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Research Article

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 *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- Nelssen 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 IS*2404*-PCR.

Method: A total of 70 clinical samples from Togo and 10 control strains of *M.ulcerans* from Benin, were 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 qPCR targeting the IS*2404* 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. Our decontamination protocol was compared to those of other studies to evaluate the pros and cons of our decontamination 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 IS*2404*-qPCR with a significantly higher rate for qPCR (p=0.001). All samples positive for Ziehl-Neelsen staining and culture were also positive for qPCR.

Conclusion: Our results show that the culture, despite it difficulty and the slow growing of the bacteria, can be carried out with basic tools of the mycobacteria culture. 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*.

Keywords: Buruli ulcer; *Mycobacterium ulcerans*; qPCR; Ziehl-Neelsen; culture

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; LJ: Löwenstein-Jensen

Introduction

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 highest 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 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 IS*2404*. According to WHO, 70% of BU cases should be confirmed by the PCR-IS*2404* 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 and 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 in particular on the detection of cases by the Ziehl-Neelsen and PCR technique on clinical and

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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) in Togo where patients were recruited. The laboratory of this center was used for direct examination of the smears. Culture was performed at the reference laboratory for mycobacteria at the Sylvanus Olympio teaching hospital. 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 clinical isolates of *M. ulcerans* were received from the Reference laboratory of mycobacteria of Benin (RLM) were used as control for identification of isolates and as quality control of culture of clinical specimens.

Clinical samples: Seventy (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 were consisted of 32 FNAs collected from non-ulcerated lesions (nodules, plaques or edema) and 38 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). The second sample was used for molecular diagnosis by qPCR was 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 reference strains and 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 were previously 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, theses cultures were subcultured onto Löwenstein-Jensen medium (Becton Dickinson Dyfco™) supplemented with glycerol prepared according to the manufacturer's instructions. The cultures on Löwenstein-Jensen medium was 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 was agitated and allowed to stand for 15 minutes at room temperature. Then the mixture was centrifuged at 3,000 rpm for 15 minutes.

After removal of the supernatant, 15 ml of sterile physiological water were added to suspend the pellet. The suspension was again centrifuged at 3,000 rpm for 15 minutes. After removal of the supernatant, the pellet was resuspended in 1 ml of sterile physiological water and 200µl were inoculated onto Löwenstein-Jensen medium and incubated at 31°C. The medium was examined weekly for identifying the growth in comparison to the growth of the reference strain from Benin (culture control). All suspected colonies of mycobacteria appearing on a tube were confirmed by IS*2404*-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 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 isolates using primers and probe targeting IS*2404* insertion sequence (Table1).

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 IS*2404* sense, 1µl of the IS*2404* primer antisense, 1µl of the Taqman probe, 8.6µl of water and 4µl of qPCR Mix Plus. The reaction was conducted in the ABI 7500 thermal cycler (Applied Biosystems) under the following conditions: 95°C-15min and 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.

Data processing and analysis

Statistical analysis was performed by SPSS (Statistical Package for Social Science, Version 24.0, SPSS Inc, and Chicago, IL). The Chi-square test was applied to determine the difference between the positive proportions observed for the different techniques and for the types of clinical samples. 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 references strains.

Culture

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

Table 1: List of Primer and Probe Sequences for Real-Time PCR Targeting IS*2404* Insertion.

Primer and Probe	Sequence (5'-3')	Position of nucleotides	Size of amplicons
IS*2404* TF	AAAGCACCACGCAGCATCT	27746-27762	59
IS*2404* TR	AGCGACCCCAGTGGATTG	27787-27804	
IS*2404* TP	6FAM-CGTCCAACGCGATC-MGBNFQ	27768-27781	

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

Samples	Ziehl-Neelsen		Culture		IS*2404* qPCR	
	Positive	Negative	Positive	Negative	Positive	Negative
Clinical (Togo) n=70	31 (44.28%)	39 (74.28%)	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%)	44 (65%)	33 (41.25%)	47 (58.75%)	64 (80%)	16 (20%)

Table 3: Comparison of the positivity rates obtained from the different types of sample.

