Molecular Detection of Buruli Ulcer among Patients with Various Degrees of Skin Ulcerative Lesions Revealed Zero Prevalence in Parts of Jigawa State, Northern Nigeria

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Abstract: The indolent, necrotizing Buruli ulcer disease is a chronic condition caused by *Mycobacterium ulcerans*, an environmental bacteria that can damage the skin, tissues, and bones. It is now recognized as one of the 20 Neglected Tropical Diseases targeted for elimination by the year 2030. Public health continues to face difficulties due to its mechanism of transmission that is yet to be completely unravel. From December 2019 to September 2023, a hospital-based study was conducted to ascertain the prevalence of Buruli ulcer in parts of Jigawa State, Nigeria. A purposive sampling technique was adopted in selecting and diagnosing 382 samples from patients with various degrees of skin ulcerative lesions across all age groups and gender. The diagnosis of all the samples initially involved the use of Ziehel Nelseen staining procedure. Thirty-five (35) samples with symptoms suspected to be Buruli ulcer were diagnosed using real-time quantitative polymerase chain reaction (qPCR) method. The results were negative for the 382 samples diagnosed using Ziehel Nelseen staining procedure as well as the 35 suspected Buruli ulcer using real-time PCR. This study concludes that despite testing samples with obvious ulcerative lesions suspected to be Buruli ulcer from patients, both Ziehel Nelseen staining procedure and real-time PCR revealed negative results. It is recommended that more samples suspected to have symptoms of Buruli ulcers should be diagnosed using real-time PCR and also make use of both IS2404 and IS2606.

Key word: Public health, Buruli ulcer, *Mycobacterium ulcerans*, Neglected Tropical Disease.

INTRODUCTION

The bacteria *Mycobacterium ulcerans* is the aetiologic agent that causes Buruli ulcer (BU), a chronic, indolent, necrotizing cutaneous illness that is categorized by the World Health Organization (WHO) as one of the skin-related Neglected Tropical Diseases (NTDS) (Mitja *et al.*, 2017). After leprosy and tuberculosis, it is the most prevalent mycobacterial illness (Keragala *et al.*, 2020). Large ulcers, typically on the limbs, are the result of the infection's devastation of soft tissue and skin (Peetermans *et al.*, 2020). Clinically speaking, a BU begins as a papule, nodule, plaque, or oedematous lesion that gradually spreads to a large area of skin ulceration (Yotsu *et al.*, 2018). Surprisingly, considering the degree of tissue loss, the lesion typically causes little to no pain. Buruli ulcer is distinct from other mycobacterial infections in that the pathophysiology of the illness can be attributed to mycolactone, a lipid-like and diffusible exotoxin, rather than the bacterium (Yotsu *et al.*, 2018). In the endemic areas, Buruli ulcer is highly feared and stigmatized due to the unsightly abnormalities it leaves behind. It is also often linked to witchcraft and curses (Osei and Duker, 2015).

Currently, PCR of IS2404, IS2606, and ER is used to detect M. ulcerans DNA for BU diagnosis confirmation. BU can also be confirmed by histology, culturing, and microscopic identification of acid-fast bacilli in lesions. The goal of the World Health Organization is to have PCR confirmation for more than 70% of cases that are reported. (Yotsu *et al.*, 2018). The difficulty in

confirming cases persists because many endemic areas lack convenient access to facilities needed for these testing. New diagnostic tools are now being developed for case management as well as early case detection, with a focus on those that might be used in the field. The loop-mediated isothermal amplification (LAMP) test is one of them. (Ablordey *et al.,* 2012; Beissner *et al.,* 2015), applications of thin-layer chromatography in mycolactone detection (Wadagni *et al.,* 2015), and tests for antigen detection (Dreyer *et al.,* 2015).

