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Research Article

Six-Year Retrospective Analysis of Buruli Ulcer Diagnosis via Real-Time PCR at the Nigerian Institute of Medical Research

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ABSTRACT

Buruli ulcer, caused by Mycobacterium ulcerans, is a neglected tropical disease that affects the skin resulting in serious disfigurements and long-term disability. The World Health Organization has set at least 70% Polymerase Chain Reaction confirmation rate for countries to meet. The Control Program in Nigeria faced significant challenges due to lack of in country molecular confirmation laboratories. Nigerian Institute of Medical Research, supported by WHO/TDR, established a unit in 2016 for the molecular diagnosis laboratory which assists the national program and eventually joined the Buruli Ulcer Laboratory Network in Africa. Clinical samples from 18 states and the Federal Capital Territory were analyzed between 2018 and 2023. Sample collection was predominantly done using dry swabs (92%), from which DNA was extracted using Ethanol -Sodium Hydroxide DNA extraction solution and Genolyse kit. The DNA was screened using qPCR, targeting the IS2404 sequence. Out of the 1386 samples screened only 58 (4.2%) were positive, indicating low prevalence. More male samples were screened (52.4%) as compared to women (47.6%). Most of the samples came from the age group 21-40 years (32%) ($P<0.001$). Infection rates in males and females were not significantly different ($p>0.05$), but age group < 50 years were significantly more infected than those above ($p<0.001$). Most samples were from southern Nigeria. The low number of samples from states and prevalence calls for more governmental sensitization efforts at the grassroots level. The Buruli Ulcer Laboratory Network in Africa ensures reliability of results from member countries, hence the need for inclusion of more countries.

Keywords: Buruli ulcer; *Mycobacterium ulcerans*; Real time PCR; IS2404 sequence; Control program; Nigerian Institute of Medical Research

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INTRODUCTION

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* (M. *ulcerans)*, is one of the skin-neglected tropical diseases. It is ranked third in the family of mycobacterial diseases in humans after tuberculosis and leprosy (De Souza *et al.,* 2012; Mitjà *et al.,* 2017). BU can cause huge skin lesions as well as underlying tissue lesions, which can result in serious disfigurements and long-term disability (Boccarossa *et al.,* 2022; Johnson *et al.,* 2005). The

exact mode of transmission is still not clear; however, studies have shown the possibility of distinct routes of transmission occurring in different geographic regions and epidemiological settings (Sakyi *et la.,* 2016), but there is no evidence so far suggesting that human-to-human transmission is possible (Leuenberger *et al.,* 2022; Yotsu *et al.,* 2018).

Buruli ulcer starts with a pre-ulcerative stage that is characterized by a hard, non-tender nodule, edema, or plaque with broad areas of indurated skin. The ulceration results from severe death of skin cells, which causes the edges to become generally undermined (Sakyi *et la.,* 2016). If left untreated, it can result in stigmatization, exclusion, impoverishment and ultimately mortality of affected individuals. Although there is a low mortality rate, morbidity and ensuing functional disabilities can be severe (Schunk *et al.,* 2009).

The main virulence factor causing the Buruli ulcer's pathophysiology is mycolactone, a cytotoxic and immunosuppressive macrocyclic polyketide. It has been suggested that this marker could be used to diagnose Buruli ulcers because it is widely present in human lesions that are infected (Sakyi *et al.,* 2016). The virulence factor is not exclusive to *M. ulcerans*, it is present in other mycobacteria, each exhibiting a distinct congener (Yotsu *et al.,* 2018). Presently, two methods are used to affirm a clinical confirmation of BU: Ziehl-Neelsen staining for identifying acid-fast bacilli and quantitative polymerase chain reaction (qPCR) for detecting pathogen-specific DNA (Marion *et al.,* 2022).Among the two methods, qPCR targeting IS2404 is the gold standard for confirmation of BU clinical samples (Marion *et al.,* 2022; Lavender *et al.,* 2013), and therefore before the commencement of BU treatment WHO recommends PCR confirmation. BU samples are obtained either through swab sticks or fine needle aspirates (FNA) depending on the stage of the infection. FNA are usually from non-ulcerative lesions like plaques, nodules, and edemas, while swabs are sampled from undermined edges of the ulcer (Eddyani *et al.,* 2009; Cassisa *et al.,* 2010).

