achieved the highest recovery rate (53%), while oxalic acid resulted in a higher contamination rate (29%) when compared to the other methods [24]. Of note, all cultures in that study were grown on LJ medium. These findings emphasize that the ideal decontamination technique might depend on the sample type (e.g., swab vs. excised tissue) being cultured. Bratschi et al. [21] also indicated that, growth on LJ supplemented with PANTA returns a higher yield and a lower contamination rate. Comparatively, all these recovery rates surpassed those reported in the present study.

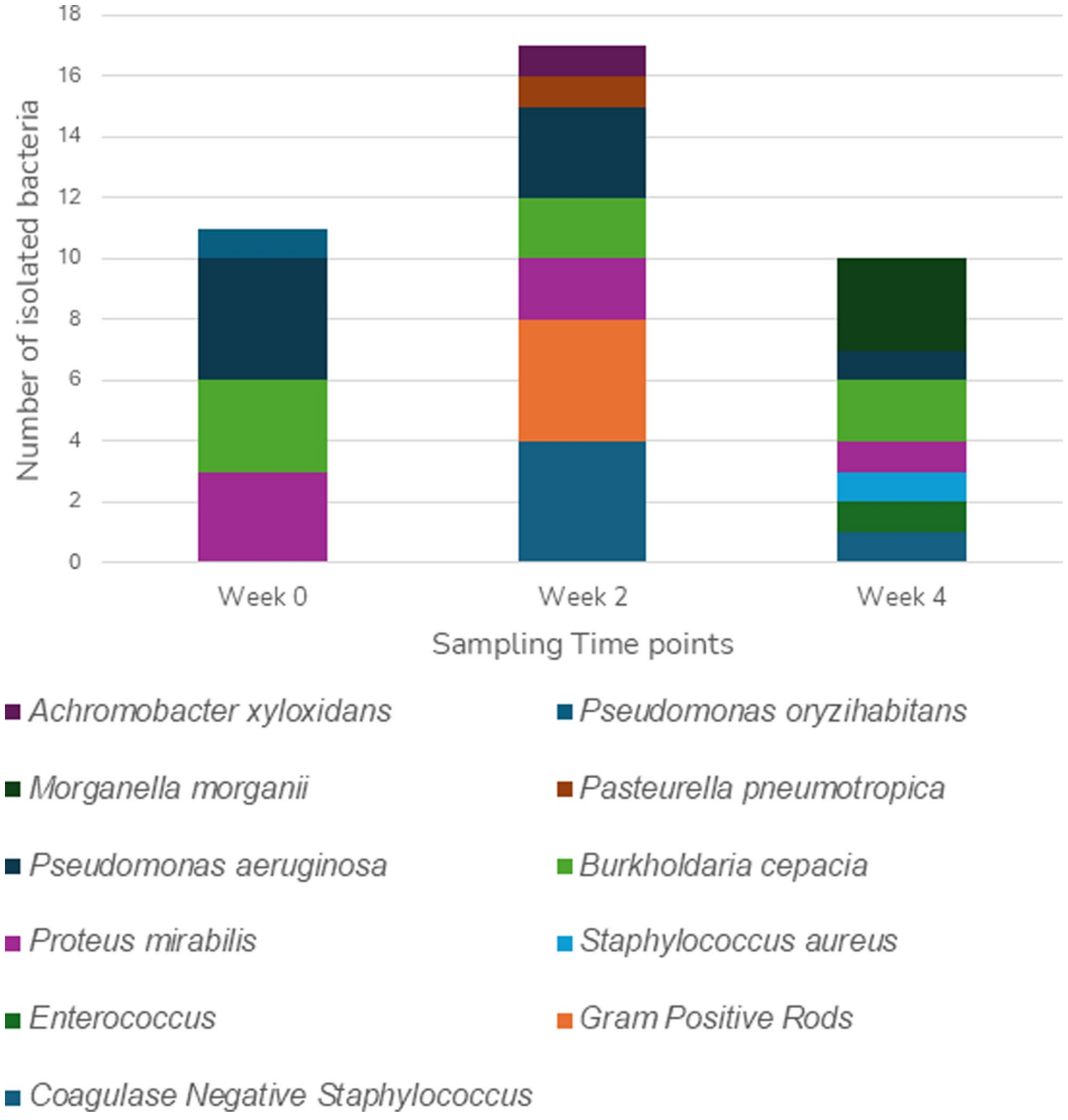

Fig. 4 comparison of isolated bacteria strains from *M. ulcerans* contaminated cultures between time points

A prevalent complication with mycobacteria cultures is contamination from other fast-growing microorganisms. This underscores the need for developing decontamination techniques to reduce their activity thereby increasing recovery rates. While Tika presented the highest recovery, it was associated with a contamination rate of 16.3% compared to 14.0% of the Petroff-MGIT method. It is very likely that the Tika decontamination is more gentle, allowing more *M. ulcerans* to survive but also allowing more contaminants to survive. The Petroff-LJ combination recorded the highest contamination rate of 25.6%. By comparison Tika-MGIT had the highest numbers of the varying bacterial strains isolated from the contaminated samples. It also required an extended incubation period (between 15 and 19 h). A good culture outcome depends on the quality of samples available. With a shift toward less-invasive sampling methods for BU over recent years, current samples include swabs from the undermined edges of ulcerated lesions and FNAs from the pre-ulcerative lesions. Although less invasive, swab

samples tend to yield less due to sample collection constraints compared to surgically obtained tissues, which offer higher baseline bacterial loads and sterility, as previously reported [24, 27, 37].

