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Diagnosis of Buruli ulcer disease in Nigeria using IS2404-Based Nested PCR

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Abstract

Background: Buruli ulcer is a chronic, indolent, necrotizing infectious disease of the skin and soft tissues characterized by the formation of large ulcers, often in the arms or legs. Nigeria is a Buruli ulcer disease (BUD) endemic country with its control programme still in infancy. As a result, samples are sent to laboratories outside the country. Some patients go to neighbouring countries with more established programmes for polymerase chain reaction (PCR) as a basis of diagnosis and treatment. Hence, this study was embarked upon to assist the national control programme in overcoming the PCR diagnosis test challenge.

Method: This was a cross-sectional and community-based type of study of Buruli ulcer patients from 15 states, mostly from southern Nigeria; Abia, Akwa Ibom, Anambra, Bayelsa, Cross Rivers, Delta, Ebonyi, Ekiti, Enugu, Imo, Lagos, Ogun, Ondo, Osun, Rivers and the Federal Capital Territory (FCT) Abuja. Swab and Fine Needle Aspiration (FNA) samples were received from January 2016 to June 2018. DNA was extracted, and each sample was subjected to IS2404-based nested PCR.

Results: Out of 920 samples received, 427 (46.4%) were IS2404-based nested PCR positive. Of which 204 were males, and 223 were females. The patients' mean age was 35.7 ± 19.94 , with 172 (18.7%) being children ≤ 15 years, while 748 (81.3%) were ≥ 15 years. During the study period, the highest number of samples, 171 (18.6%) and 170 (18.5%) were received from Cross Rivers and Delta states, respectively. In contrast, the least number of samples, 2 (0.2%) and 3 (0.3%), came from Ekiti and Lagos states, respectively.

Conclusion: This is the first in-country PCR confirmed diagnosis of a large cohort of BU patients. The results show a high prevalence in southern Nigeria, which is an indicator of high transmission. Our findings suggest the need for a prompt intervention by the government by providing the needed health facilities and education for the communities.

Keywords: *Mycobacterium ulcerans*, IS2402-Based Nested PCR, Confirmatory laboratory diagnosis

1. INTRODUCTION

Mycobacterium ulcerans, the causative pathogen of Buruli ulcer disease (BUD), ranks third among the most common mycobacteria infection worldwide, after tuberculosis and leprosy [1]. Though in some affected communities in the west and central Africa, BUD incidence rates surpass the other two [2]. With early cases first described in Buruli county of Uganda [3], the World Health Organization (WHO) in 1998 declared Buruli ulcer an emerging skin disease of public concern. The disease has now been reported in over 33 countries in Africa, Asia, America, and West Pacific [1], with most cases currently in West Africa [4].

Buruli ulcer is a disfiguring and occasionally disabling cutaneous disease characterized by chronic necrosis of subcutaneous tissue, with bones affected in some cases. At the onset of infection, it appears as a painless insect bite or pimple which progresses slowly destroying subcutaneous tissues leading to an extensive skin peel off, then the characteristic ulcer with undermined edges [5]. The cytotoxic and immunosuppressive activities of mycolactone, a polyketide toxin produced by *M. ulcerans* is responsible for the necrosis of tissue which distinguishes it from other mycobacteria. This has been suggested as a possible diagnostic target but for its lipid nature, making it poorly immunogenic [6].

M. ulcerans is an environmental bacterium often found in swampy and humid areas with stagnant lakes or slow-flowing streams in tropical and subtropical regions globally [7,8]. However, some cases have been reported without exposure to wetlands. The establishment of a specific mode of transmission to humans remains elusive, with many theories proposed. However, established risk factors include; living in BU endemic areas, previous trauma to the skin, and lack of wearing protective footwear [9, 10]. *M. ulcerans* have been found in aquatic bugs and mosquito, fish, aquatic plants, and *Acanthamoeba* species, but no conclusion about the transmission mode has been drawn yet [11, 12, 13].

