

Fine-Needle Aspiration, an Efficient Sampling Technique for Bacteriological Diagnosis of Nonulcerative Buruli Ulcer[∇]

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Invasive punch or incisional skin biopsy specimens are currently employed for the bacteriological confirmation of the clinical diagnosis of Buruli ulcer (BU), a cutaneous infectious disease caused by *Mycobacterium ulcerans*. The efficacy of fine-needle aspirates (FNA) using fine-gauge needles (23G by 25 mm) for the laboratory confirmation of BU was compared with that of skin tissue fragments obtained in parallel by excision or punch biopsy. In three BU treatment centers in Benin, both types of diagnostic material were obtained from 33 clinically suspected cases of BU and subjected to the same laboratory analyses: i.e., direct smear examination, IS2404 PCR, and in vitro culture. Twenty-three patients, demonstrating 17 ulcerative and 6 nonulcerative lesions, were positive by at least two tests and were therefore confirmed to have active BU. A total of 68 aspirates and 68 parallel tissue specimens were available from these confirmed patients. When comparing the sensitivities of the three confirmation tests between FNA and tissue specimens, the latter yielded more positive results, but only for PCR was this significant. When only nonulcerative BU lesions were considered, however, the sensitivities of the confirmation tests using FNA and tissue specimens were not significantly different. Our results show that the minimally invasive FNA technique offers enough sensitivity to be used for the diagnosis of BU in nonulcerative lesions.

Mycobacterium ulcerans disease, or Buruli ulcer (BU), a devastating skin disease that may affect bone, is the third most common mycobacteriosis after tuberculosis and leprosy. The disease is focally endemic in Africa, the Americas, Australia, and Asia, where rural populations are the most affected (14, 28, 32).

For many years, the standard treatment of BU was surgical excision of affected tissue (1, 29). Now, however, specific antibiotic therapy for 8 weeks without surgery has been increasingly implemented, especially in patients with small lesions, whether closed or ulcerative (6, 9, 31). This recent development increases the importance of confirming the clinical diagnosis of BU by laboratory techniques to avoid therapy with antimycobacterial drugs when the lesion is not caused by *M. ulcerans* (30). However, with the decreasing importance of surgery as a therapeutic intervention, tissues for microbiological diagnosis are often not available. Alternatively, punch bi-

opsy specimens, requiring local anesthesia, have been employed for confirmation of the clinical diagnosis of BU (23).

In fact, while microbiological tests on ulcerative forms of BU can be performed on multiple swabs of exudates taken from the undermined edges of ulcers, for nonulcerative forms the microbiological diagnosis must be carried out on tissue fragments, i.e., collected by incisional or punch biopsy (30). As a minimal invasive technique, fine-needle aspiration (FNA), commonly used for the cytologic diagnosis of tumors (10, 11), lymphadenopathies caused by trypanosomiasis (17) and tuberculosis (8, 15, 27), leprosy osteitis (20), or leprotic neuritis (16), would seem to offer an advantageous alternative.

In addition, FNA could be valuable for the follow-up of the infection, during and after treatment with antimycobacterial drugs. Compared to punch biopsy specimens, FNA includes less risk of ulceration or possible subsequent reactivation of healing lesions since it is a minimally invasive procedure. Very recently, Phillips et al. (24) also reported the use of FNA for the diagnosis of BU.

In three BU treatment centers in Benin (Centres de Dépistage et de Traitement de l'Ulcère de Buruli [CDTUB] at Zagnanado, Allada, and Lalo), FNA was applied to clinically suspected cases of BU. The efficacy of this technique, as a source of diagnostic material, was compared in parallel with

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that of tissue fragments obtained by excision or punch biopsy, concerning the laboratory confirmation of both ulcerative and nonulcerative BU lesions.