	Ziehl Neelsen			Culture			qPCR		
	FNA	Swab	*p*-value	FNA	Swab	*p*-value	FNA	Swab	*p*-value
Percentage of Positivity	19	12	0.13	15	10	0.23	24	30	0.3
(n=70)	(27.14)	(17.14)		(21.42)	(14.28)		(34.28)	(42.85)	
Total	44.28			35.71			77.14		

Table 4: Best decontamination method achieved by other studies compared to our.

Study	Decontamination method	Contamination rate	Culture medium
Our study	Modified Petroff method (NaCl 9%, NaOH 4%)	17%	LJ
Owusu et al 2017 [18]	2% cetylpyridinium chloride/4% sodium chloride	14%	LJ
Coulibaly et al 2009 [9]	1% cetylpyridinium chloride - Modified Petroff method (NaCl 9%, NaOH 4%)	15.80%	LJ
Yeboah Manu et al 2004 [19]	Oxalic acid	2.40%	LJ
Palomino and F. Portaels 1997 [20]	Mild HCl	NA*	BACTEC 12B medium
Yeboah Manu et al 2011 [11]	Oxalic acid	5%	LJ
Aboagye et al 2016 [21]	-1M NaOH	15.60%	LJ
	- 5% oxalic acid		

NA: Not Available

The colonies appear yellowish, rough and well defined on board. The overall contamination rate achieved by the modified Petroff decontamination method was 17%. This contamination rate was compared to those obtained in other studies (Table 4).

IS*2404*-qPCR

Of 80 samples tested the overall positivity was 64/80 (80%) with 54/70 (77%) for clinical samples and 10/10 (100%) for reference strains. All the clinical isolates 25/25 (100%) were tested positive for IS*2404*. A significant difference was observed between the proportions of qPCR, microscopy and culture (p=0, 001). All the results obtained from all the techniques are summarize in Table 2 and the comparison of the positivity rates obtained from the different types of sample analyzed is displayed in Table 3.

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 IS*2404*-PCR as *M.ulcerans*. The positivity rate observed in this study is similar to the WHO recommendation. However, in other studies some author found a positivity rate high [18,19] or low to compare to our rate [9,20-22]. 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 Middlebrooks 7H9 supplemented with PANTA and OADC which conserve the mycobacteria in the sample for a long time [6,23]. 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 [24,25]. However, the recovery rate (35.71%) of our decontamination protocol is optimal compared to other studies which used the oxalic acid or the method

with 2% cetylpyridinium chloride/4% sodium chloride [19,26,27] with a contamination rate similar to those obtained in these studies (Table 3). It confirm that our decontamination protocol can be used in the culture of *M.ulcerans*.

The culture has been compared to the Ziehl Nelssen and IS*2404*-qPCR. The positivity rate of the direct examination was not significantly different compared to the culture (p=0.29) and the rate of the two techniques were lower than the qPCR (p=0.001). This observation is online with some studies and also the recommendations of WHO [9,22,23,28]. This is explained by the capacity of IS*2404*-qPCR to reliably detect low genome copies of the bacteria in samples containing live or dead bacteria [29]. No significant difference was observed in our study between the positivity rates of FNA and swab samples from one technique to the other.

In our study, the incubation time required to obtain positive cultures of *M. ulcerans* from clinical specimens was 8 weeks compare to subcultures of reference strains (3weeks). 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 basic tools in mycobacteria culture. A particular attention has been given to the respect of the protocols required from the sampling to the execution of the different techniques used in this study for confirmation. 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 ant it can lead to the discovery of new strains of *Mycobacterium ulcerans*. 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.

Ethics Approval and Consent to Participate

The study protocol was approved by the National Program for Buruli ulcer Control, 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 Centre. The objective of the study was explained to the participants and their inclusion was voluntary. For each participant, we obtained a consent. As for children, parents or legal representatives gave consent on their behalf.

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