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Primary Culture of *Mycobacterium ulcerans* from Human Tissue Specimens after Storage in Semisolid Transport Medium▽

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Tissue specimens collected from patients with clinically suspected Buruli ulcer treated in two Buruli ulcer treatment centers in Benin between 1998 and 2004 were placed in semisolid transport medium and transported at ambient temperature for microbiological analysis at the Institute of Tropical Medicine in Antwerp, Belgium. The impact of the delay before microbiological analysis on primary culture of *Mycobacterium ulcerans* was investigated. The length of storage in semisolid transport medium varied from 6 days to 26 weeks. Of the 1,273 tissue fragments positive for *M. ulcerans* DNA by an IS*2404*-specific PCR, 576 (45.2%) yielded positive culture results. The sensitivity of direct smear examination was 64.6% (822/1,273 tissue fragments). The median time required to obtain a positive culture result was 11 weeks. Positive cultures were obtained even from samples kept for more than 2 months at ambient temperatures. Moreover, there was no reduction in the viability of *M. ulcerans*, as detected by culture, when specimens remained in semisolid transport medium for long periods of time (up to 26 weeks). We can conclude that the method with semisolid transport medium is very robust for clinical specimens from patients with Buruli ulcer that, due to circumstances, cannot be analyzed in a timely manner. This transport medium is thus very useful for the confirmation of a diagnosis of Buruli ulcer with specimens collected in the field.

Mycobacterium ulcerans disease, or Buruli ulcer (BU), is a devastating disease affecting the skin and bones. BU is the third most common mycobacterial disease after tuberculosis and leprosy and is endemic in foci throughout Africa, the Americas, Australia, and Asia, with impoverished, rural populations being the main victims (4, 12).

The transport of clinical specimens for laboratory confirmation to local reference laboratories or abroad usually takes time and cannot always be done under refrigerated conditions. Several studies on successful conditions for the transport of sputa from tuberculosis patients collected at remote settings and sent to reference laboratories have been published (6, 8). However, until now there has been no published report on the effect of a delay between the time of collection of clinical specimens from patients with clinically suspected BU and the time of their analysis in the laboratory.

In a known area of endemicity, an experienced person can make the clinical diagnosis of BU. For confirmation, any two of the following findings are required: (i) acid-fast bacilli (AFB) in a smear stained by the Ziehl-Neelsen (ZN) or the auramine staining technique, (ii) culture of *M. ulcerans*, (iii) pathognomonic histopathology (contiguous coagulation necrosis and the presence of AFB), and (iv) the presence of IS*2404 M. ulcerans* DNA as detected by PCR (17).

Since most BU patients are treated in health care centers or hospitals lacking suitable laboratory facilities, a diagnosis is most often made locally on clinical grounds and on the basis of the presence of AFB in a smear. A swab or a tissue specimen is then transported to a national or international reference laboratory for microbiological confirmation of BU disease. Samples analyzed within 24 h are preferentially kept at 4°C in a sterile vial without additive. For longer transportation times, tissue samples should be introduced into a transport medium: Middlebrook 7H9 broth supplemented with polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (Becton Dickinson, Sparks, MD); oleic acid, albumin, dextrose, and catalase (Difco Laboratories, Detroit, MI); and 0.5% agar, also named semisolid transport medium (STM). This medium has been recommended for use, since specimens kept in it for up to 21 days were still culture positive (17).

Primary cultures from clinical specimens are usually positive within 6 to 12 weeks of incubation at 29 to 33°C, although much longer incubation times of up to 9 months have been observed (17).

In vitro culture is important for the management of BU for the same reasons that it is important for the management of tuberculosis (14, 15): (i) the use of antibiotics is now recommended for the treatment of BU. This necessitates surveillance of the drug susceptibilities of the *M. ulcerans* isolates, which, in general, is done with cultured bacteria. (ii) In vitro culture gives information on the viability of the bacilli in the lesion. (iii) For determination of the molecular epidemiology of the disease, cultured bacteria are needed as well, since specimens do not always contain sufficient numbers of bacilli for fingerprinting analysis.

Difficulties with transportation in regions where BU is endemic often result in delays before the samples can be handled in the laboratory. We collected data on the impact of the delay on primary cultures from tissue specimens obtained from the

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TABLE 1. Number of positive cultures by time of delay before processing and median incubation times

No. of days in STM	No. of positive cultures/total no. of specimens (%)	Median (minimum–maximum) incubation time (wk) required to obtain a positive culture
6–7	49/108 (45.4)	10 (6–28)
8–14	160/354 (45.2)	10 (4–30)
15–21	184/379 (48.5)[a]	11 (3–44)
6–21	393/841 (46.7)	10 (3–44)[b]
22–42	126/287 (43.9)	11 (6–32)
43–63	35/96 (36.5)[a]	12 (6–36)
>63	22/49 (44.9)	15 (6–29)
>21	183/432 (42.4)	11 (6–36)[b]
Total	576/1,273 (45.2)	11 (3–44)

[a] $P = 0.04$.
[b] $P < 0.001$.