At least 27 nations in Africa, Asia, South America, and the western Pacific region have patchy foci of Buruli ulcer (Joshi *et al.,* 2021). Most of the cases that have been recorded are from West and Central Africa (Singh, 2019). Earlier research had shown that Buruli ulcer is endemic in Nigeria, particularly among those who live close to bodies of water. However, most investigations were all conducted in Nigeria's southern regions. There is no documented study on Buruli ulcer in Jigawa State, Northern Nigeria, particularly among the people that live near water features. It is against this background that this study was conducted to determine the prevalence of Buruli ulcer in parts of Jigawa State, Nigeria.

MATERIALS AND METHODS

Study area: Jigawa State was created on 28th August, 1991 from the old Kano Stae, Nigeria. The state is situated between latitudes 11.00°N and longitudes 8.00°E to 10.15°E, and ies in the northwest part of Nigeria. It is bordered on the west by Katsina and Kano States, on the east by Yobe and Bauchi States, and on the north by the international boundary with the Niger Republic. As shown in Figure, 1 the state is divided into three senatorial Districts namely: Northwest, Central and Northeast. The study was conducted in secondary health facilities in Northwest and Central Senatorial districts. The state has two distinct seasons: the dry season, which runs from October to May, and the rainy season, which runs from June to September. The hot temperature during the rainy season is approximately 42°C, while the low temperature is 10°C (Okereke *et al.,* 2015). The state ranks eighth in terms of population according to the 2006 census.

The state has three types of health facilities: the primary health care which is under the purview of the Local Government, secondary health care is overseen by the State Government (the state has sixteen general hospitals and one specialty hospital), and tertiary health care is under the purview of the Federal Government and there is only one Federal Medical Center (Dogara and Ocheje, 2016; Makinde *et al.,* 2018). State-level health policy development and execution, as well as translation of Federal health policies for implementation, fall under the purview of the Ministry of Health and the Agency for Primary Health Care. A primary health center is intended to be available in every ward of the state, as the state currently functions under a single roof system (Makinde *et al.,* 2018). Patients identified with various degrees of ulcerative lesions are admitted into General Hospitals for care.

Six secondary healthcare facilities were selected, they include General Hospital Dutse, General Hospital Birnin Kudu, General Hospital Jahun, General Hospital Ringim, General Hospital Gumel and General Hospital Kazaure, as shown in Figure 1.

Study design: A cross-sectional hospital-based study was conducted. Two out of the three senatorial districts were selected for the study. The samples were collected from selected secondary healthcare facilities within the two senatorial districts of Northwest and Central of the state.

Sample size determination: The study adopted a purposive sampling technique in the selection of the patients whose samples were collected from the study population. A total of three hundred and eighty-two (382) skin related ulcer patients were screened. Specifically, thirty-five (35) samples were collected from patients who presented

symptoms mimicking Buruli ulcer for the molecular analyses (Portaels and World Health Organization, 2014). Samples were collected from the patients between December 2019 and September 2023.

Screening of samples for the detection of *Mycobacterium ulcerans*

Microscopic examination: Microscopic diagnoses by direct smear examination with Ziehl-Neelsen staining to detect the presence of acid-fast bacilli (AFB) was done using the quantification of smears by the method locally used for the diagnosis of tuberculosis (Portaels, 2001). The positive control sample was collected from the tuberculosis (Tb) positive stock at the Tb Unit of Dutse General Hospital, Jigawa State, Nigeria. Self-sputum was collected and smeared, stained, and subsequently used as the negative control. Using a sterile swab stick and fine needle aspirate (FNA), ulcerative lesions, papules and nodules were collected respectively from suspected patients and then smeared on a clean grease free slide using sterile technique. The smear was air dried, and heat fixed by passing through an open blue f lame. The smear was covered with the carbol fuchsin stain. The stain was heated until vapour rose for 5 minutes. The smear was washed with clean water and 3% v/v acid alcohol was applied to the smear for 5 minutes to decolorize the smear to pale pink colour. The smear was rinsed with clean water. Malachite green stain was applied to the smear and allowed to stain for 2 minutes. The stain was rinsed with clean water. Back of the slide was wiped to clean and then placed in the rack for the smear to air-dry. The smear was examined microscopically, using the ×100 oil immersion objective. Confirmed positive and Negative tuberculosis samples were used as controls. Negative Microscopy confirms the absence of acid-fast bacilli.

