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Microscopy for Acid Fast Bacilli: A Useful but Neglected Tool in Routine Laboratory Diagnosis of Buruli Ulcer

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Abstract

Background: Laboratory diagnosis of Buruli ulcer disease has become vital with the introduction of antibiotic treatment. Polymerase chain reaction (PCR) for the IS2404 repeat sequence of *Mycobacterium ulcerans* is the gold standard for laboratory diagnosis. This is expensive and only carried out in reference laboratories in endemic countries in Africa. In order to improve the efficiency of diagnosis at the point of care and reduce the total cost of patient management, we decided to evaluate Ziehl-Neelsen (ZN) staining for acid-fast bacilli (AFB) as an inexpensive diagnostic tool.

Methods: Two smears directly prepared at the point of care were examined under oil-immersion microscopy after ZN staining for AFB and compared the results to PCR samples from the same patients.

Results: Good quality smears were obtained from all subjects and our results showed that when a second smear was added the sensitivity of microscopy for AFB was increased from 52 to 55% for FNA samples and from 51 to 57 % for swabs.

Conclusion: If PCR were to be omitted in all patients with suspected Buruli ulcer disease when AFB were detected it would result in a considerable saving.

Keywords: *Mycobacterium ulcerans,* Buruli ulcer, Ziehl-Neelsen staining, Polymerase chain reaction

Introduction

Buruli ulcer disease (BUD) is the third most common mycobacterial infection after tuberculosis and leprosy in immunocompetent individuals [1,2]. It has been reported in over 30 countries across the world but the highest burden is in West Africa affecting mostly children living in poor rural communities [3]. Large ulcers and physical deformities are associated with this disease due to late reporting of patients to medical centers. Hence control strategies rely largely on early case detection and treatment. Laboratory confirmation of clinically suspected cases has become an important step in management of the disease since the introduction of antibiotic therapy with rifampicin and streptomycin for 8 weeks as recommended by the WHO [4] as the first line of treatment. Accepted laboratory techniques available now for the confirmation of BUD are *M. ulcerans* isolation by culture, histopathology [5], smear microscopy for acid-fast bacilli (AFB) and polymerase chain reaction (PCR) for detection of the *M. ulcerans* specific insertion sequence IS2404 [6-8]. Culture for *M. ulcerans* takes several weeks so it cannot be used to make treatment decisions and histopathology is not available in most countries where BUD is endemic so smear microscopy for AFB and IS2404 PCR are the investigations most commonly used for case confirmation [9]. IS2404 PCR is regarded as the gold standard due to its high sensitivity and specificity but it is expensive and needs a sophisticated laboratory setup and technical expertise, which are not always available in resource-poor endemic communities. The need to transport diagnostic samples to specialized laboratories for case confirmation delays treatment and increases the cost of case management. The cost of standard PCR is in the range of $11 to $20 per sample [6,10]. The WHO has set up reference facilities but the cost of such services is mainly covered by research projects and non-governmental organizations [11] and alternative cheaper and easy-to-perform diagnostic tests are a high priority.

In the meantime a stepwise approach in diagnosis with microscopy as the first step on the ladder has been proposed [10] but the role of microscopy has been limited by its low sensitivity ranging from 40% to 70% [5, 12-14]. Several approaches have been proposed to increase sensitivity such as obtaining at least two samples per lesion [15], concentration of sample before smearing [16] and good microscopy practice, taking care to read at least 100 high-power fields before declaring a slide negative [13]. Improving the transport of samples may further improve detection rate as many samples arrive at the laboratory 'dry' having spent between two days and one month in transit [10]. Therefore this study has evaluated the usefulness of preparing two direct smear slides for microscopy on site before transport to the laboratory as a means to improve the sensitivity of AFB detection in swab and fine-needle aspirate (FNA) samples from a large cohort of clinically diagnosed BUD patients.

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Materials and Methods

Study participants

Between March 2010 and February 2013, 236 clinically suspected Buruli ulcer patients were recruited from two districts in the Ashanti Region of Ghana, the Ahafo-Ano North District and Asante Akim-North District. Buruli ulcer treatment centres have been set up in these districts with coordinated supervision from a team of specialists from the Komfo Anokye Teaching Hospital (KATH) and the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) at the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. Ethical approval was obtained from the Committee on Human Research, Publications and Ethics (CHRPE), School of Medical Sciences (SMS) at KNUST (CHRPE/91/10). All patients (or their parents or guardians, for children younger than 18 years) gave written informed consent to participate in the study. Clinically suspected Buruli ulcer lesions were grouped as non-ulcerative (nodules, plaques, oedema) and ulcerative lesions in accordance with the WHO classification [4].