Centre Sanitaire et Nutritionel Gbemoten in Zagnanado, Benin, and the Centre de Dépistage et de Traitement de l'Ulcère de Buruli in Lalo, Benin, and processed at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium.

MATERIALS AND METHODS

Collection of specimens. During a 7-year period from 1998 to 2004, 576 (45.2%) cultures of *M. ulcerans* were obtained from 1,273 IS*2404*-specific PCR (IS*2404* PCR)-positive clinical specimens from surgically excised lesions from patients in Zagnanado and Lalo, Benin. Immediately after collection, the specimens (weight, approximately 1 g) were placed in 2 ml STM. In general, the specimens were kept at 4°C until they were transported to Belgium at ambient temperature.

Microbiological analysis. Upon arrival at ITM, the tissue fragments were cut into small pieces, ground aseptically with a mortar and pestle, and suspended in 2 ml phosphate-buffered saline.

One drop of this suspension was smeared for microscopy, stained by the ZN technique, and read and given a grade according to the American Thoracic Society scale (1a). The rest of the suspension was decontaminated by the "Fortep" technique (7) by adding 3 ml 1 N HCl for 20 min, neutralizing the suspension with 1 N NaOH, and centrifuging the mixture at 3,500 × *g* for 20 min. One-tenth milliliter of the pellet, suspended in 1 ml distilled water, was inoculated onto Löwenstein-Jensen (LJ) medium, incubated at 32°C, and examined weekly for growth (11). The colonies were identified to the species level on the basis of phenotypic characteristics: growth rate, pigmentation, photoreactivity, enzymatic characteristics, morphology, and growth in the presence of some inhibitory agents (16). LJ media that did not show growth after 12 months were considered negative. The number of weeks required to obtain a positive culture as well as the number of colonies observed on the LJ medium was recorded.

The IS*2404* PCR, which is specific for *M. ulcerans* DNA, was performed with the decontaminated suspensions (3). Only IS*2404* PCR-positive specimens, which were considered the reference material, were included in the present analysis.

Statistical analysis. Data were analyzed with EpiInfo (version 3.3.2) and SPSS (version 14.0) software. The usual Pearson chi-square test was used to compare proportions, and the Mann-Whitney and Kruskal-Wallis nonparametric tests were applied to compare the medians of the asymmetric distributions.

RESULTS

Sensitivity of in vitro culture and direct smear examination. The 1,273 tissue fragments positive for *M. ulcerans* DNA by IS*2404* PCR yielded 576 (45.2%) cultures of *M. ulcerans*. No other mycobacterial species were cultivated. The sensitivity of direct smear examination for IS*2404* PCR-positive specimens was 64.6% (822/1,273).

Incubation time of in vitro culture. No *M. ulcerans* isolate showed visible growth during the first 3 weeks of incubation at 32°C. The median time for cultures was 11 weeks (Table 1). One smear-negative specimen that had spent 19 days in STM showed growth after 44 weeks (Table 1 and Fig. 1).

For specimens stored for 14 days or less, the median incubation time was 10 weeks; for specimens that had resided in STM for a longer period of time, the median incubation time increased from 11 to 15 weeks (Table 1). There was a significant difference in the incubation times between tissue specimens stored for more than 21 days and those stored for a shorter period of time ($P < 0.001$).

Influence of delay before processing of specimens. Positive cultures were obtained even from samples stored in STM for more than 9 weeks and up to 26 weeks at ambient temperature (Table 1). Striking was the observation that there did not seem to be a reduction in the viability of the *M. ulcerans* isolates detected by culture when specimens remained in STM for long periods of time. This was also observed when the number of colonies was plotted versus the time delay in STM. No reduction in viability, as detected by the numbers of colonies that grew, was observed (data not shown). As shown in Table 1, there was no statistically significant difference between the proportions of culture-positive specimens (36.5% to 48.5%)