Molecular analyses: The analyses were performed at the Molecular Parasitology Research Laboratory of the Nigerian Institute of Medical Research (NIMR), Yaba Lagos State, Nigeria, a reference laboratory for Buruli ulcer diagnosis and research. Deoxyribonucleic Acid (DNA) extraction was performed by bacterial lysis with the use of Genolysis kit (reference; 51610. Hain LifeScience).

***Sample collection and processing*:** Thirty-three (33) swab and two (2) fine needle aspiration (FNA) samples were aseptically collected into collection tubes. Each tube was labelled for ease of identification. Subsequently each swab sample collected from lesion on the patients' limbs (upper or lower) was transferred into 15 ml conical tube, was rehydrated with 2 ml of sterile water and vortexed. This was left to stand for 5 minutes and re-vortexed to increase the concentration of the sample. The swabs were gently discarded from the 15 ml tube leaving the content. Thereafter, 400 µl content obtained from 2 ml swab content was measured and transferred into a 1.5 ml tube with screw cap for DNA extraction and purification. The FNA samples, were vortexed and a 400 µl each was taken and transferred into a 1.5 ml tube with screw cap for DNA extraction (Nigerian Institute of Medical Research, 2023).

Deoxyribonucleic acid (DNA) extraction and purification procedure: The DNA extraction was performed using the Genolysis kit (Hains Lifescience, Nehren, Germany). A 400 µl of specimen suspension was placed into 1.5 ml tubes with screw cap for each sample, Thereafter, tubes were centrifuged at 12000 g for 15 minutes at room temperature. The supernatants were carefully discarded using a P1000 filter tip leaving the pellets at the bottom of the conical tubes. The pellets were re-suspended in 400 µl of distilled water and centrifuged at 12000 g for 15 minutes at room temperature. The supernatant was carefully discarded, and the pellets were re-suspended in 50 µl of A-LYS buffer and incubated for 10 minutes at 95°C. The tubes were centrifuged for 10 seconds to pellet the suspension. Then, 50 µl of buffer A-NB was added and the tubes were kept at 4°C prior to PCR/amplification procedure (Nigerian Institute of Medical Research, 2023).

Real-time quantitative PCR (RT qPCR): The RT qPCR was performed as described by Fyfe and Lavender (2022). The method is recommended by the WHO for *M. ulcerans* detection (Bretzel and Beissner, 2018), based on primers IS2404TF and IS2404TR. The primers set employed for quantification is depicted in Table 1.

Quantitative PCR (qPCR)/amplification condition/procedure: The qPCR/amplification reaction was performed in a total volume of 20 µl reaction in Bio-Rad thermal cycler machine (Bio-Rad Laboratories, Marnes- la- Coquette, France). Five micro liter (5 µl) of 5x Hot fire Pol probe qPCR mix (Master mix) was added to 11.5 µl water (PPI), followed by 1.5 µl of forward primer and 1.5 µl of reverse primer including 0.5 µl template DNA. The qPCR amplification procedure is presented as follows: The sample was heated at initial denaturation temperature of 95°C for 10 minutes at 1 cycle, followed by final heating at 95°C for 15 seconds at 60°C for 1 minutes at 40 cycles. The detection was made through Taqman probe. The constitution of total reaction volume for the qPCR reaction is shown in Table 2.

Statistical analysis of data: Results of the analysis were converted to percentages and presented on tables.

Ethical approval: The study was conducted under human right ethical approval sought from Jigawa State Ministry of Health with reference number: MOH/SEC.3/S/819/1, prior to commencement. Study protocol was submitted for approval by the ministry. Permission was sought from the Medical Directors of all the six (6) hospitals where this research was conducted. Sample collection from the participants was conducted following verbal and documented informed consent by each of the participant.

RESULTS

Prevalence of Buruli ulcer in parts of Jigawa State, Nigeria

The observations from both microscopic examination (Ziehel Nelseen staining procedure) and molecular analyses (Real-Time PCR) which were all negative are shown in the Tables 3 and Table 4.