Baseline samples had higher *M. ulcerans* recovery rates than samples collected at week 2 and week 4. Antibiotic therapy for BU causes killing of *M. ulcerans* within BU lesions and this explains the reduced yield in the cultures taken after commencement of treatment. Similar observations were made in a study conducted in Australia [38].

An outcome of the study was the isolation of bacteria that contaminated *M. ulcerans* cultures. Isolated bacteria from contaminated samples included *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Pasteurella pneumotropic*, *Proteus mirabilis*, *Morganella morganii*, *Achromobacter xyloxidans*, *Staphylococcus aureus*, *Enterococcus* (*Streptococcus* group D) Coagulase Negative *Staphylococcus* and Gram-positive rods.

These are known pathogenic bacteria that cause various diseases in humans and some animals. Most of these are known secondary colonising bacteria in infected wounds and have been isolated from BU wounds [39–41]. Their presence in wounds is likely to delay healing of these BU lesions and increase cost of treatment. The persistence of these organisms (particularly those that are resistant to first line antibiotics used in treating BU and other wound infections) in wounds is alarming in the context of rising antimicrobial resistance. Such persistence underscores the necessity for interventions targeting these wound microbiomes during BU treatment. It was observed that while the antibiotic therapy decreased *M. ulcerans* levels, the load of secondary infecting bacteria changed, with a broader variety present in samples from week 2 and 4 compared to the baseline samples.

Optimization of decontamination techniques in the culture of *M. ulcerans* is essential to improve the recovery rates. Nevertheless, some research indicates that the vigorousness of certain decontamination procedures can inadvertently have detrimental effects on the viability of *M. ulcerans* [21, 23, 42]. The Tika-Kic medium has successfully navigated this issue, achieving superior *M. ulcerans* yields while preserving the organisms' viability. In this study TiKa-Kic decontamination was marginally less successful than the standard Petroff method suggesting that more optimisation may be necessary. This could be partly due to the absence of antimicrobials in their transport medium (PBS) which unlike the Petroff samples incorporated PANTA antibiotics in its transport medium. A study by Eddyani et al.. in 2008 suggested that the transport medium used may influence recovery rate [43]. Moreover, augmenting the MGIT growth medium with PANTA and other growth supplements did not seem to reduce contamination levels. Other studies, however, confirm otherwise [20, 21].

Interestingly, the study demonstrated an increased *M. ulcerans* growth in the liquid medium, contrary to previous findings that solid Löwenstein-Jensen media were superior for cultivating *M. ulcerans* [21, 24, 27, 44]. Considering the efficiency and cost-effectiveness in resource limited settings, future recommendations should encourage the use of liquid media to achieve greater recovery rates of *M. ulcerans*.

Conclusion

We have demonstrated that the Tika system offers improvement over conventional methods in recovery and isolation of *M. ulcerans* from Buruli ulcer clinical samples. Effective decontamination is paramount for successful isolation of *M. ulcerans* and further improvements in sample transport media, decontamination techniques and growth media are needed to reduce overgrowth by other contaminating fast growing microorganisms and optimize recovery rates. Further research is needed to understand the role and impact of secondary colonizing organisms on outcomes in BU.

Abbreviations

BU	Buruli Ulcer
NTD	Neglected Tropical Disease
MGIT	Mycobacterium Growth Indicator Tube
LJ	Löwenstein-Jensen
MU	*Mycobacterium Ulcerans*
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, and Azlocillin
WHO	World Health Organisation
FNA	Fine Needle Aspirates
OADC	Oleic Albumin Dextrose Catalase
AFB	Acid-Fast Bacilli
KCCR	Kumasi Centre for Collaborative Research
KNUST	Kwame Nkrumah University of Science and Technology
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide

Supplementary Information

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Supplementary Material 1

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Author contributions

BA, MWJ, TJB, YAA and ROP conceptualised and designed the study. RAA, AA, DKA, CW-A, CA-A, KMA, EO, GA, and YAA collected data and were involved in patient management. BA and YAA performed data analysis. BA and YAA wrote the initial draft of the manuscript. KB, MGA, TT, DD, MWJ, TJB and ROP reviewed the manuscript for important intellectual content. All authors read and approved the final manuscript.

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and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethical approval
was sought from the Committee on Human Research, Publications and Ethics (CHRPE) (CHRPE/AP/472/17) of the Kwame Nkrumah University of Science and Technology (KNUST). Written informed consent was obtained from willing participants. Full disclosure and explanation of the study procedures was provided to all participants in a language of their choice (this was largely English or the local language, Twi). For individuals who could not read or understand English, consenting was done via an interpreter in the presence of a witness of the individual's choice. Following this, persons could sign or were thumb-printed to provide consent for inclusion in the study. For young children (< 18 years), written consent was obtained from parents or legal guardians. All the study processes were conducted in accordance with the principles guiding research in human subjects as set out in the Declaration of Helsinki [45].

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Clinical trial number
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