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Control of Mycobacterium Ulcerans Infection in Endemic Countries: An Approach to Increase the Biological Confirmation Rate among Suspected Skin Lesions

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Abstract **Background** Buruli ulcer is a chronic necrotizing infectious paniculitis caused by Mycobacterium ulcerans which is prevalent in tropical countries with hot and humid climates. In Côte d'Ivoire, there is a problem of biological confirmation despite the availability of an efficient technical platform and well-trained laboratory staff. Biological confirmation tests carried out from 2005 to 2014 revealed the presence of M. ulcerans only in 61% of suspected cases. Conversely, in 39% of cases no etiology was identified. To understand the reasons for this under-reporting, a study was carried out in 3 endemic health districts in Côte d'Ivoire. The objectives were to improve the quality of clinical samples for microbiological and molecular analysis, to optimize the biological confirmation of Buruli ulcer, and to improve results management. **Methods** Buruli ulcer suspected patients were enrolled in three endemic health districts from 2016 to 2022. Clinical samples were taken in health centers and transferred to the national reference laboratory. Three confirmation methods were carried out: PCR targeting the IS2404 insertion sequence, direct-smear examination by microscopy after Ziehl-Neelsen staining and culture on Löwenstein-Jensen medium. When the first PCR result was negative, a second sample was taken 10 to 15 days later. When the result of the second PCR test was negative, a third sample was taken 10 to 15 days later and analyzed according to the same protocol. Test results were reported to healthcare providers within 3-5 days. An external evaluation of the quality of the PCR tests was carried out by the Institute of Tropical Medicine in Antwerp and by the Swiss Institute of Public and Tropical Health. **Results** 270 patients with suspected cutaneous lesion were recruited in the health districts of Tiassalé (55.2%), Oumé (21.9%) and Sinfra (22.9%). The study population was divided into four age groups, with a predominance of subjects aged under 17 (53%). Ziehl-Neelsen staining revealed the presence of Acid-fast bacilli in 38.1% of cases. The insertion sequence IS2404 was detected in 80% of the samples and mycobacterial isolates were detected in 44.8% of cases. The combination of the three diagnostic methods gave a positivity rate of 81.5% throughout the study period. The culture-PCR coupling optimized the confirmation rate to 81.5%, with a detection of 3% of cases not diagnosed by PCR. **Conclusion** During the study 18.5% of suspicious lesions were not confirmed as Buruli ulcer cases. This approach has optimized the biological confirmation of M. ulcerans infection in suspected BU cases. The interaction between the peripheral centers and the reference laboratory was improved in patient follow-up. The delay in reporting results was reduced and the quality of the data collection was improved. At the end of this study, a Buruli ulcer management approach was proposed, which could help endemic countries to strengthen the quality of case management.

Keywords: *M. ulcerans infection control, confirmation rate, insertion sequence, microscopy, culture*