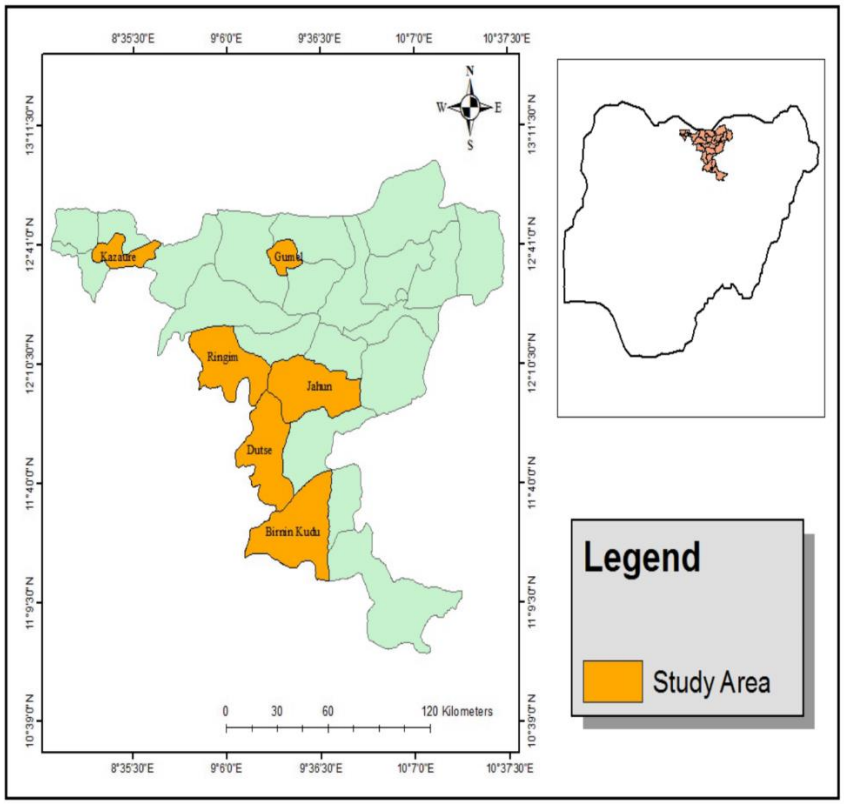

Figure 1: Map of the study area. Source: Geographic Information System (GIS) Unit, Department of Environmental Management and Toxicology, Federal University Dutse, Dutse, Nigeria

Table 1: Primer Set Information for IS2404 Gene

Primer	Primer Sequence (5′–3′)	Primer Length	Fragment Size (bp)
IS2404TF (5′)	AAAGCACCACGCAGCATCT	19	276
IS2404TR (3′)	AGCGACCCCAGTGGATTG	18	330

Fyfe and Lavender (2022)

Table 2: Constitution of total reaction volume for the qPCR reaction

Component	**Volume (µl)**
Master mix (5x)	5
F Primer	1.5
Reverse Primer	1.5
DNA template	0.5
Water	11
Total volume	20

Table 3: Prevalence of Buruli ulcer in relation to age group, gender and health facility

Variable	Number Tested	Number of Positive	Percentage Positive (%)
Age group (Years)			
0 – 5	13	0	0
6 – 11	48	0	0
12 – 17	127	0	0
18 and above	194	0	0
Gender			
Male	265	0	0
Female	117	0	0
Health Facility			
Birnin Kudu GH	67	0	0
Dutse GH	60	0	0
Gumel GH	60	0	0
Jahun GH	65	0	0
Kazaure GH	65	0	0
Ringim GH	65	0	0