The first time Nigeria notified WHO of BUD cases was in 2006, after a collaborative assessment by a team from Benin republic and WHO, which investigated the BUD situation in the country with the aim of establishing endemic areas [14]. Historically, Gray et al in 1967 described the first cases of BUD in Nigeria in four patients residing in Benue state. Then in 1976 another 24

patients were described around Ibadan in Oyo state [15]. Since then, over two decades passed before investigations on BUD picked up again in the country between 1998 and 2000 when samples from the Leprosy and Tuberculosis Hospital in Moniya-Ogoja, Cross-Rivers state, were sent to the Institute of Tropical Medicine in Belgium for PCR confirmation.

Diagnosis of BUD is currently done using four methods: PCR, usually IS2404 amplification, microscopy (ZN stain) for acid-fast bacilli detection, culture for the isolation of viable organisms, and histopathology. The IS2404 PCR is the gold standard, and WHO recommends that at least 70% of reported cases be confirmed using it in any survey [16]. After the 1998 International Conference on Buruli ulcer Control and Research in Yamoussoukro, Cote d' Ivoire [17], many endemic countries kicked-off their national control programmes. Unlike other nations, the BUD control programme in Nigeria is in its infancy and is yet to cover all endemic areas [17]; hence there is a gross information dearth about BUD among the populace and some healthcare practitioners. The programme suffers inadequate health infrastructure, low surveillance, under-reporting, and poor measures geared towards prevention and control [18]. This situation led to Nigerians seeking medical assistance in neighbouring countries [19]. The lack of health infrastructure in the country includes the non-existence of a standard laboratory for PCR diagnosis of BUD. Hence, this study was embarked upon to assist the national control programme to overcome this challenge.

Most of the cases reported in the country had limitations, including using a purely descriptive approach, with most diagnoses being retrospective or prospective based only on clinical presentation [20, 21]. This led to the speculation that BUD may be underdiagnosed hence under-reported in Nigeria compared to its two endemic neighbours on the east (Cameroon) and west (Benin republic). Therefore there is an urgent need to establish a PCR diagnosis laboratory to address this speculation, hence the need for this study.

2.0 METHODOLOGY

2.1 Study Population

From January 2016 to June 2018, samples were received from fifteen states from Southern Nigeria, with a few

coming from the Federal Capital Territory (FCT), the country's central zone. The states were; Abia, Akwa Ibom, Anambra, Bayelsa, Cross Rivers, Delta, Ebonyi, Ekiti, Enugu, Imo, Lagos, Ogun, Ondo, Osun, and Rivers. A total of 920 samples from BUD suspected cases were screened during the study period.

2.2. Study design

The study was cross-sectional and community-based. Community Health Workers (CHWs) from the states were trained on clinical sample collection, storage and transportation to the laboratory. Before sample collection, there was outreach and sensitization of the communities, including distribution of information, education and communication (IEC) materials, interactive talks, and occasionally video shows. Patients were interviewed, and their details recorded before samples were collected. Trained CHWs took a swab and Fine Needle Aspiration (FNA) specimens. Collected samples were stored in appropriate transport media and sent to the laboratory for analysis.

2.3. Ethics Statement

Approval for the study was obtained from the Nigerian Institute of Medical Research, Institutional Review Board (IRB) (NO: IRB/15/314). The project was done in collaboration with the national BU control programme. Approval and participants' consent was also obtained at the state level where samples were received.

2.4. Sample preparation and DNA isolation

Specimens received on swab sticks were cut to size to fit into 2ml tubes, then 500µl of phosphate-buffered saline (PBS) was added and mixed vigorously for 5 minutes. One hundred microlitres of the solution was aliquoted into another sterile screw cap tube ready for DNA extraction. Fine Needle Aspirates (FNA), if dry on arrival, 200µl of PBS was added and mixed for 5 minutes, and 100µl aliquoted to sterile tubes. If FNA was in alcohol on arrival, it was centrifuged, the supernatant decanted, and 200µl of PBS added and mixed briefly, and 100µl aliquoted to a sterile tube, ready for DNA extraction.