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1. Introduction

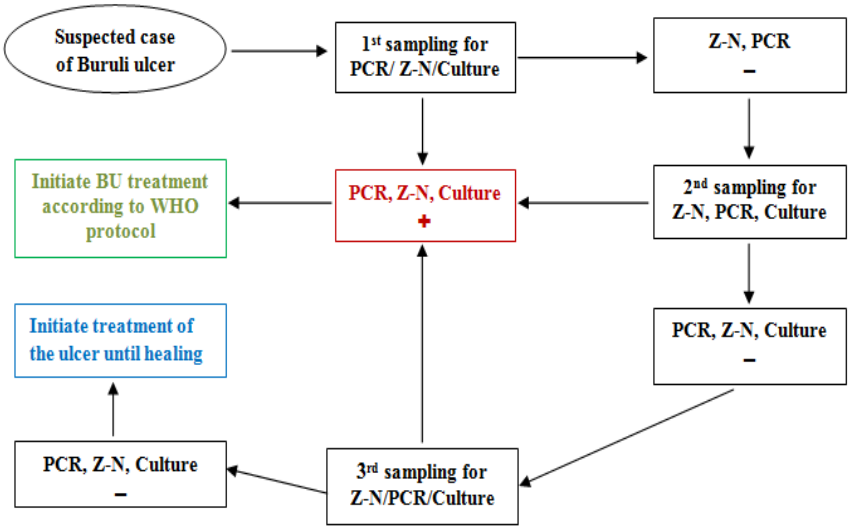
Buruli ulcer is a necrotizing infectious paniculitis of chronic evolution which represents in Côte d'Ivoire the third human mycobacterial disease after tuberculosis and leprosy. This endemic disease caused by *Mycobacterium ulcerans* is prevalent in countries with a humid tropical climate [1,2,3,4,22] Biological confirmation of Buruli ulcer is done by three biological methods which are culture on specific medium, gene amplification by PCR and pathological anatomy examination [5,6,7,8]. Since 2008, the WHO has recommended that endemic countries use PCR targeting the IS2404 insertion sequence to perform biological confirmation. The objective was that at least 80% of suspected cases benefit from biological confirmation [9,10,11]. To do this, service providers at Buruli ulcer diagnosis and treatment centers have been trained to collect samples from suspicious lesions. These samples were transferred to the Institut Pasteur of Côte d'Ivoire by the national Buruli ulcer control program (PNLUB). The Buruli National Reference Center (BU-NRC) carried out the confirmation tests and the results were sent to the treatment centers. In this context, from 2005 to 2014, the BU-NRC performed PCR on 5850 pathological products taken from suspected cutaneous lesions. The IS2404 insertion sequence was found in 3574 samples, representing 61% of suspected cases. Conversely, in 39% of cases, this target was not detected [12,13]. The annual confirmation rate was lower than 80% recommended by the WHO. However, all suspected patients were systematically treated as Buruli ulcer cases, whether or not they benefited from biological confirmation [14]. This low confirmation rate could mean that several patients in previous years received the WHO antibiotic protocol without actually needing it. The negative PCR tests raised several questions: were suspected cutaneous lesions true negative cases or were they declared negative by default? Is it appropriate to institute antibiotic treatment according to the WHO protocol in any suspected case before biological confirmation by PCR? Is PCR as practiced in endemic context sufficient to ensure exhaustive confirmation of BU cases?

To elucidate these questions a study was initiated and conducted by the Buruli national reference center in three health endemic districts for Buruli ulcer in Côte d'Ivoire. Its objectives were to improve the quality of clinical samples intended for microbiological and molecular analysis, to optimize the biological confirmation of Buruli ulcer and to improve the management of biological confirmation results.

2. Patients and Methods

2.1. Patient's Enrollment and Sample Collection

The study took place from 2016 to 2022 in the health districts of Tiassalé, Oumé and Sinfra. These districts had been reporting numerous cases of Buruli ulcer for several years and had qualified health workers to carry out the diagnosis and treatment of Buruli ulcer. In order to standardize practices, capacity building was organized for service providers at the study sites. This training focused on the criteria for defining BU cases and on the procedures for collecting, storing and transporting biological samples [12]. The study subjects were selected among patients presenting a plaque, a nodule, an edema or a chronic ulceration raising suspicion of Buruli ulcer [15,16,17]. The epidemiological and clinical data from patients were collected using the WHO UB01 form. For any BU suspected case, a sample collection algorithm was adopted as following (Figure 1). A sample collection was done during the first contact with the patient. A direct-smear examination [9,18,12], a gene amplification targeting IS2404 [6,7,8,13,17], and a culture on Löwenstein-Jensen medium were performed [5,17,19,12]. If the PCR result is negative, a second sample is taken 10 to 15 days later. If the second PCR result is negative, a third sample is taken within 10 to 15 days and analysed using the same protocol (Figure 1).



PCR: polymerase chain reaction; Z-N: Ziehl Nielsen stain; +: Positive result; –: Negative result.

Figure 1. Sampling and testing algorithm

It was agreed to collect and transfer the samples to the health districts within a maximum of 2 days. The samples were stored at low temperature (-20°C) at the district laboratory and transferred within 2 to 3 days to the BU national reference center at Institut Pasteur de Côte d'Ivoire. The BU reference center had 5 days to carry out the Ziehl-Neelsen staining, the molecular tests, and to communicate the biological data to the health districts by call or by e-mail.