Key**:** GH = General Hospital

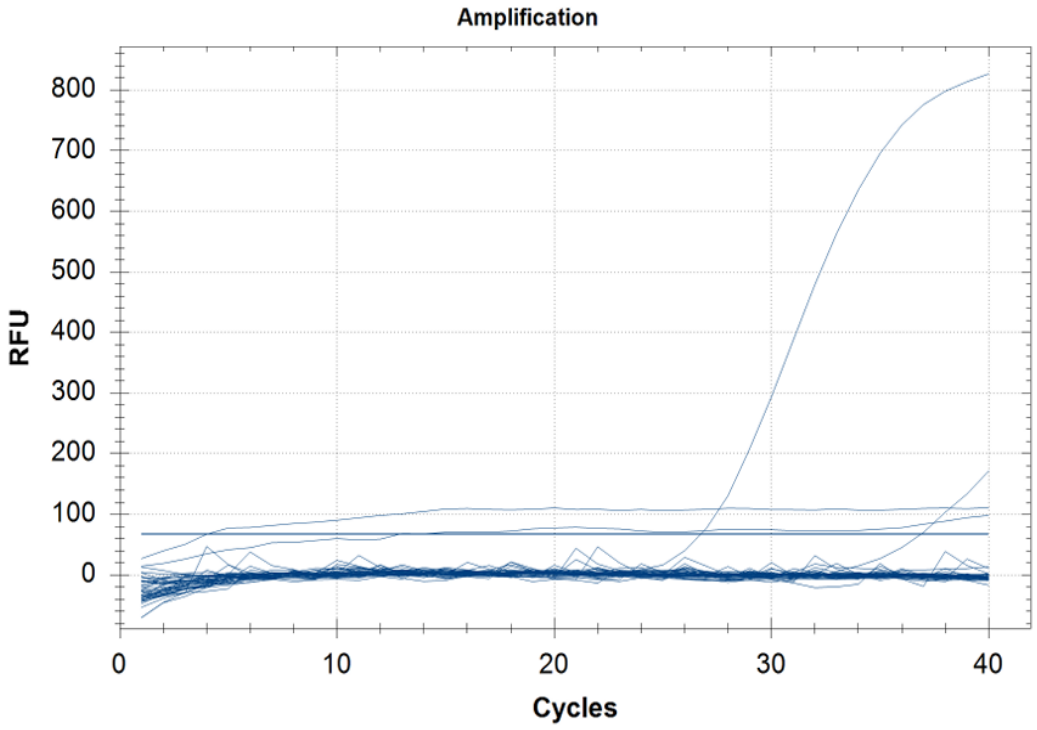

Figure 2: The qPCR amplification plot

Table 4: The qPCR Quantification Parameters

S/N	Sample ID	Type of Specimen	Target Gene	C_q	C_q Mean	PCR Status
1	3449	Swab	IS2404	N/A	0.00	Negative
2	3450	Swab	IS2404	N/A	0.00	Negative
3	3451	Swab	IS2404	N/A	0.00	Negative
4	3452	Swab	IS2404	N/A	0.00	Negative
5	3453	Swab	IS2404	N/A	0.00	Negative
6	3454	Swab	IS2404	N/A	0.00	Negative
7	3455	Swab	IS2404	N/A	0.00	Negative
8	3456	Swab	IS2404	N/A	0.00	Negative
9	3457	Swab	IS2404	N/A	0.00	Negative
10	3458	Swab	IS2404	N/A	0.00	Negative
11	3459	Swab	IS2404	N/A	0.00	Negative
12	3460	Swab	IS2404	N/A	0.00	Negative
13	3461	Swab	IS2404	N/A	0.00	Negative
14	3462	Swab	IS2404	N/A	0.00	Negative
15	3463	Swab	IS2404	N/A	0.00	Negative
16	3464	Swab	IS2404	N/A	0.00	Negative
17	3465	Swab	IS2404	N/A	0.00	Negative
18	3466	FNA	IS2404	N/A	0.00	Negative
19	3467	Swab	IS2404	N/A	0.00	Negative
20	3468	FNA	IS2404	N/A	0.00	Negative
21	3469	Swab	IS2404	N/A	0.00	Negative
22	3470	Swab	IS2404	N/A	0.00	Negative
23	3471	Swab	IS2404	N/A	0.00	Negative
24	3472	Swab	IS2404	N/A	0.00	Negative
25	3473	Swab	IS2404	N/A	0.00	Negative
26	3474	Swab	IS2404	N/A	0.00	Negative
27	3475	Swab	IS2404	N/A	0.00	Negative
28	3476	Swab	IS2404	N/A	0.00	Negative
29	3477	Swab	IS2404	N/A	0.00	Negative
30	3478	Swab	IS2404	N/A	0.00	Negative
31	3479	Swab	IS2404	N/A	0.00	Negative
32	3480	Swab	IS2404	N/A	0.00	Negative
33	3481	Swab	IS2404	N/A	0.00	Negative
34	3482	Swab	IS2404	N/A	0.00	Negative
35	3483	Swab	IS2404	N/A	0.00	Negative
36	Water	N/A	IS2404	N/A	0.00	Negative
37	Negative Control	N/A	IS2404	N/A	0.00	Negative
38	Negative Control	N/A	IS2404	N/A	0.00	Negative
39	Positive Control	N/A	IS2404	26.75	26.75	Positive