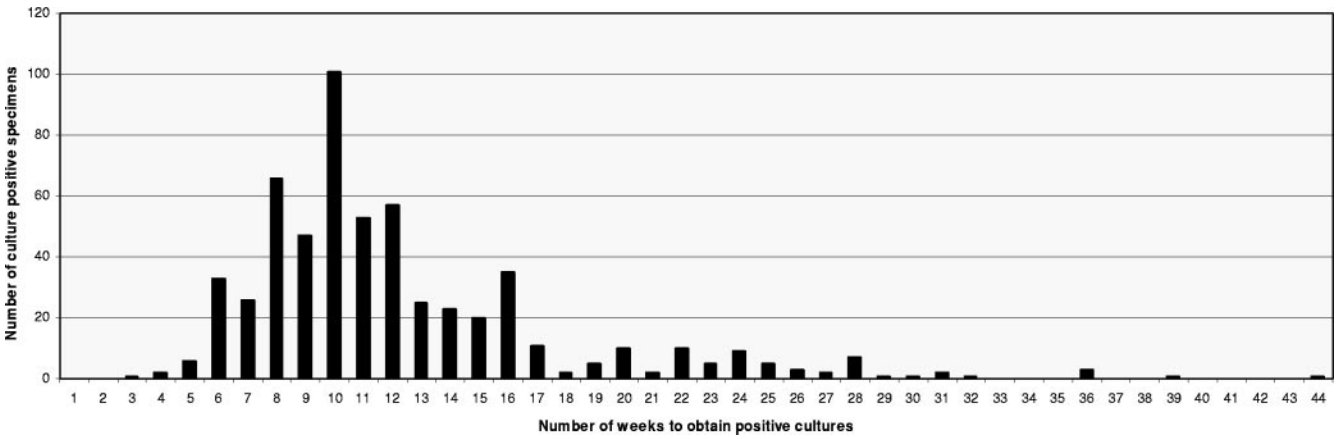

FIG. 1. Incubation time required to obtain positive cultures of *M. ulcerans* from clinical specimens ($n = 576$).

TABLE 2. Number of positive cultures by positivity for AFB and median incubation times

Positivity score for AFB	No. of positive cultures/total no. of specimens (%)	Median (minimum–maximum) incubation time (wk) required to obtain a positive culture
−	106/451 (23.5)[a]	14 (6–44)[b]
1+	175/389 (45.0)[c]	11 (3–36)[d]
2+	102/162 (63.0)	10 (5–36)
3+	100/151 (66.2)	10 (5–30)
4+	93/120 (77.5)[c]	9 (4–39)[d]
Total smear positive	470/822 (57.2)[a]	10 (3–39)[b]
Total	576/1,273 (45.2)	11 (3–44)

[a] $P < 0.001$.
[b] $P < 0.001$.
[c] $P < 0.001$.
[d] $P < 0.001$.

TABLE 3. Number of positive cultures by number of colonies grown, median incubation times, and the presence of AFB in the respective specimens

No. of colonies grown or type of growth	No. of positive cultures obtained from smear-positive specimens/ total no. of positive cultures (%)	Median (minimum–maximum) incubation time (wk) required to obtain a positive culture
1–50	180/268 (67.2)[a]	13 (5–44)[b]
51–100	136/151 (90.1)	10 (4–39)[b]
101–200	42/43 (97.7)	8 (3–24)[b]
201–300	85/87 (97.7)	9 (5–17)[b]
Confluent	27/27 (100.0)	8 (4–14)[b]
Total	470/576 (81.6)	11 (3–44)[b]

[a] The rate of smear positivity for culture-positive specimens showing 1 to 50 colonies is significantly lower than that for culture-positive specimens showing more growth ($P < 0.001$).
[b] $P < 0.0001$.

with various periods of time in STM. Only between the categories of specimens stored for 15 to 21 days and specimens stored for 43 to 63 days was there a slightly significant difference ($P = 0.04$) in culture positivity. Smear positivity (59.4% to 70.4%) also did not differ significantly for different storage intervals (data not shown).

Number of bacteria measured by direct smear examination and number of colonies that grew. Smear-positive specimens yielded a greater proportion of positive cultures with a shorter incubation time than AFB-negative specimens (Table 2). Among the 451 smear-negative specimens, only 106 (23.5%) were culture positive, while among the 822 smear-positive specimens, 470 (57.2%) were culture positive for *M. ulcerans* ($P < 0.001$). The yields of in vitro culture for specimens with 4+ and 1+ scores by microscopy were 77.5% (93/120) and 45.0% (175/389), respectively ($P < 0.001$).

Among the smear-positive specimens, the median time for growth was 10 weeks, while that for the smear-negative specimens was 14 weeks ($P < 0.001$) (Table 2). The median incubation times for specimens with 4+ and 1+ scores by microscopy were 9 and 11 weeks, respectively ($P < 0.001$). The same reduction in the time to grow was observed when the number of colonies was counted. Specimens containing more viable bacilli, as detected by the number of colonies, needed a shorter time to grow, with specimens showing 1 to 50 colonies having a median incubation time of 13 weeks and those showing confluent growth having a median incubation time of 8 weeks ($P < 0.001$) (Table 3). Specimens showing 1 to 50 colonies were also significantly less frequently smear positive than specimens showing more growth ($P < 0.001$).