Buruli ulcer cases have been reported in tropics and sub-tropical regions in the world especially in Africa, the Americas, Asia, and the Western Pacific (Ukwaja *et al.,* 2016). It is endemic in West and Central Africa (Omansen *et al.,* 2019; Pluschke *et al.,* 2019), usually found among individuals residing near stagnant water and low-lying wet fields that are either inundated or floodable (Kenu *et al.,* 2014; Aboagye *et al.,* 2017). Although there appears to be a decrease in BU cases, probably due to underdiagnosis or underreporting (Yotsu *et al.,* 2018), recent reported cases came come from countries bordering the Gulf of Guinea in West Africa including Benin, Cote d'Ivoire, Ghana, Guinea, Liberia, Nigeria, Sierra Leone, and Togo (Boccarossa *et al.,* 2022). Populations generally affected by BU in West and Central Africa have either restricted or no access to water treatment facilities or clean drinking water (Marion *et al.,* 2011; Bratschi *et al.,* 2013; Degnonvi *et al.,* 2019). This disease infects individuals of any age and gender, but it is more pervasive in children younger than fifteen; accounting for half of the new cases in endemic regions in Africa (Yotsu *et al.,* 2015). Some studies showed women to be more infected than men (Vincent *et al.,* 2014), while the reverse is the case in others (Kenu *et al.,* 2014). The closeness to stagnant water or slow-following water bodies has been shown to be a risk factor for Buruli ulcer, also there is a possible association between aquatic insects and transmission of *M. ulcerans* (Boccarossa *et al.,* 2022; Johnson *et al.,* 2005; Ukwaja *et al.,* 2016).

Nigeria, despite sharing borders with highly endemic countries, reported cases of BU have declined. In 1967, Benue was the site of primary known cases of BU (Gray *et al.,* 1976). In 1976, twenty four cases were reported in Ibadan (Oluwasanmi *et al.,* 1976), while in 2006, fourteen clinically thought to be BU cases were recorded (Chukwuekezie *et al.,* 2007). From 2006 to 2012, nine types of *M. ulcerans* strains were isolated from patients in Oyo, Anambra, Cross River, Enugu, Ebonyi, and Ogun states (Vandelannoote *et al.,* 2014). All PCR confirmations were performed in neighboring countries due to the continued absence of in-country PCR diagnosis/confirmation for BU cases. The National BU Control Program faced significant challenges due to the time, cost, and efforts required to ship clinical samples to the WHO Collaborating Centre in Belgium for PCR confirmation. Consequently, there was a pressing need for standardized, well-equipped laboratories, and trained scientists within the country for timely PCR confirmation of BU cases, which would reduce diagnosis delays and ensure prompt treatment of patients.

In view of this, Molecular Parasitology Research Laboratory (MPRL), at Nigerian Institute of Medical Research (NIMR), established a BU molecular diagnosis laboratory in 2016. Prior to qPCR for BU diagnosis in the laboratory, nested PCR was used, which was time consuming and prone to contamination. MPRL changed to quantitative PCR (qPCR) for BU diagnosis in December 2018 and has been maintained till date. The laboratory is a member of the Buruli Ulcer Laboratory Network (BULABNET) in Africa and participates in their annual external quality assessment programs

(EQA), of which the results have been very good. This report gives a retrospective analysis of M. *ulcerans* diagnosis at MPRL during the specified period.

MATERIALS AND METHODS

Study Area

Southern Nigeria has thick vegetation with lots of water bodies and marshy environments and a tropical rainforest type of climate, while northern Nigeria is characterized by less vegetation with tropical dry climate.