An external quality control of PCR tests was carried out by the laboratories of the Antwerp Institute of Tropical Medicine and the Swiss Tropical Institute of Public Health, involving 189 samples collected during the three patient contacts.

The study protocol was approved by the National Committee for Ethics in Life and Health Sciences (CNESVS). Patients and minor patient parents were informed about the content of the study by the healthcare providers. A copy of the information letter signed by the investigator was given to them. The inclusion in the study was done after obtaining agreement, either by the patient himself or by the parents of minor patients.

The data collection form was anonymized by a unique number. The announcement of biological results was done by the healthcare providers under conditions of confidentiality. Data entry was carried out on an Excel table listing all the sections of the standardized data collection tools used during the study. Epidemiological and clinical data were entered as the samples were received. Biological data were integrated as soon as the laboratory test results were delivered. Data were erased when they were incomplete or insufficient and patients could not be reached. After consolidation, the data were transferred to EPI-INFO software version 6/7 for analysis.

3. Results

3.1. Socio-demographic Characteristics of Patients

During the study, 270 BU suspected patients were enrolled in the health districts of Tiassalé (55.2%), Oumé (21.9%) and Sinfra (22.9%). 57.4% of patients were male compared to 42.6% female, with a male/female sex ratio of 1.3. This population was divided into four age groups of different sizes (Table 1).

3.2. Clinical Characteristics of Patients

The study population consisted of patients with suspected Buruli ulcer who had not yet received the WHO protocol. Pre-ulcerative lesions represented 34.4% of cases with 9.8% of plaque, 7.9% of nodule and 7% of edema. Ulcerative lesions represented 65.6% of cases, respectively 46.5% of category 1, 21% of category 2 and 11% of category 3 (Table 2).

3.3. Diagnosis of Buruli Ulcer Disease in Suspected Patients

The three methods used for the BU diagnosis showed a confirmation rate of 81.5% during the study period. Ziehl-Neelsen staining detected the presence of Acid-Alcohol-Fast Bacilli in 38.1% of cases. The positivity rate was 34.4% at first contact and 11.5% at second contact. But no AFB was observed at third contact. The insertion sequence IS2404 was detected in 80% of cases, respectively a rate of 64.4% at first contact, 40.2% at second contact and 14.3% at third contact. Mycobacterial isolates were detected in 44.8% of cases, respectively a rate of 33.7% at first contact, 27.6% at second contact and 12.3% at third contact (Table 3).

3.4. Results of Microscopy and PCR Coupling

The coupling of microscopy with PCR showed a sensitivity of 45.8% (positive concordant results), a specificity of 92.6% (negative concordant results), a positive predictive value of 96.1% and a negative predictive value of 30% (Table 4).

Four microscopy-positive cases were not confirmed by PCR as cases of M. ulcerans infection since the IS2404 sequence was not detected.

Table 1. Distribution of patients according to sex and age

Health District	Effective	Sex		Age groups (Years)			
		Male	Female	[1-17[	[18-45[	[46-65[	[66-90]
Tiassalé	149	81 (30.0%)	68 (25.2%)	71 (26.3%)	52 (19.3%)	22 (8.1%)	4 (1.5%)
Oumé	59	35 (13.0%)	24 (8.9%)	29 (10.7%)	23 (8.5%)	7 (2.6%)	1 (0.4%)
Sinfra	62	39 (14.4%)	23 (8.5%)	43 (15.9%)	15 (5.5%)	3 (1.1%)	1 (0.4%)
Total	270 (100%)	155 (57.4%)	115 (42.6%)	143 (53.0%)	90 (33.3%)	32 (11.8%)	6 (2.2%)

Table 2. Patients distribution according to skin lesion

Lésions cutanées	Number	Proportion (%)
Non-ulcerative lesions	93	34.4%
Nodule	46	17%
Plaque	33	12.2%
Oedema	14	5.2%
Ulcer	177	65.6%
Total	270	100 %