MATERIALS AND METHODS

Collection of specimens in Benin. Sampling was carried out in November 2006. During anesthesia and before surgery, 84 FNA samples were taken from lesions of 33 consecutively enrolled patients with a clinical diagnosis of BU. Sampling was carried out by the same study physician (Y.B.) in all BU treatment centers. Patients were under treatment for various periods of time (see Table 3) with streptomycin and rifampin combined with surgery (31). Depending on the size of the lesion, up to six aspirates were taken from each lesion using fine-gauge needles (23G by 25 mm) attached to 10-ml syringes. An optimal aspiration technique is very important to obtain sufficient material (12). The needle is advanced through the skin without applying suction. For nonulcerative forms, the middle of the lesion is sampled; for ulcerative forms, the undermined edges are sampled (30). Suction is applied while moving the needle back and forth repeatedly through the lesion in multiple directions. Aspiration is terminated when fluid first appears at the needle hub. With cessation of aspiration and with the needle still inside the skin, negative pressure within the syringe returns the syringe piston to the base of the syringe barrel automatically. The reason for releasing suction before the needle is withdrawn is to leave the maximum amount of aspirated material inside the needle. This is so because if the needle is withdrawn from the lesion under negative pressure in the syringe, the aspirated specimen is drawn into the syringe and is dispersed into numerous small droplets. These droplets would stick to the walls of the syringe barrel and would be virtually impossible to recover (10). After withdrawal from the lesion, the needle is detached from the syringe, and the syringe is allowed to fill with air. The needle is then placed back onto the syringe, uncovered, and the specimen is dispersed into the transport medium.

Aspirates were stored at 4°C in a liquid transport medium (LTM) containing Middlebrook 7H9 broth supplemented with polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA, Becton Dickinson, Sparks, MD) and OADC (oleic acid, albumin, dextrose, and catalase) (Difco Laboratories, Detroit, MI). The needle was detached from the syringe, and the content of the needle hub was smeared on a slide for direct smear examination (DSE).

During surgery, parallel tissues were removed at the same sites from which the aspirates were taken. In the CDTUB of Zagnanado, fragments of ± 1 g from surgically excised tissues were taken, while in the CDTUB of Allada and Lalo, 4-mm punch biopsy specimens were used. Tissue specimens were stored at 4°C in a sterile vial with 1 ml of a semisolid transport medium with the same components as LTM plus 0.5% agar. This medium is efficient for storage of clinical specimens from patients with BU (7).

All specimens were transported to Belgium at ambient temperature and processed within 2 weeks.

Microbiological analysis of specimens in Belgium. Smears prepared in Benin were stained with auramine and read using fluorescence microscopy according to the American Thoracic Society scale (4). The LTM containing the aspirate was processed for IS2404 PCR (13) and in vitro culture after decontamination using the reversed Petroff technique (22).

Tissue fragments were cut into small pieces, ground aseptically with a mortar and pestle, and suspended in 2 ml phosphate-buffered saline as previously described (30). One drop of this suspension was dried on a slide, stained with auramine, and read using fluorescence microscopy. IS2404 PCR and in vitro culture were performed on the suspensions.

When culture tubes of specimens with positive results by PCR and DSE were contaminated by other microorganisms, the specimens were processed again for in vitro culture after decontamination with oxalic acid as previously described by Portaels et al. (25) and Yeboah-Manu et al. (33). Briefly, 0.5 ml of 0.2% malachite green, 0.5 ml NaOH, and 0.1 ml of 0.8% actidione were added to 0.5 ml of the specimen suspended in phosphate-buffered saline. This mixture was left for 30 min at room temperature and then centrifuged for 15 min at $3,500 \times g$. The supernatant was discarded, and 1 ml of 5% oxalic acid was added to the sediment and left at room temperature for 20 min. The suspension was then centrifuged again for 15 min at $3,500 \times g$, after which 0.1 ml of the pellet, suspended in 1 ml distilled water, was inoculated onto Löwenstein-Jensen medium, incubated at 30°C, and examined weekly for growth. Positive cultures were identified to the species level based on phenotypic characteristics (18).