For DNA extraction, Ethanol-Sodium Hydroxide (EtNa) {200mM NaOH, 61% ethanol and 2.25mM EDTA} extraction protocol was used. Briefly, to 100µl of the bacterial suspension in PBS, 455µl of EtNa DNA extraction reagent was added and mixed briefly. The mixture was

then heated at 95°C for 10 minutes, spun at 16,000 rpm (using MIKRO 200, Hettich Zentrifugen centrifuge) for 10 minutes, and the supernatant was removed. The pellet was re-suspended in 100µl of DNA suspension solution. One-tenth volume of Sodium Acetate (3M, pH 5.2) was added and mixed, then 2X the volume of ice cold 100% ethanol was added and vortex for 10 seconds. It was then incubated overnight at -20°C. After the incubation, it was centrifuged at 13,000 rpm for 30 minutes, the supernatant discarded, rinsed by adding 250µl of ice-cold 70% ethanol, and centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded, and the pellet dissolved in 50µl of TE and used for PCR.

2.5 DNA amplification

10µl of the extracted DNA was amplified in a 20µl reaction mixture containing 25pmol of each primer (pGp1 and pGp2), 1X FIREPol® Master mix ready to load, FIREPol® DNA Polymerase and 5X reaction buffer (0.4M Tris-HCl, 0.1M (NH₄)₂SO₄, 0.1% w/v Tween-20, 7.5mM MgCl₂, 1mM dNTPs, Blue dye: 3.5-4.5 kb DNA fragment, Yellow dye: 35-45bp DNA fragment, a compound that increases sample density for direct loading). Primers used directed at IS2404 were pGp1 (5'-AGGGCAGCGCGGTGATACGG 3') and pGp2 (5'-CAGTGGATTGGTGCCGATCGAG 3'), with a thermocycling profile thus: denaturation at 95°C for 15 minutes; 40 cycles of 94°C for 30 seconds, 64°C for 1 minute, and 72°C for 1 minute 30 seconds and final elongation at 72°C for 10 minutes. The heated lid function was used at 105°C throughout the cycling time. Before the following step of the nested PCR, the status of the negative and positive controls was confirmed through agarose gel electrophoresis as indicated by the absence/presence of 549-bp amplicon, which was a fragment indicating the presence of *M. ulcerans* genomic DNA. For the nested PCR, 3µl of the first amplicon was used in a 20µl reaction mixture with primers pGp3 (5'-GGCGCAGATCAACTTCGCGGT-3') and pGp4 (5'-CTGCGTGGTGCTTTACGCGC-3'). Other reaction components were added at similar concentrations with the first PCR. The thermocycling profile was also identical with the first except that the number of cycles was changed to 35.

2.6. Gel electrophoresis

Two percent agarose gel in 0.5X TBE buffer was prepared with ethidium bromide. Ten microliters of each

PCR product were loaded into the wells of the gel. On a row on the gel were a molecular weight standard (marker), positive and negative controls and test sample amplicons. The gels were run at 100V for 1hr and were viewed in a photo documentation chamber (Clinx, USA) using UV light and captured on a laptop. Any 217-bp fragment appearing from the second PCR round was considered positive for *M. ulcerans* [22].

2.7. Statistical analysis

Categorical variables were summarized using frequencies. A Chi-squared test evaluated proportions of infection based on gender, age group, and states. Data analysis was done using SPSS 23.0

3. RESULTS

A total of 920 specimens were received during the study period (January 2016 – June 2018). The samples included those collected from microscopically positive and negative patients and also from clinically suspected cases. The demographic characteristics are shown in Table 1. Patients' distribution based on sex showed that 458 (49.7%) were females while 462 (50.2%) were males, indicating almost a balanced sex distribution. The infection rates were significantly different ($p < 0.01$) between gender, with females being more infected. Patients older than 15 years were 748 (81.3%), while 172 (18.7%) were children ≤ 15 years. Infection rates were also significantly different between the age groups ($p < 0.05$), with age groups > 15 yrs having more infection.

Table 1: Demographic characteristics of patients infected with *M. ulcerans*

Variable	Infection rate		Chi-squared	p-value
	n	(%)		
Gender				
Female (n = 458)	223	48.7	181.7	0.000*
Male (n = 462)	204	44.2		
Age group				
<15 years (n= 172)	99	57.6	17.94	0.022*
16-25 years (n= 186)	80	43.0		
26-35 years (n= 113)	54	48.2		
36-45 years (n=151)	59	39.1		
>45 years (n= 298)	135	45.3		
Total	427	100.0		

* $p < 0.05$ is considered significant

During the study period, the highest number of samples; 171 (18.6%) and 170 (18.5%) were received from Cross Rivers and Delta states, respectively. While the least number of samples; 2 (0.2%) and 3 (0.3%) came from Ekiti and Lagos states respectively, as shown in Figure 2

Figure 1. Distribution of *M. ulcerans* by states.