Key**:** N/A = Not Applicable; FNA = Fine Needle Aspirates; C_q = Cycle of quantification; ID = Identity; PCR = Polymerase Chain Reaction

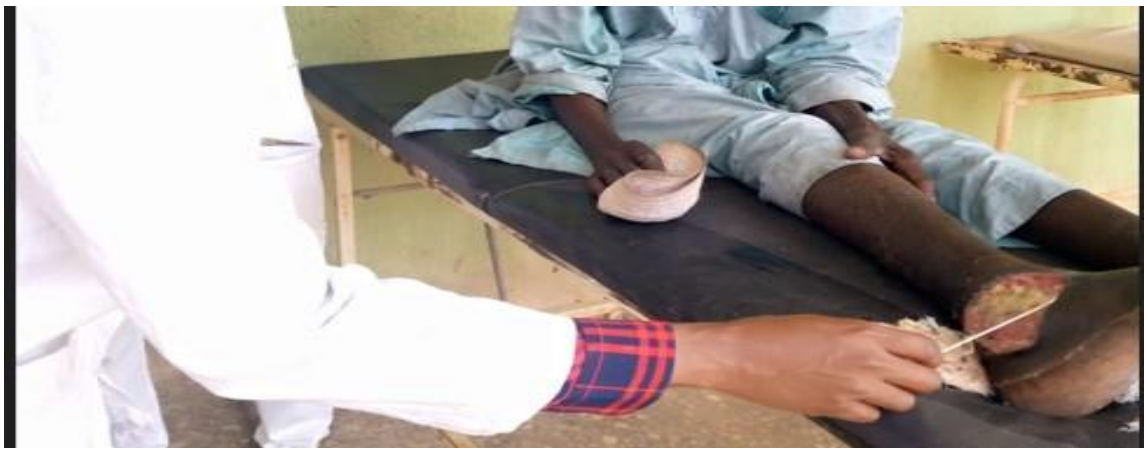

Plate 1: Sample of Patient from General Hospital Dutse

Plate 2: Sample of Patient from General Hospital Ringim

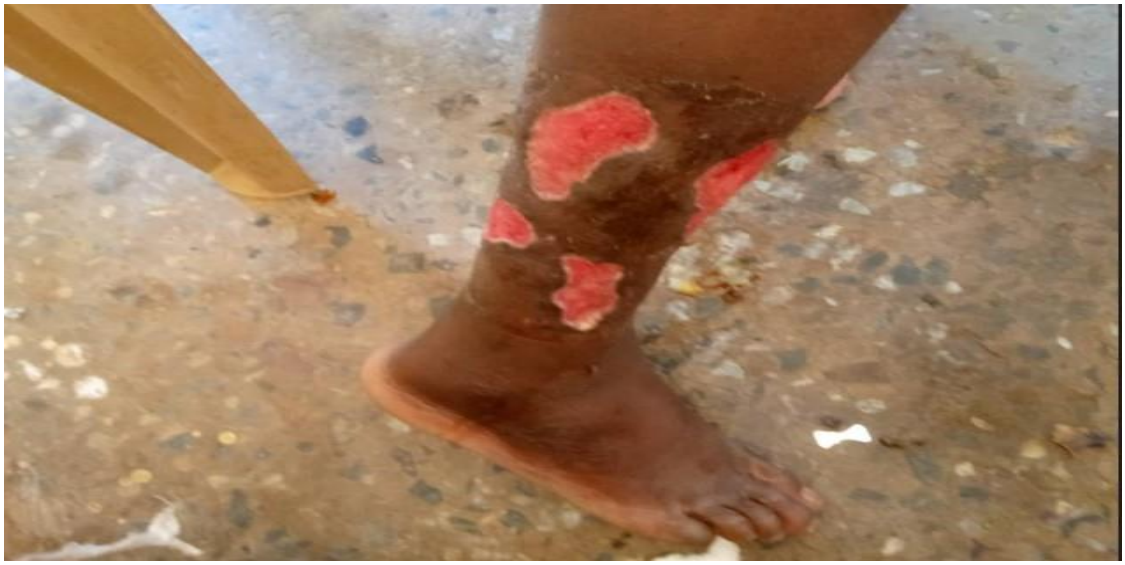

Plate 3: Sample of Patient from General Hospital Kazaure

Plate 4: Sample of Patient from General Hospital Gumel

Plate 5: Sample of Patient from General Hospital Birnin Kudu

Plate 6: Sample of Patient from General Hospital Jahun

DISCUSSION

Finding from this study is different from that of Ukwaja *et al.* (2016) who reported the crude prevalence of 18.7 per 100,000 in their study communities and much higher rate 41.4 per 100,000 in Ogoja. However, this study agrees with one of the commonly held notions by heath policymakers in Nigeria that Buruli ulcer disease is not endemic in the country anymore (Ukwaja *et al.,* 2016).

The findings from this study also differs from the number of cases from the following regions, Nkpo Hamida village, Igbo-Eze North Local Government Area of Enugu State (1 case); Iburu village, Ohaozora Local Government Area of Ebonyi State (1 case), Akoju village, Ikwo Local Government Area of Ebonyi State (1 case); Amazunze village, Nkanu East Local Government Area of Enugu State (1 case); Okro Mbokho village, Eastern obolo, Akwa Ibom State (1 case); Oron village, oron Local Government Area of Akwa Ibom State. (1 case); and (1 case) in Ugwu Tank, Awka South Local Government Area of Anambra State (Okechukwu *et al.,* 2007).