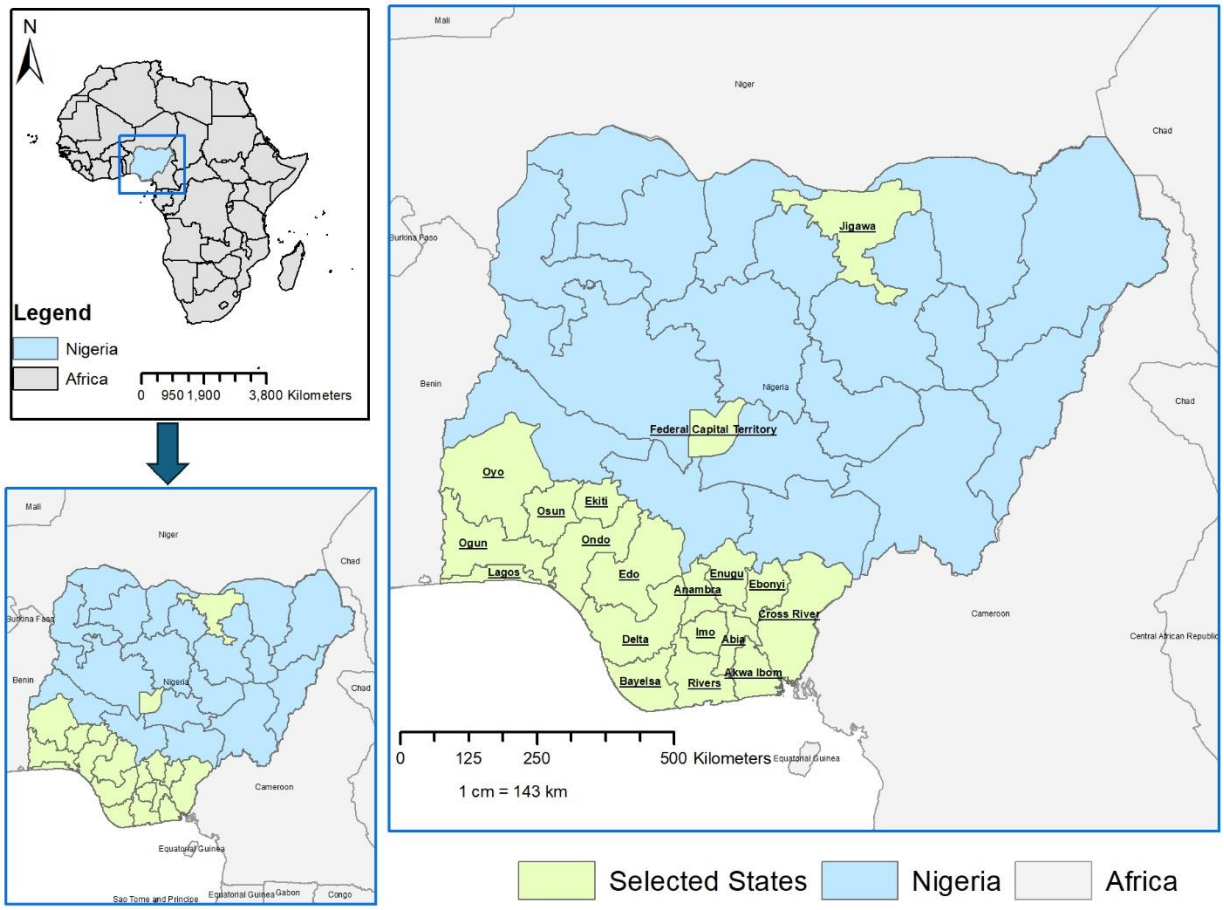

Fig. 1 Map showing states where samples were collected

Sample preparation

All samples were transported to the laboratory on ice packs, adhering to the Standard Operating Procedure of the BU-LabNet (Marion *et al.,* 2022). They came either on swab sticks or as Fine Needle Aspirates (FNA). Specimens that were received on swab sticks, were first cut to size to fit into 2ml tubes, then 500 ml of PBS was added and the tubes were vortexed vigorously for 5 minutes. 100ul of the solution was then aliquoted from each tube into new sterile screw cap tubes ready for DNA extraction. For samples that came as Fine Needle Aspirates (FNA), if dry on arrival, 200ul of PBS was added and vortexed for 5 minutes, then 100ul aliquoted to sterile tubes. If FNA was in alcohol on arrival, it was centrifuged, the supernatant decanted, then 200ul of PBS was added and mixed briefly, after which 100ul was aliquoted to sterile tubes and stored in a refrigerator waiting for DNA extraction.

DNA extraction

Two DNA extraction methods were used: EtNa DNA extraction solution, and Genolyse kit (Ref 51610, Hain LifeScience). The EtNa (Et for ethanol and Na for NaOH) DNA extraction solution was an in-house method used prior to the introduction of Genolyse kit by BU-LabNet Africa. The procedure starts with the addition of 455ul of EtNa DNA extraction solution to 100ul of specimen suspension prepared. It was mixed briefly, heated at 95oC for 10 minutes, and spun at 16,000 x g for another 10 minutes. The supernatant was discarded, and the pellet resuspended in 100ul of DNA suspension solution (Vingataramin and Frost, 2015).