A total of 427 (46.4%) out of 920 samples received were positive for *M. ulcerans* by PCR. Out of which, 204 were males, and 223 were females. An example of a gel showing both positive and negative samples is shown in Figure 2. In the age group ≤ 15 years, a total of 99 (57.6%) out of 172 specimens were positive. While in ages above 15 years, out of 747 samples, 329 (44%) were positive. Age group > 45 years had the highest number (298; 32.4%), of samples, of which 136 (45.6%) were positive (Table 1).

Figure 2. PCR for *Mycobacterium ulcerans* in clinical samples. Gel picture showing positive and negative results after second PCR. Lane 1 = 100bp marker, lane 2 = positive sample, lanes 3-6 = negative samples, lanes 7-10 = positive samples, lane 11 = negative sample, lane 12 = positive control, lane 13 = negative control.

4. Discussion

Buruli Ulcer Disease (BUD) is a global health issue due to the pain, deformation, and gross economic burden on patients' families. In our study, out of the 920 specimens

received, almost half, 427(46%), were positive for *M. ulcerans*. Out of this number, 204 were males while 223 were females. This 46% prevalence recorded agrees with previous studies, which also showed that Southern Nigeria was BUD endemic [14, 19]. Females were more infected than males (48.7% and 44.2%, respectively), which is in disagreement with reports elsewhere in Africa [23] and Japan [24]. Such gender variation has been attributed to differential exposure to *M. ulcerans* and immune responses [23, 25]. Many studies have suggested that age group <15 years are mostly affected by BUD in sub-Saharan Africa [19, 23, 26, 27], but this is not in line with our study. Only 19% (172) were under 15 years, which is the same as what was recorded in a study in Japan [24]. Over 80% of our study patients were aged ≥ 15 years, with most samples coming from the age group ≥ 45 years (32.4%). A study by Ayelo et al, [28], also showed a similar outcome, but it cannot be generalized that most age group ≥ 15 years are affected by BUD in Nigeria because other studies had contrary reports [19].

All samples examined were from the southern part of the country, except Abuja (in the central region). The southern region has similar climatic conditions with other BU endemic areas of sub-Saharan Africa, a tropical rainforest type. Other studies have also shown that this region is endemic [29, 30]. Northern Nigeria is characterized by a tropical dry climate which has not been reported to be associated with BUD endemicity. The highest numbers of samples were from Cross Rivers (171) and Delta (170) states. Going by their names literally, these are areas with lots of water bodies and marshy environments known to support BU presence. Ekiti (2) and Lagos (3) had the lowest number of samples attributed to a lack of proper awareness of BU by the populace.

This study reports the first in-country nested PCR confirmed BU cases of a large cohort of patients. Before now, the national control programme cooperated with the TB and Leprosy control program called "National Tuberculosis, Buruli ulcer, and Leprosy Control Programme (NTBLCP)", sending samples of suspected cases to a WHO laboratory in Antwerp, Belgium, for PCR confirmation. This consumed a lot of time, leading to treatment delays, and required a lot of logistics. The IS2404 PCR for BU used in this project is considered the gold standard diagnostic tool [31]. It has high specificity

and sensitivity with low detection limits [32-34].

4.1. Conclusion

This study has shown that southern Nigeria is endemic for BU, which agrees with other previous studies [14, 19] and supports the notion that BU's prevalence in Nigeria might be more than previously thought. The outcome of this study stresses the need for more government involvement in providing the needed health facilities to help alleviate the agony of BU patients.

Declaration of conflict of interest

The Authors declare that there is no conflict of interest.

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Authors Contribution

VPG contributed to study design, performed data collection, analysis and wrote the manuscript. **TW** performed data collection and analysis. **SIA** contributed to study design and data analysis. **AO** performed data collection and analysis. **OPA** conceived, designed and supervised the work. All authors approved the final version of the manuscript

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