Orujyan *et al.* (2022), reported the protective effect of neonatal Bacille Calmette-Guérin (BCG) vaccination against severe forms of BU disease, although there are conflicting reports on its effectiveness. The zero prevalence of BU in parts of Jigawa state may be attributed to the administration of BCG vaccine as a component of routine immunization schedule. This findings also revealed that Buruli ulcer is not endemic in Jigawa state, Northern Nigeria, despite the availability of

various degrees of skin ulcerative lesions in the study facilities.

Although reports of the community's attitude towards Buruli ulcer illness as not being a medical condition exist, the zero prevalence may possibly be a result of the study being conducted entirely in hospitals (Kashim *et al.,* 2024). Such a bad impression might have discouraged many BU patients from visiting medical facilities, which would have limited the number of opportunities to interact with BU patients. Another common belief regarding buruli ulcers is that they are spiritual or paranormal illnesses brought on by witchcraft. (Osei and Duker, 2015). These beliefs may have also caused BU patients to seek care from native or herbalist doctors, so obstructing their access to healthcare institutions and potentially making it more difficult to include them in this study.

CONCLUSION

This study diagnosed Buruli ulcer using molecular and microscopic approaches, but nonetheless, the findings show that there is no Buruli ulcer in the study area. Sampling in this study was hospital-based, future researcher should focus on community sampling. More samples suspected to have symptoms of Buruli ulcers should be diagnosed using real-time PCR and make use of both IS2404 and IS2606.

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