First isolation of *Mycobacterium ulcerans* from Swabs and Fine-Needle-Aspiration Specimens in Togo.

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Abstract

Background Buruli ulcer is a skin disease caused by a mycobacterium called *Mycobacterium ulcerans*. It is prevalent in more than 33 countries on several continents but West Africa is the most affected. The isolation in culture of the bacteria is difficult because of its slow growth and the facilities required. In Togo, studies have been done on the risk factors for *Mycobacterium ulcerans* infection and the detection of cases by the Ziehl-Neelsen and PCR technique on clinical and environmental samples, but to date no data of isolates from clinical samples are available. The purpose of this study was to perform an in vitro culture of *M. ulcerans* from swab and fine needle aspiration samples through the confirmation stages of direct examination and IS2404 -PCR.

Method A total of 70 clinical samples from Togo and 10 clinical isolates from Benin are analyzed by the three techniques indicated in the diagnosis, in particular the direct examination of acid-fast bacilli (AFB) using the Ziehl-Neelsen staining, the PCR targeting the IS2404 sequence, and the culture after transport of the samples in a transport medium made of Middlebrook 7H9 medium supplemented with a mixture of PANTA and OADC and decontamination by the modified Petroff method.

Results The application of the three techniques of diagnosis for clinical samples yielded 44.28% of positivity rates on direct examination of AFB, 35.71% on culture and 77.14% on qPCR IS2404 with a significantly higher rate for qPCR (0.0001). All samples positive for Ziehl-Neelsen staining and culture were also positive for qPCR.

Conclusion : Our results show that the culture, despite its difficulty and the slow growth of the bacteria, can be carried out with recommended tools of the mycobacteria culture and a good method of decontamination of the samples can improve the positivity rates. Its realization will allow the assessment of the in vitro sensitivity to the antibiotics used in the treatment and the discovery of new strains of *Mycobacterium ulcerans*.

Background

Buruli ulcer is a serious skin disease caused by *Mycobacterium ulcerans*. To date, Buruli ulcer has been identified by the WHO in more than 33 countries on several continents (Africa, America, Asia, Oceania) [1]. However the most burden is found in Africa and particularly in sub-Saharan Africa [1]. The outbreaks are geographically almost always circumscribed around an aquatic ecosystem (river, artificial or natural lake, marsh area, irrigation system) [2,3]. This disease is the third most common mycobacterial infection in human after tuberculosis and leprosy [4]

The notification of Buruli ulcer cases is based on confirmation in the laboratory by the WHO recommended tests for the diagnosis of the disease, including direct examination of smears for acid-fast bacilli (AFB); in vitro culture and gene amplification (PCR) targeting the genome sequence *IS2404*. According to WHO, 70% of BU cases should be confirmed by the PCR-*IS2404* gene amplification technique [5, 6]. The isolation of *M. ulcerans* from clinical specimens is a slow and difficult process due

to many factors, including bacteria growing extremely slowly [6–8 weeks] and growing on media that are often contaminated by other fast-growing bacteria. This makes the culture technique difficult to rapid confirmation of BU cases in the laboratory [7–11]. Despite this, the culture of *M. ulcerans* is of great epidemiological interest because it makes it possible to characterize the circulating strains, an essential step in the determination of the resistance of *M. ulcerans* to antibiotics. There are applications of the culture of *M. ulcerans* in several studies [12–15].

In Togo, studies have been done on the risk factors for *Mycobacterium ulcerans* infection and the detection of cases by the Ziehl-Neelsen and PCR technique on clinical and environmental samples [2, 16, 17], and to date no data of isolates from clinical samples are available. The objective of this study is to perform an in vitro culture method to isolate circulating clinical strains of *M. ulcerans* in Togo from fine needle aspiration (FNA) and swabs samples.

Methods

Study sites

The study was conducted from January 2018 to September 2019 at the National Reference Center for the Treatment of Buruli Ulcer located at the regional hospital of Tsévie (CNRT-UB) where patients were samples were collected. The laboratory of this center was used for direct examination of the smears. The culture was performed at the reference laboratory for mycobacteria at the Sylvanus Olympio teaching hospital. The DNA amplification using PCR technique was conducted at the national reference laboratory for Buruli ulcer at the National Institute of Hygiene (INH).

Sampling

Control strains of *M. ulcerans*

Ten strains of *M. ulcerans* received from the Reference laboratory of mycobacteria of Benin (RLM) were used as control for identification of our isolates and as quality of culture of clinical specimens.

Clinical samples

70 samples were collected from suspected patients of Buruli ulcer who visited the national center for treatment of BU according to WHO criteria. These samples are the FNAs

collected from non-ulcerated lesions (nodules, plaques or edema) and swabs from ulcers [5, 6]. For each type of lesion, three samples were collected. The first was used for culture and put in a screw-cap tube containing a transport medium consisted of 2 mL of Middlebrook 7H9 broth medium (Becton Dickinson) supplemented with a mixture of PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin) and OADC (Oleic acid, Albumin, Dextrose and Catalase) (Becton Dickinson). The second sample was used for DNA amplification by qPCR is collected in a tube containing cell lysis solution (CLS, Qiagen Germany) and the third for a smear for Ziehl-Neelsen staining. All samples were taken after the consent of the patients was obtained [5, 6].