Statistical analysis. Data were analyzed statistically with Epi Info v. 3.3.2 and SPSS v. 16.0. Pearson's chi-square test was used to compare proportions, and Student's *t* test was applied to compare means of symmetric distributions. Sen-

TABLE 1. Treating CDTUB, categorization according to WHO, clinical presentation, and type of specimen with at least one positive result for confirmed BU patients

Patient no.	CDTUB location	Categorization by WHO	Ulceration ^a	Confirmation	No. of specimens
06-213	Zagnanado	I	U	FNA, tissue	3
L06-217	Lalo	I	U	FNA, tissue	1
L06-220	Lalo	I	U	FNA	2
06-182	Zagnanado	II	U	Tissue	2
06-196	Zagnanado	II	U	FNA, tissue	3
06-212	Zagnanado	II	U	FNA, tissue	6
06-214	Zagnanado	II	NU	FNA, tissue	2
06-216	Zagnanado	II	U	Tissue	1
06-223	Zagnanado	II	NU	FNA, tissue	6
A239/10/6	Allada	II	U	Tissue	4
A252/10/6	Allada	II	U	FNA, tissue	3
A365/11/6	Allada	II	U	FNA, tissue	2
A366/11/6	Allada	II	NU	FNA, tissue	2
A369/11/6	Allada	II	U	FNA, tissue	4
A375/11/6	Allada	II	NU	FNA, tissue	2
A378/11/6	Allada	II	U	Tissue	3
A379/11/6	Allada	II	U	FNA, tissue	3
L06-117	Lalo	II	U	Tissue	4
L06-195	Lalo	II	NU	FNA, tissue	2
L06-213	Lalo	II	U	Tissue	1
04-36	Zagnanado	III	U	Tissue	5
06-186	Zagnanado	III	U	FNA, tissue	4
06-221	Zagnanado	III	NU	FNA, tissue	3

^a U, ulcerative lesion; NU, nonulcerative lesion.

sitivity were calculated as the ratio of positives for a certain test to the total number of specimens from confirmed patients.

Ethical provisions. This study had the agreement of the Ministry of Health of Benin. All patients (or their parents or guardians, for children younger than 15 years) gave their oral informed consent to participate in the study.

RESULTS

Among the 33 patients, 23 were confirmed to have active BU according to WHO guidelines (30): at least two positive tests among the DSE, PCR, and culture methods (Table 1). Six patients did not have BU, and four were found to have inactive BU (only one positive test by either DSE or PCR). Among the 23 confirmed BU patients, 17 had ulcers and 6 had nonulcerative lesions: plaques (3), edema (2), or osteomyelitis (1). When classified according to WHO guidelines (31), 3 patients had early lesions (category I), 17 had large lesions (category II), and 3 had disseminated lesions (category III) (Table 1). Altogether 68 aspirates and 68 parallel tissue specimens were available from these confirmed patients.

DSE. Sensitivity of DSE by FNA was 32.4% (22/68) (Table 2). This is not significantly lower than the sensitivity on tissue specimens, which was 38.2% (26/68) ($P = 0.59$). A comparison of the sensitivities of DSE by FNA of ulcerative and nonulcerative lesions revealed that nonulcerative lesions were significantly more often positive for acid-fast bacilli (AFB) than ulcerative lesions (64.7% versus 21.6%; $P = 0.003$). In tissue specimens, the sensitivity of DSE was also higher for nonulcerative lesions, but this was not significant ($P = 0.08$). The sensitivity of DSE was higher by FNA than on tissue specimens from nonulcerative lesions, but this was not statistically significant (64.7% versus 58.8%; $P = 0.70$).

IS2404 PCR. In general, the sensitivity of PCR for the total number of FNA specimens was 48.5% (33/68) (Table 2). This is significantly lower than the sensitivity for tissue specimens, which was 85.3% (58/68) ($P < 0.0001$). When comparing the sensitivities of PCR on FNA of ulcerative and nonulcerative

TABLE 2. Results from DSE, PCR, and in vitro culture of FNA and tissue specimens of ulcerative and nonulcerative BU lesions

Parameter	No. (%) of specimens ^a					
	Tissue			FNA		
	Ulcerative (17 patients)	Nonulcerative (6 patients)	Total (23 patients)	Ulcerative (17 patients)	Nonulcerative (6 patients)	Total (23 patients)
DSE positive	16 (31.4)	10 (58.8)	26 (38.2)	11 (21.6)*	11 (64.7)*	22 (32.4)
PCR positive	43 (84.3)	15 (88.2)	58 (85.3)†	18 (35.3)†	15 (88.2)†	33 (48.5)†
Culture positive	7 (13.7)§	10 (58.8)§	17 (25.0)	5 (9.8)¶	7 (41.2)¶	12 (17.6)
Total no. of specimens	51	17	68	51	17	68