DISCUSSION

When tissue specimens from patients with clinically suspected BU are transported in STM, positive cultures are obtained after a wide range of times that vary from 3 to 44 weeks. Yeboah-Manu et al. (18) observed an incubation time that averaged 10 weeks (minimum, 5 weeks; maximum, 19 weeks) when they processed biopsy specimens stored in STM for a maximum of 1 week after surgical excision. We observed the same incubation time for specimens stored for 6 to 7 days in STM (Table 1). Bacilli residing for a long time in STM may have entered into a dormant phase, resulting in long incubation times for the cultures.

The number of bacilli present in tissue has an influence on the growth rate. Our observations show that for specimens with smaller numbers of bacteria, reflected by the rate of positivity of the smears as well as the number of colonies that grew, the level of recovery of bacteria in primary culture is decreased. This was demonstrated by our results presented in Table 2 and 3. The correlation between the number of AFB observed and positivity by culture is not absolute, however. Dead bacilli may keep their acid-fast properties for some time and may thus still produce positive results in smear examinations. For this reason, 24.7% (23/93) of the specimens with a 4+ microscopy score showed only 1 to 50 colonies (data not shown). A similar correlation between smear positivity and the number of colonies that grew on the medium was observed for patients with tuberculosis (5).

The overall sensitivity of primary culture in this study was 45.2% (576/1,273 specimens), and that for direct smear examination was 64.6% (822/1,273 specimens). The difference was statistically significant ($P < 0.001$). For direct smear examination, Phillips et al. (10) obtained a positivity rate of 41.8% when they analyzed 55 punch biopsy specimens. This is significantly lower than the positivity rate of 64.6% in our study ($P = 0.006$). This discrepancy may be due to differences in the sizes of the tissue specimens taken in the two studies (±1 g in this study versus 4- or 6-mm punch biopsy specimens in the study of Phillips et al. [10]).

For culture positivity, our results do not differ significantly from those obtained by Phillips et al. (10). The rate of positivity of culture with these specimens, which were kept at 4°C and analyzed 24 h after sampling, was 49.1%. Several years ago we used other growth media for the isolation of *M. ulcerans*, namely, Ogawa medium, Ogawa medium supplemented with mycobactin, and Bactec 12B vials (Becton Dickinson, Sparks, Maryland); and they had sensitivities even lower than the sensitivity of the LJ medium used in this study and by Phillips and colleagues (10) (33.5%, 38.3%, and 16.9% respectively) (unpublished results). Yeboah-Manu et al. (18) also observed a higher sensitivity for LJ medium than for Ogawa medium.

Some of the specimens analyzed in our study were cut into

two pieces, one for analysis in the Mycobacteriology Reference Laboratory in Cotonou, Benin, and one for analysis at ITM in Antwerp, Belgium. The fragments analyzed in Benin were processed before the fragments were sent to ITM, but there was still no difference in the culture positivity rates (1). The delay before analysis, as experienced in this study, did not influence the viability of the bacilli, as detected by the incubation time of the culture as well as the number of colonies that grew. STM contains Middlebrook 7H9 medium and is supplemented with oleic acid, albumin, dextrose, and catalase. This medium could allow the multiplication of *M. ulcerans*, in particular, if it is kept at ambient temperature, which is usually about 30°C in tropical areas. This may explain the presence of viable AFB that grow on LJ medium even after a long period of time in STM.

The long delay between the collection of some specimens and their analysis results from the difficult working conditions in some regions where BU is endemic. The swabs and tissue fragments from patients with clinically suspected BU sent to ITM in STM allowed us to confirm that the disease is still present in areas of Gabon, Uganda (13), the Democratic Republic of Congo (9), and Nigeria (2) where BU was previously endemic and to discover a new focus of endemicity in southern Sudan (13).

Finally, appropriate sampling of the specimens from BU lesions is very important for microbiological diagnosis and strongly influences the detection of *M. ulcerans* by in vitro culture, microscopy, as well as PCR and histopathology, as recommended by the World Health Organization (17).

The cultivation of *M. ulcerans* from tissue specimens transported in STM gives results identical to those for specimens kept at 4°C and processed within 24 h, as was done by Phillips et al. (10). Other advantageous features of STM for use in the field are as follows: (i) the STM tubes are small (2 ml), (ii) the STM tubes can be used for swabs as well as for very small tissue specimens, (iii) the screw-cap tubes are sealed and are thus safe, (iv) the tubes may be transported at ambient temperature, (v) ZN staining and PCR analyses can be done with specimens transported in STM, and (vi) the entire system is inexpensive.

We conclude that our transportation method with STM is very robust for clinical specimens that, due to circumstances, cannot be examined immediately. Thus, STM is very useful for the confirmation of a diagnosis of BU in remote areas devoid of laboratory facilities.

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