Table 3. Results of BU diagnostic tests

Methods	Effective at 1st Contact N1 = 270			Effective at 2nd Contact N2 = 87			Effective 3rd Contact N3 = 49			Overall rate of positivity (%)
	+	-	Positivity rate (%)	+	-	Positivity rate (%)	+	-	Positivity rate (%)	
Ziehl-Neelsen staining	88	182	30.7	15	72	17.2	0	49	0	38.2%
PCR IS2404	174	96	64.4	35	52	40.2	7	42	14,3	80%
Culture	91	179	33.7	24	63	27.6	6	43	12,3	44.8%
BU confirmed patients	177		65.5%	35		40.2%	8		16.2%	81.5%

Table 4. Contingency table of Ziehl-Neelsen staining and IS2404 detection

		IS2404 amplification		Predictive values
		IS2404 detected	IS2404 not detected	
Ziehl-Neelsen staining	Acid-fast bacilli detected	99 (45.8%)	4	96.1%
	Acid-fast bacilli not detected	117	50 (92.1%)	30%
Total		216 (80%)	54	270

3.5. Results of Culture/PCR Coupling

Coupling of culture with PCR confirmed *M. ulcerans* infection in 53.2% of suspected cases. This association showed a specificity of 88.8%, a positive predictive value of 95% and a negative predictive value of 32,2%.

Mycobacterial colonies were detected in 6 cases (2%) where the amplification of IS2404 was negative. The PCR analysis of the extracted DNA revealed the IS2404 insertion sequence, confirming *M. ulcerans* infection (Table 5).

Table 5. Results of the culture-PCR coupling for BU diagnosis

		IS2404 amplification		Predictive values
		IS2404 detected	IS2404 not detected	
Culture	*MU* colonies detected	115 (53.2%)	6	95%
	MU colonies not detected	101	48 (88.9%)	32.2%
	Total	216 (80%)	54	270

3.6. External Assessment of the Quality of PCR Tests (Table 6)

Table 6. Results of PCR external assessment

		PCR tests performed by supra national laboratories N = 189		
		IS2404 detected	IS2404 not detected	Proportion
PCR tests performed by Institut Pasteur N = 189	IS2404 +	109 (57.7%)	20	129 (68.3%)
	IS2404 –	22	38 (20%)	60 (31.7%)
Total		131 (69.3%)	58 (31.7%)	189 (100%)

The external quality assessment of the IS2404 amplification was carried out by the laboratories of the Institute of Tropical Medicine in Antwerp and the Swiss Tropical and Public Health Institute. This evaluation focused on 189 samples collected respectively at first contact (81.4%), second contact (11.6%) and third contact (7%).

Cross-referencing the results of PCR tests carried out at Institut Pasteur de Côte d'Ivoire with those of supranational laboratories gave concordant positive results in 57.7% of cases (109/189), concordant negative results in 20% (38/189) and discordant results in 22.2% of suspected cases (42/189).

4. Discussion

This study was initiated following an observation that the confirmation rate was still below WHO forecasts, which recommended a rate of 80%. Despite the efforts made by the national program to optimize the confirmation by PCR, the positivity rate had barely exceeded 50%. Healthcare providers at Buruli ulcer treatment centers in Côte d'Ivoire had many questions regarding this low confirmation rate. Did this low rate represent the true proportion of cutaneous lesions caused by *M. ulcerans* or was it due to under-reporting? What should be done when faced with suspected cutaneous lesions that are not confirmed by PCR?

The following practical arrangements were made during the study: health districts were chosen based on the level of Buruli ulcer endemicity, the number of cases reported in previous years and the availability of transport companies able to ensure the transfer of biological samples. To standardize practices, healthcare providers were trained on the inclusion criteria, the samples collect, storage and packaging procedures for biological samples [12].

A total of 270 patients were enrolled in three health districts. The majority of cases were recorded in the health district of Tiassalé, representing 55.2% of patients (Table 1). This frequency confirms the persistence of Buruli ulcer in this area despite control efforts deployed by well-trained and experienced healthcare providers. The M/F sex ratio of the study population was 1.3 with a predominance of subjects aged less than 17 years. These data corroborate those of several studies which had shown a male and childhood predominance of Buruli ulcer in Côte d'Ivoire [2,3,13,16,20].