The Genolyse kit procedure starts with taking 400 µl of specimen suspension prepared, then

centrifuging at 12000 g for 15min. The supernatants were discarded, each pellet was re-suspended in 400 µl of sterile water, centrifugation was repeated, and the supernatant was also discarded. Thereafter, 50 µl of A-LYS buffer was added to each pellet and incubated at 95°C for 10 minutes, then centrifuged for 10 seconds to pellet the suspension before 50 µl of buffer A-NB was added to neutralize the A-LYS buffer. Negative controls (sterile water) were added at a frequency of 10% (1 control per batch of 10 extractions) to monitor potential cross-contaminations among the samples. (World Health Organization, 2020)

Mycobacterium ulcerans IS2404 qPCR assay

The assay detects *M. ulcerans* DNA (IS2404) from clinical samples or culture suspension. 2µl of extracted DNA templates were amplified in 18ul reaction mixture in 96 well multiplates containing primers, Taqman MGB Probe and TaqMan Fast Advances Master Mix. Primers used directed at IS2404 were ULC5 (5'GTCGCCGAGAAAAGCAATGA'3) (Applied Biosystems UK) and ULC6 (5'GACTTCAAGGTGGCGCAGAT'3), the Taqman MGB Probe was ULCS01 (5'FAM-ATGCGATGCATACCCA-MGBNFQ'3) (Applied Biosystems UK). The thermocycling profile was thus: 1 cycle of UNG activation at 50°C for 2 minutes, 1 cycle of Polymerase activation at 95°C for 15 minutes; 40 cycles of denaturation at 95°C for 15 seconds, Annealing & Extension at 60°C for 60 seconds; activating detection of channel FAM for all wells following each cycle after step 4. A Bio-Rad CFX96 real-time PCR detection system was used for analysis. Any sample with FAM signal positive and Cq Value <40 was interpreted as positive for M. *ulcerans* DNA (IS2404).

Ethics Statement

Nigerian Institute of Medical Research, Institutional Review Board (IRB) (NO: IRB/15/314) gave approval for this study. The national BU control program collaborated in the study and approval was also obtained from the participating states.

RESULT

A total of 1,386 samples from patients were received in MPRL between December 2018 and November 2023, the study period. Gender distribution shows a slightly higher representation of males (52.4%) compared to females (47.6%) (Table 1). Participants were distributed across various age groups with the highest percentage in the 21-40 age group (32%). Most of the samples were dry swabs (92%). Out of the 1386 samples screened only 58 (4.2%) were positive, indicating low prevalence in the 18 states from which samples were received and the Federal Capital Territory (FCT)

Table 2 shows the distribution of samples by state in the country. Ebonyi has the highest representation with 14.9%, followed by Bayelsa (8.8%) and Akwa-Ibom (8.2%). States like Ondo, Edo, and FCT have lower percentages, each below 2%.

Table 3 presents infection rates according to variables (Gender, Age Group, Sample Type) Chi-square test showed no significant association between gender and age groups with Buruli ulcer infection ($p>0.05$), Although infection is significantly more in age groups less than 50 years ($p<0.001$). There was also a significant association between type of sample collected (dry swab or FNA) and infection rates ($p<0.05$).

Table 1: Distribution of samples received based on gender, age and sample type

Variable	Frequency (N)	Percent (%)
Gender		
Male	726	52.4
Female	660	47.6
Age group		
Less than 20	305	22
21 - 40	444	32
41 - 60	414	29.9
61 - 80	202	14.6
81 and above	21	1.5
Sample Type		
Dry swab	1275	92
FNA	111	8

Table 2: Distribution of samples received by states

State	Frequency	Percent
Abia	63	4.5
Akwa-Ibom	114	8.2
Anambra	34	2.5
Bayelsa	122	8.8
Cross River	94	6.8
Delta	85	6.1
Ebonyi	206	14.9
Edo	22	1.6
Ekiti	23	1.7
Enugu	76	5.5
Imo	96	6.9
Jigawa	39	2.8
Lagos	67	4.8
Ogun	95	6.9
Ondo	13	0.9
Osun	68	4.9
Oyo	41	3
Rivers	98	7.1
FCT	30	2.2

Table 3: Infection rates in the study

Variable	Number (n)	Positive	chi-square	P-value
Gender				
Male	696	30	0.01	0.92
Female	632	28		
Age group				
< 50	920	55	17.39	0.001
> 50	408	3		
Sample type				
DRY SWAB	1217	58	5.27	0.02
FNA	111	0		