Specimen collection using swab and FNA samples

Swab samples were collected by clinicians using standard procedures as previously described [13]. Briefly, four swabs were collected by circling the entire undermined edge of ulcers where the pathogens are more likely to be found. Two swabs were used to prepare direct smears on separate slides and the remaining two were transferred into separate tubes containing 700 µl cell lysis solution (CLS, Qiagen, Germany) for dry-reagent-based (DRB) IS2404 PCR. For patients with non-ulcerative lesions four fine needle aspirate (FNA) samples were collected using a 21G needle as described elsewhere [6]. Two FNA samples were smeared on slides, allowed to dry, transferred into a slide case (Fisher Scientific GmbH, Schwerte, Germany) and transported to the KCCR for microscopy. The remaining two were transferred into separate tubes containing 300 µl CLS for IS2404 PCR.

Dry-reagent-based IS2404 PCR (DRB-PCR)

Duplicate samples were taken from each patient for PCR targeting the *M. ulcerans* IS2404 repeat sequence as described previously [17]. In brief, DNA was prepared from swabs and FNA specimens using the Puregene DNA isolation kit (Genomic DNA Purification Kit, Gentra Systems). For DRB-PCR, oligonucleotides MU5 (5' AGCGACCCCAGTGGATTGGT 3') and MU6 (5' CGGTGATCAAGCGTTCACGA 3') were lyophilized in reaction tubes. PuReTaq Ready-To-Go PCR beads and water were added followed by the DNA extracted from patient samples. A negative extraction control and positive, negative and inhibition controls were included. The thermal cycling protocol was as follows: 94°C for 10 min, followed by 40 cycles at 94°C for 10 s, 58°C for 10 s, and 72°C for 30 s, with a final cycle at 72°C for 15 min. Amplification products were kept at 4°C until they were processed further by agarose gel electrophoresis.

Smear microscopy

Slides were passed over a Bunsen burner to fix the mycobacteria and then flooded with filtered carbol fuchsin (alcoholic fuchsin in 3% phenol solution) to cover the whole slide. The underside of the slides was heated using an ignited cotton swab soaked in 70% ethanol and the heated stain was left on the slide for 5 minutes. Slides were gently rinsed with water and decolourised with 20% H_2SO_4 solution for 5 minutes. After rinsing with water, methylene blue was added for 1 minute and rinsed away with water. Slides were wiped on the underside and allowed to air dry on a draining rack. They were examined under a 100-fold oil immersion objective. At least 100 fields were examined to declare a slide negative. Positive slides showing red rod-like bacilli were graded on the number of AFB seen according to the WHO recommended system [18].

Quality control

Microscopy slides were reexamined and DNA extracts re-evaluated independently by the Department for Infectious Diseases and Tropical Medicine at the Ludwig-Maximilians-University of Munich, Germany, for external quality assurance.

Statistical methods

The raw data was entered using Microsoft Excel. GraphPad Prism 5 software was used for data analysis. Descriptive statistics were used to obtain general descriptive information such as the median and inter-quartile ranges. One sample analysis (Fisher's exact test) was used to compare two proportions or groups. Contingency tables were used to calculate the sensitivity of the various laboratory techniques employed.

Results

Table 1 shows the characteristics of 236 patients with suspected Buruli ulcer lesions of which there were 111 pre-ulcerative and 125 ulcerative forms. There were 49 (44%) nodules, 41 (37%) plaques and 21 (19%) edematous lesions. The median age of those with pre-ulcerative lesions was 13 years (range 2-68) and 16 years (range 2-80) for those with ulcerative lesions.

Using standard procedures 96 (86%) pre-ulcerative and 109 (87%) ulcerative lesions were IS2404 PCR positive. All smears prepared and stained on site were of sufficient quality to be examined for AFB by microscopy and of those from pre-ulcerative lesions (FNA samples) 55 of 111 (50%) were positive. Similarly 63 of 125 smears swabs taken from ulcerative lesions (50%) were positive for AFB (Table 1). When two smears were prepared in clinic (on-site) from FNA samples of 111 patients the sensitivity of AFB detection was 52% (41-62, 95% CI) for the first slide increasing to 55% (45-66, 95% CI) when a second

				No. (%) positive samples	
	No. (%) of Pre-ulcerative lesion (n=111)	No. (%) of ulcerative lesions (n=125)	No. (%) of total lesions (n=236)	PCR	ZN Microscopy
Sex					
Male	44 (40)	60 (48)	104 (44)	92 (88)	51 (49)
Female	67 (60)	65 (52)	132 (56)	113 (86)	67 (51)
Age in years					
Median (range)	13 (2-68)	16 (2-80)	14 (2-80)	_	_
Type of Lesion					
Nodule	49 (44)	_	49 (21)	41 (84)	20 (41)
Plaque	41 (37)	_	41 (17)	36 (88)	23 (56)
Edema	21 (19)	_	21 (9)	19 (91)	12 (57)
Ulcer	_	125 (100)	125 (53)	109 (87)	63 (50)
Category of Lesion					
I	64 (58)	57 (45)	121 (51)	103 (85)	61 (50)
II	25 (22)	41 (33)	66 (28)	57 (86)	36 (55)
III	22 (20)	27 (22)	49 (21)	45 (92)	21 (43)