All the patients enrolled were BU suspected cases who haven't received any specific antibiotic treatment. They mainly presented ulcers (65.6%), with a predominance of category 1 lesions (46.5%). The predominance of recent lesions would probably be linked to the active door-to-door search carried out during the study (Table 2). On the one hand, this approach discovered many patients who

were receiving traditional care at home. On the other hand, it contributed to discovering more lesions at an early progressive stage. According to data from the national Buruli ulcer control program, a gradual decline in annual incident cases has been observed for several years [20,13]. However, active research has uncovered authentic cases of Buruli ulcer that probably would not have come willingly to receive appropriate care in a hospital. It therefore appears necessary for national BU control programs to strengthen passive case detection in health centers through periodic active search campaigns in endemic areas.

The biological diagnosis of *M. ulcerans* infection was carried out by combining microscopy, culture and PCR (Table 3). The results of microscopic examination showed acid-fast bacilli in 38.1% of cases, allowing suspicion of *M. ulcerans* infection. The sensitivity of microscopy was improved in the present experiment compared to previous studies where it was low, rarely exceeding 30% [15,21]. However, this sensitivity was lower than that reported by Herbinger et al. in a study in 2009. The direct-smear examination carried out under optimal conditions could constitute a good presumptive test for *M. ulcerans* infection in peripheral centers. Optimizing its performance requires the provision of quality samples and well-trained laboratory staff. In african context, microscopy has many advantages: the direct-smear examination can be performed by any health center with a laboratory; it's very easy to implement and the result is available in less than twenty minutes; the cost is accessible to all budgets.

Mycobacterial isolation was obtained in 44.8% of cases. The culture sensitivity in our study was lower than that of 51% reported by Herbinger [19]. Comparison of the isolation rate to that of PCR did not show a sensitivity improvement in BU diagnosis (Table 5). However, the combination of both methods showed interesting aspects. On the one hand, it showed relatively good convergence in excluding non-BU among suspected cases (specificity of 88.8%). On the other hand, at least 2% of *M. ulcerans* infection cases were diagnosed by culture among PCR-negative samples. These results revealed that routine coupling of culture with PCR could reduce the number of false negative cases and thus improve the confirmation rate of *M. ulcerans* infection.

At the end of this study, some lessons were learned. Their implementation in the Buruli ulcer control strategy could help national programs in improving the quality of Buruli ulcer control activities:

- Improving interaction between health centers and the Buruli National Reference Center is necessary. We have strengthened communication between healthcare providers and the reference laboratory through phone calls, electronic messages (e-mail, SMS). This interaction helped sustain provider interest, improve patient data collection, and ensure rapid reporting of test results within 2 to 5 days.
- Organizing the transfer of samples within a short time frame is necessary. The involvement of inter-urban transport companies has considerably reduced the cost and time of transferring samples from the health districts to the reference laboratory.
- The combination of different methods is necessary. We improved the confirmation rate by combining bacteriological and molecular methods. This approach optimized the confirmation rate by detecting cases of *M. ulcerans* infection that could not have been identified by PCR only (Table 5).
- It is necessary to take samples at different evolutionary periods. Analysis of samples taken at intervals revealed BU cases that were PCR-negative at previous contact.
- At least 18.5% of BU suspected patients would probably not need antibiotic treatment according to the WHO protocol, because they were not confirmed as *M. ulcerans* infection (Table 3).
- It is not efficient to analyze swabs from the same lesion separately. The results of the external assessment of PCR tests suggested collecting several samples per lesion, mixing them before analyzing them simultaneously (Table 6). This approach would increase the sensitivity of PCR and thus reduce discrepancies between laboratories.

5. Conclusion

The strategy used during this study has optimized the confirmation rate of Buruli ulcer among suspected skin lesions. Communication has been improved between peripheral centers and the reference laboratory in patient monitoring. This approach has reduced the turnaround time for PCR results and has improved the quality of patient data collected. This management model was effective and could be proposed to any national program for Buruli ulcer control.

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