DISCUSSION

Buruli ulcer, just like other neglected tropical diseases (NTD) has been overlooked for a long time but is now attracting global attention due to the pain, deformation and huge economic burden the disease places on families of patients. Accurate diagnosis of BU is key in the struggle to eliminate this NTD, which prompted the World Health Organization (WHO) to set at least a 70% Polymerase Chain Reaction (PCR) confirmation rate as a target for countries to meet, of which most are yet to achieve (WHO, 2020). This retrospective study shows a BU prevalence of 4.2% during the period studied (December 2018 to November 2023) when qPCR was used, which is much lower than the 46.4% prevalence reported in the same laboratory when Nested PCR was used (January 2016 to June 2018) (Gyang *et al.,* 2021). This difference could most likely be due to the different methods used in diagnosis. Such different results from different laboratories in different countries led to the formation of a solid network of Buruli ulcer laboratories in October 2019, with an acronym; the BU-LABNET, having representatives from 11 laboratories of 9 endemic countries (Benin, Cameroon, Ivory Coast, Ghana, Democratic Republic of Congo, Togo, Liberia, Nigeria and Gabon) and external experts (World Health Organization, 2020). One of the major objectives of the network is the harmonization of standard operating procedures, which led to qPCR being recommended for all BU laboratories within the network. The Molecular Parasitology Research Laboratory (MPRL) in NIMR is now a reference laboratory for BU diagnosis in the country and works in collaboration with the National Tuberculosis, Buruli ulcer and Leprosy Control Program (NTBLCP).

Many predisposing variables have been considered as risk factors for BU infection, but no consensus yet reached (Fevereiro *et al.,* 2019; Jacobsen *et al.,* 2010; Röltgen *et al.,* 2019). Gender and age are some factors considered since the disease is commonly seen among children, particularly males,

and in the elderly, as frequently reported (Vincent *et al.,* 2014; Marion *et al.,* 2015; Ayelo *et al.,* 2018). In this analysis of data, there was no significant difference between infection rates in males and females which with other studies (Röltgen *et al.,* 2019), while the elderly (>50 years and above) were significantly less infected (table 3). Since most of our samples came on swab sticks and not as Fine Needle Aspirates (FNA), analysis showed swab sticks correlating significantly with infection, which disagrees with Eddyani *et al.* (2009), and Fajloun *et al.* (2023), who showed Fine Needle Aspiration (FNA) to be a better sample type for BU confirmation due to its positivity rate, even though swabs were the most frequently tested.

The spread of received samples still showed that almost all samples came from southern Nigeria, with only a few from the north, as reported in our previous publication, reaffirming the endemicity of the region due to its tropical rainforest type of climatic conditions that allow for wet and marshy environments that have been attributed to BU infection (Gyang *et al.,* 2021). The highest numbers of samples came from Bayelsa (122) and Akwa-Ibom (114) states. These are states that have large numbers of water bodies and marshy environments known to support the spread of BU. Ondo (13) and Edo (22) states had the lowest number of samples sent to the laboratory in NIMR, this could be attributed to a lack of proper awareness of BU by residents and probably the environment having fewer water bodies as compared to the highest two.

CONCLUSION

This study showed that with the adoption of real-time PCR in diagnosing BU by members of BULABNET, the prevalence dropped. Indicating that previous methods of diagnosis might have been prone to contamination resulting in false positive cases. Most suspected cases are still from southern Nigeria, reaffirming the endemicity of the region due to its environmental and climatic conditions. We recommend that suspected cases that turn out to be negative for BU should be further screened for other skin NTDs like yaws, which in some cases appear similar to BU, and there is the need for more support from the Ministry of Health to help in alleviating the pains and sufferings of patients and their families.

Declaration of Conflict of Interest

The Authors declare that no conflict of interest could have influenced the work presented in this study.

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Authors' Contribution

GPV contributed to the study design, conducted molecular analysis, collected data, and wrote the manuscript. NT was responsible for conducting molecular analysis, data collection, and analysis and contributed to writing the manuscript. OKA participated in the study design and data analysis. NM was involved in data collection. EI contributed to the study design. QL handled data analysis. TO participated in both the study design and analysis. APO conceived the study, designed the methodology, and supervised the entire work. All authors approved the final version of the manuscript.

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