^a Statistically significant differences between the results of the analyses as evaluated by Pearson chi-square test are indicated as follows for the values compared: *, $P = 0.003$; †, $P < 0.0001$; ‡, $P = 0.0005$; §, $P = 0.0006$; and ¶, $P = 0.007$.

lesions, significantly more PCR-positive samples were found in nonulcerative lesions (88.2% versus 35.3%; $P < 0.001$). In tissue specimens, the sensitivities of PCR for ulcerative and nonulcerative lesions were not significantly different ($P = 1.00$). For nonulcerated lesions alone, the sensitivities of PCR on FNA and tissue specimens were identical (88.2%).

In vitro culture. The sensitivity of in vitro culture with FNA specimens was 17.6% (12/68) (Table 2). This is not significantly lower than the sensitivity on tissue specimens, which was 25.0% (17/68) ($P = 0.40$). When comparing the sensitivities of in vitro culture of FNA of ulcerative and nonulcerative lesions, the latter were significantly more often positive (9.8% versus 41.2%; $P = 0.007$). Also in tissue specimens, nonulcerative lesions yielded more positive cultures ($P < 0.001$). The sensitivities of in vitro culture in nonulcerative lesions with FNA and tissue specimens were not significantly different (41.2% versus 58.8%; $P = 0.30$).

Influence of chemotherapy on positivity of bacteriological tests. In specimens from patients under treatment, DSE and PCR remained positive, although a decrease in positivity was observed both for FNA and tissue specimens (Table 3). In contrast, FNA and tissue specimens from patients under treatment for 4 weeks or more did not yield any positive cultures.

TABLE 3. Results of DSE, PCR, and in vitro culture of FNA and tissue specimens among patients with different durations of chemotherapy

Method and specimen type	No. (%) of patients positive during chemotherapy at:		
	wk 0	wk 1-3	wk 4-6
DSE			
FNA	2 (100.0)	6 (42.9)	2 (40.0)
Tissue	2 (100.0)	8 (57.1)	2 (40.0)
PCR			
FNA	2 (100.0)	9 (64.3)	3 (60.0)
Tissue	2 (100.0)	14 (100.0)	3 (60.0)
Culture			
FNA	2 (100.0)	4 (28.6)	0 (0)
Tissue	2 (100.0)	7 (50.0)	0 (0)
Total no. of patients	2	14	5

DISCUSSION

The first attempt to confirm BU diagnosis by puncturing a lesion with a needle was made in 1957 in the Democratic Republic of Congo on an extensive edematous lesion of the arm. A smear of the exudate stained by the Ziehl-Neelsen method revealed AFB (14).

In a study by Singh et al. (27), the sensitivity of laboratory tests for the confirmation of tuberculous lymphadenitis was higher with biopsy specimens than with FNA material. In our study, when the sensitivities of laboratory tests obtained for the total number of FNA and tissue specimens (regardless of clinical form) were compared, tissue specimens yielded more positive results—but only by PCR, the most sensitive test. Importantly, when considering only nonulcerative BU lesions, the sensitivities obtained from FNA and tissue specimens were not significantly different by PCR or by any of the other confirmatory tests (Table 2). These results emphasize the potential of FNA for confirmation of the diagnosis of nonulcerative forms of BU since ulcers can easily be confirmed on swabs. With nonulcerative forms of BU, however, an invasive sampling technique is currently necessary, and FNA is a much less traumatic approach.

In the present study, three bacteriological confirmation tests were performed and PCR was found to be the most sensitive technique, for both FNA and tissue specimens, for ulcerative as well as for nonulcerative lesions (Table 2). The second most sensitive confirmation test was DSE, followed by in vitro culture. Comparable observations have been reported in other studies (5, 7, 23). These findings are analogous to those reported for the use of FNA for the diagnosis of tuberculous lymphadenitis (2, 8, 15, 27). Also, a recent study by Phillips et al. (24) reports PCR as the most sensitive technique for confirming the presence of *M. ulcerans* in FNA of nonulcerative BU lesions.