Laboratory analysis

Ziehl-Neelsen staining

Direct smears for microscopy were prepared from swab/FNA samples and control strains then subjected to Ziehl-Neelsen staining for detection of acid fast bacilli. Slides were analyzed by microscopy according to the WHO recommended grading system [5].

Culture

Control strains

Benin strains used as growth control previously stored at -80°C were thawed and then subcultured in Middlebrook 7H9 liquid medium (Becton Dickinson) supplemented with a mixture of PANTA and OADC. After four weeks of incubation at 31°C, these cultures were subcultured onto Lowenstein-Jensen medium (Becton Dickinson Dyfco™) supplemented with glycerol prepared according to the manufacturer's instructions. The cultures on Lowenstein-Jensen medium are considered negative after 12 weeks of incubation at 31°C.

Clinical samples

All the samples have been decontaminated by the modified Petroff method.

Prior to the decontamination process swabs specimens were vortexed for 2 min to disperse as much as possible all the bacteria attached to the swab. The decontamination consisted to

add 2 mL of 4% NaOH solution to 2 mL of the samples of swab or FNA. The mixture is agitated time to time and allowed to stand for 15 minutes at room temperature. Then the mixture was centrifuged at 3000 rpm for 15 minutes. After removal of the supernatant, 15 mL of sterile physiological water were added to resuspend the pellet. The suspension is again centrifuged at 3000 rpm for 15 minutes. After removal of the supernatant, the pellet is resuspended in 1 mL of sterile physiological water and 200 µL are inoculated onto Lowenstein-Jensen medium and incubated at 31°C. The medium is examined weekly for identifying the growth in comparison to the growth of the control strain. All suspected colonies of mycobacteria appearing on a tube are confirmed by *IS2404*-qPCR. The cultures was considered negative after 12 weeks of incubation at 31°C [5].

Real-time PCR (qPCR)

DNA was extracted from clinical samples and control strains using Qiagen kits according to the manufacturer's instructions and the protocols of Bretzel et al. (2011), Beissner et al. (2013) [16, 17].

Real-time PCR (qPCR) was performed on clinical samples and control isolates using primer and probe targeting *IS2404* insertion sequence.

The amplification reaction was performed with a volume of 2µL of the extracted DNA from a sample and 18µL of the mastermix. The mastermix was consisted of 0.4µL of internal control DNA (IPC), 2µL of the IPC control mix, 1µL of the primer *IS2404* sense, 1µL of the *IS2404* primer antisense, 1µL of the Taqman probe, 8.6 µL of water and 4 µL of qPCR Mix Plus. The reaction was conducted in the ABI 7500 thermal cycler (Applied Biosystems) under the following conditions: 95 ° C-15min, 40 cycles of 95 ° C-15s and 60 °C-60s. Extraction control, negative control and positive control, as well as inhibition control, were introduced into the reaction. The primers targeting the *IS2404* sequence was used with a probe that targets the *IS2404* insertion sequence (Table 1).

Table 1: List of Primer and Probe Sequences for Real-Time PCR Targeting *IS2404*

Insertion

Primer and Probe	Sequence (5'-3')	Position of nucleotides	Size of amplicons
IS2404 TF	AAAGCACCACGCAGCATCT	27746-27762	59
IS2404 TR	AGCGACCCCAGTGGATTG	27787-27804	
IS2404 TP	6FAM-CGTCCAACGCGATC-MGBNFQ	27768-27781	

Data Processing and Analysis

Statistical analysis was performed by SPSS (Statistical Package for Social Science, Version 24.0, SPSS Inc., Chicago, IL). The Chi-square test was applied to determine the difference between the observed proportions. This difference was considered significant if the *P*-value ≤ 0.05 .

Results

ZN staining

The overall positivity for AFB was 41/80 (51.25%) with 31/70 (44.28%) for clinical samples and 100% for control strains.

Culture

Of the 70 clinical samples cultured, 25/70 (36%) exhibited growth of mycobacteria on Lowenstein-Jensen Medium slants after 8 weeks of incubation and for 8/10 (80%) of the control strains, growth was observed after 3 weeks.

For both strains, colonies appear yellowish, rough and well defined on board (Figure 1). The overall contamination rate achieved by the modified Petroff decontamination method was 17%.

Figure1: slants of Lowenstein-Jensen Media showing colonies of *M.ulcerans* with two control strains B04 and B318.

IS2404-qPCR

Of 80 samples tested the overall positivity was 64/80 (80%) with 54/70 (77%) for clinical samples and 10/10 (100%) for control strains. All the clinical isolates 25/25 (100%) were tested positive for *IS2404*. A significant difference was observed between the proportions of qPCR, microscopy and culture ($P=0.0001$). All the results obtained from all the techniques are summarize in table 2.