Table 1: Patient characteristics and laboratory results.

slide was examined (Table 2). Similarly when swab samples from 125 patients were examined the sensitivity of microscopy was 51% (42-61, 95% CI) but increased to 57% (48-67, 95% CI) when a second sample was examined. However, this increase did not reach statistical significance. A blinded independent external scientist from the DITM/LMU confirmed these results.

Discussion

There is an urgent need for national programmes to establish laboratory diagnosis as a part of the routine care of patients with *M. ulcerans* infection especially in an era of antibiotic treatment but reliance on PCR in resource poor countries of Africa is not sustainable due to the cost of the investigation. Until a point of care diagnostic test that can be used in primary and secondary health facilities is developed, the most suitable option would be to improve the sensitivity of AFB detection by ZN microscopy as a first-line laboratory-confirmation technique for Buruli ulcer as it is applied for TB. Our study has demonstrated that obtaining two samples and immediately preparing smears on slides for later microscopy in the laboratory increased the sensitivity from 52% to 55% for FNA samples and from 51% to 57% for swab samples. These sensitivities are comparable to those obtained recently when sample concentration techniques such as pooling or 3mm bead-beating with vortexing were used to release bacteria from swabs [10].

The advantage of this technique over the concentration method is that it did not require expensive equipment and the processing time was significantly reduced. Slides smeared and stained on site were of better quality than those prepared in the laboratory and they could be kept securely in slide cases for review by an independent person for quality assurance purposes. This is a larger study of the use of microscopy and employs a simpler technique than the concentration method in a retrospective study from Ghana and Togo, when similar sensitivity of microscopy of 58% for 36 FNAs and 46% for 69 swabs was reported [17].

The sensitivity of PCR (86-87%) was significantly higher than that of ZN microscopic examination (55-57%) ($P < 0.01$) which is comparable to an earlier study [10]. Further stratification by lesion type made the results more interesting since the sensitivity at 63% for oedemas remained the same whether one slide or two was examined. These results show that it may not be necessary to take multiple samples from an edematous lesion, and that one sample is as good as processing two. Plaques and nodules however, showed significant improvement in terms of sensitivity when two samples were processed. Notably, the sensitivity of FNA from plaques increased from 56% to 62% after examining two slides instead of one. A similar trend was observed for nodules where the sensitivity increased from 43% to 48%. The higher sensitivity in plaques and edemas could imply higher bacterial load in these disease forms. These results support the recommendation for two samples to be processed per case thereby increasing the sensitivity of the ZN microscopy for Buruli ulcer confirmation.

	First microscopy sample	**Second microscopy sample**
Sample type	Sensitivity (% [95% CI)]	Sensitivity (% [95% CI)]
Pre ulcerative		
FNA (n=111)	52 (41-62)	55 (45-66)
Ulcerative		
Swab (n=125)	51 (42-61)	57 (48-67)
Pre-ulcerative		
Nodule	43 (27-59)	48 (32-64)
Plaque	56 (38-73)	62 (44-78)
Edema	63 (38-84)	63 (38-84)

Table 2: Sensitivity of immediate slide smear microscopy in the districts improves detection of *M. ulcerans* when a second slide smear is examined.

PCR currently presents a series of challenges especially in resource poor setting. These have to do with adequate three-room space for separation of the various stages in sample processing, equipment acquisition, technical expertise, and shipment. In an attempt to overcome these challenges a rapid and sensitive amplification platform for DNA, the Loop mediated isothermal amplification method (LAMP) has been developed [19-22]. However challenges of obtaining pure DNA extracts from clinical specimens as well as the use of a pocket warmer capable of maintaining 65°C for 1 hour need to be addressed in order to improve the performance of the assay [23].

Conclusion

Laboratory confirmation of clinical diagnosis of Buruli ulcer disease is vital; first to prevent the situation of miss-diagnosis and also to have epidemiological data on new infections. The evaluation of the usefulness of preparing two direct smears for each case instead of one on-site for ZN microscopy can easily be implemented in treatment centers with little technical expertise. Our finding that examining two slides for each case improves the overall sensitivity of the technique to over 50% comparable to other methods clearly shows that about half of clinically diagnosed cases of Buruli ulcer could be laboratory confirmed on site. If PCR were to be omitted in all patients with suspected Buruli ulcer when AFB were detected it would result in a considerable saving and reduce delays in early treatment.

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