In the present study, FNA samples from nonulcerative lesions had significantly higher positivity rates than FNA samples from ulcerative lesions for all of the tested laboratory methods. This may be due to the sampling technique, since in ulcerative lesions it is probably more difficult to find the most productive sampling site. In the ulcerative lesions that have not been debrided, the necrotic slough in the base of the ulcer contains the majority of AFB. After debridement, the largest numbers of AFB are seen at the base of the ulcer in the undermined

edges. Using tissue fragments in a larger study, we have also observed a higher positivity rate in the confirmation tests for nonulcerative lesions (plaques and edemas) than for ulcerative lesions (F. Portaels et al., unpublished data). Positivity of microbiological confirmation tests also varies in tuberculous lymphadenitis, depending on the type of lesion (19, 21).

Recent unpublished studies by our group of more than 1,000 BU patients show that at least two tissue specimens from the same lesion must be analyzed to achieve acceptable sensitivity for the bacteriological confirmation of BU (F. Portaels, unpublished data). The taking of multiple FNA aspirations from different sites of the lesion may thus be justified to increase the sensitivity of the laboratory tests. In the present study, patients were anesthetized before the sampling procedures because these preceded surgery. FNA is routinely used for cytologic diagnoses without anesthesia (12). The pain when taking five to seven FNA in breast lesions of 79 patients has been reported to be 3.19 on a scale of 0 (no pain) to 10 (worst possible pain) (34). It is therefore expected that for sampling of diagnostic FNA specimens from BU lesions, anesthesia would also not be necessary.

In both FNA and tissue specimens, DSE and PCR remained positive during treatment (Table 3). This type of phenomenon has been observed in patients with tuberculosis, whose sputum smears may remain positive for AFB after up to 36 weeks of treatment while cultures became negative much earlier (3). Moreover, the overall sensitivities of in vitro culture with FNA as well as tissue specimens were lower in this study than those in previous studies (7, 23). The reason for this is probably that most of the patients included in this study were undergoing treatment when the specimens were collected, therefore reducing the number of viable bacilli in the lesion. Thus, this study also demonstrates the importance of FNA for follow-up of chemotherapy when the use of more invasive sampling techniques could be deemed unethical. Indeed, according to our observations, the outcome of in vitro culture from nonulcerative BU lesions could be best assessed in FNA specimens.

We have evaluated several small collections of FNA specimens taken by other teams of investigators who used 19G needles and found them much less productive in the confirmation of BU (data not shown). The types of lesions sampled and the techniques employed were not uniform; however, direct comparisons of samples taken by 19G and 23G needles seem warranted.

Siegmund et al. (26) found a great variation in positivity rates of bacteriological tests between BU treatment centers, suggesting that the quality of the clinical diagnosis and of sampling specimens differed between centers. We therefore recommend the development of training sessions on the different sampling techniques for the confirmation of BU: tissue or swab specimens from ulcerative lesions and FNA from nonulcerative lesions.

In conclusion, our results support the use of FNA, a minimally invasive technique, as a sensitive sampling method for the diagnosis and follow-up of BU in nonulcerative lesions. The facts that the available sampling techniques for confirmation of BU diagnosis cause high levels of discomfort and that BU affects primarily children reinforce the need to develop less invasive sampling methods as proposed here. Additionally, due to the present sampling difficulties, antibiotic treatment is of-

ten performed without bacteriological confirmation of BU, implying the risk of submitting patients to long chemotherapeutic protocols in skin lesions not caused by *M. ulcerans*. Moreover, in these cases, confirmation of BU diagnosis may prevent potentially harmful surgery. Our findings have therefore a potential impact in benefit of patients with nonulcerative lesions. However, studies in larger case series of patients with nonulcerative lesions are warranted before making any recommendations to replace the more invasive techniques currently used for the diagnosis of BU. In addition, further studies are needed to optimize the sensitivity of BU confirmation tests on FNA taken with needles of different sizes and on the number of FNA aspirates that would be ideal to obtain an optimal sensitivity for the laboratory confirmation of BU diagnosis.

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