Table 2: Rate of positivity of culture and the results of the other techniques

Samples	Ziehl-Neelsen		Culture		<i>IS2404</i> qPCR	
	Positive	Negative	Positive	Negative	Positive	Negative
Clinical (Togo) n=70	31 (44.28%)	39 (55.71%)	25 (35.71%)	45 (64.28%)	54 (77.14%)	16 (22.85%)
Control Strains n=10	10 (100%)	0 (0%)	8 (80%)	2 (20%)	10 (100%)	0 (0%)
Total	41(51.25%)	39 (48.75%)	33 (41.25%)	47 (58.75%)	64 (80%)	16 (20%)

Discussion

Culturing *M.ulcerans* is one of the recommended method to confirm BU disease. However, due to the long time require to obtain isolates, this technique, is not useful for the immediate care of patients. However the culture of *M. ulcerans*, provides an essential basis for research, for example on the resistance of circulating strains to conventional antibiotics used in the treatment of the disease. So we realize this first study that focused on the culture and isolation of *M.ulcerans* from clinical samples in Togo.

From 70 clinical samples, 25 (36%) showed growth of mycobacteria compared to the control strains. The colonies obtained were confirmed by real time *IS2404*-PCR as *M.ulcerans*. The rate observed in this study is similar to the WHO recommendation. However in other studies some authors found a positivity rate high [18, 19] or low to compare to our rate [9, 20]. The different of positivity rate observed could be explained by the number of samples cultured but especially by the decontamination method. Indeed, the transport medium used in our study is the Middlebrook 7H9 supplemented with PANTA and OADC which conserve the mycobacteria in the sample for a long time [6, 21]. However, the method of decontamination with sodium hydroxide 4% and Sodium Chloride 0.85% (modified Petroff) used has a great impact on the viability not only for the non-acid-fast contaminants bacteria but it also kills 60–70 % of the mycobacteria present in the sample due to NaOH toxicity [22,23]. Compared to our decontamination

method described above, other studies have employed the oxalic acid or the method with 2% cetylpyridinium chloride/4% sodium chloride which provided more positive culture and less contamination rate [19, 24, 25].

The culture has been compared to the Ziehl Nelssen and *IS2404*-qPCR. There is no difference between the positivity rate of the direct examination (44.28%) and culture (36%) ($P = 0.37$) but the positivity rate of the two techniques were lower than the qPCR. These rates are online with some studies and also the recommendations of WHO [9, 20, 21, 26]. This is explained by the capacity of *IS2404*-qPCR to reliably detect low genome copies of the bacteria in samples containing live or dead bacteria [27].

In our study, the incubation time required to obtain positive cultures of *M. ulcerans* from clinical specimens was 8 weeks compare to subcultures of control strains (3 weeks). In both cases, the colonies observed were yellowish, rough corresponding to the African strains of *M. ulcerans* more yellowish than the Australian strains [5, 6].

Conclusion

The culture of *M. ulcerans* has been possible in our context with recommended tools of the mycobacteria culture. A particular attention has been given to the respect of the protocols required from the sampling for the different techniques to the confirmation analysis. The in vitro culture of *M. ulcerans* is important for the management of BU since the use of antibiotics recommended for treatment requires monitoring the susceptibility of *M. ulcerans* strains to the molecules used and it can lead to the discovery of new strains. This is generally possible only with bacteria in culture. In vitro culture also provides information on the viability of *M. ulcerans* in BU lesions post antibiotic treatment, indicative of treatment successes or failures and for the determination of molecular epidemiology.

Declarations

Ethics approval and consent to participate The study protocol was approved by the National Program for Buruli Ulcer Control, and the Ministry of Health as an integral part of the surveillance of the disease and all activities fall under routine patient management. However, this study did not require a review of the ethics committee. In accordance with the usual practice at the National Buruli Ulcer Reference Center, The objectives of the study were explained to the participants and their inclusion was voluntary. For each participant, we obtained a consent. For children, parents or legal representatives gave consent on their behalf.

List of abbreviations

CNRT-UB: National Reference Center for the Treatment of Buruli Ulcer

OADC: Oleic acid, Albumin, Dextrose and Catalase.

PANTA: Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin.

FNA: Fine Needle Aspiration

Consent for publication

Not applicable

Competing interests

All authors declare that they have no competing interests.

Availability of data and materials

The majority of the data generated or analyzed during this study are included in this article. However, the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

Tchalare Kondi Makagni, Issaka Maman, Tchacondo Tchadjobo and Damintoti Simplicie Karou contributed to the study design, statistical analyses of data, wrote the paper and performed critical review of the manuscript for important scientific content. Piten Ebekalisai was involved in the clinical diagnosis and sample collection. Dagnra Anoumou provides advice and contributed to the facilitation of the project. Tchalare Kondi Makagni, Issaka Maman, Disse Kodjo, Kadanga A. Essossimna, Tchao Sambiani, Lowa Pya-Abalo Madimili, Eza Ayabavi were involved in laboratory techniques. All authors read and approved the final manuscript.

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Figures

Figure 1

slants of Lowenstein-Jensen Media showing colonies of *M.ulcerans* with two control strains B04 and B318.