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Short Communication

Effects of grinding surgical tissue specimens and smear staining methods on Buruli ulcer microscopic diagnosis

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Summary To optimize Buruli ulcer (BU) microscopic diagnosis, we compared two smear preparation methods from tissue specimens: smears made with tissue suspension after grinding and smears made directly with unground tissue. We also compared two smear staining methods: auramine and Ziehl–Neelsen (ZN). IS *2404*-PCR was used as reference method. One hundred and thirty-one surgical tissue specimens from patients suspected of having BU were analyzed. Both smear preparation methods and both staining methods were equivalent in any combination. Thus we recommend ZN stained smears of unground tissue for peripheral treatment centres.

keywords Buruli ulcer, smear, staining, tissue

Introduction

Among the tests recommended for the confirmation of a suspected case of Buruli ulcer (BU), only microscopy is technically simple, less costly and within the technical competence of peripheral treatment centres (van der Werf *et al.* 2005). Microscopy requires smear preparation and staining. Tissue specimens yield the most positive results and are therefore the preferred diagnostic specimen (N'Guessan *et al.* 2001; Bretzel *et al.* 2007). For microscopic examination, smears are usually made from suspensions of ground or diced tissue specimens and stained by Ziehl–Neelsen (ZN) method (World Health Organization 2001). For peripheral treatment centres, tissue grinding is labour intensive, time consuming and potentially hazardous. The ZN method is also time-consuming and less sensitive than auramine staining, which improves sensitivity and turnaround time for detection of acid fast bacilli (AFB) (Kent & Kubica 1985; American Thoracic Society Diagnostic 2000; Shinnick *et al.* 2005).

We compared two smear preparation methods from tissue: smears made with a tissue suspension after grinding and smears made directly with unground tissue, in combination with two smear staining methods (auramine and ZN). IS*2404*-PCR was used as reference method to confirm the presence of *Mycobacterium ulcerans* in tissues.

Materials and methods

All patients gave informed consent before surgery. The study was approved by the Benin National BU Program board.

Specimens were received from Allada, Lalo and Zagnanado peripheral BU treatment centres in Benin. Between October 2005 and April 2006, tissue fragments were obtained from 131 patients after surgery in the treatment centres. Tissue fragments were collected according to the WHO recommendations, i.e. for non-ulcerative forms from the centre of the surgically excised tissues, and for ulcerative forms from the undermined edges of ulcers (World Health Organization 2001). Only subcutaneous tissues were taken. This piece of tissue was split into two equal parts. One part was randomly used by a local technician to make two smears directly (referred to as direct smears: DS). Smears were air dried and stored in a box at ambient temperature. The second part of the tissue

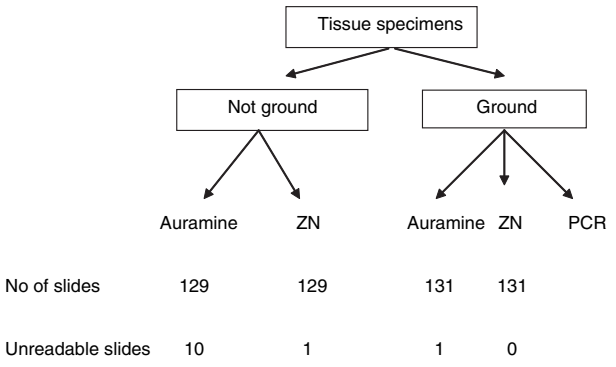

Figure 1 Summary of samples process.

was stored at 4 °C. Every fortnight, these second parts and DS were sent to the National Reference Laboratory (Laboratoire de Référence des Mycobactéries: LRM) at Cotonou. At the LRM, the tissue specimens were ground and resuspended in 1 ml of sterile distilled water. This suspension was used to prepare two other smears (referred to as ground smears: GS) which were air dried. The size of the smears (DS and GS) was approximately 10 × 20 mm. All smears were heat-fixed.

One DS and one GS were stained by auramine staining (0.1% auramine O for 10 min, 1% acid-alcohol for 4 min and 0.1% potassium permanganate for 30 s). The other DS and GS were stained by hot ZN (1% carbolfuchsin for 15 min, 20% sulphuric acid for 5 min, methylene blue for 1 min). Each slide was read by two technicians, who knew neither the smear preparation method used nor the result of the other technician's reading. Results were compared between the two technicians, and in case of discrepancy, the reading was repeated by both technicians. Only consensus results were considered. Before declaring a smear negative, at least 300 fields for ZN and 30 fields for auramine were read. Results were interpreted according to the WHO scale (World Health Organization 1998).

IS*2404*-PCR was performed on portions of the suspension used for GS (Guimaraes-Peres *et al.* 1999). The PCR was done at the Institute of Tropical Medicine (ITM), Antwerp. (The sampling process is summarized in Figure 1). Statistical analysis was carried out using EPIINFO 3.3.2.

Results and discussion

Twelve slides were unreadable: 10 (7.8%) DS stained by auramine, 1 (0.8%) DS stained by ZN, 1 (0.8%) GS stained by auramine and none for GS stained by ZN. There was no difference between the proportion of unreadable DS stained by ZN and unreadable GS stained by ZN. But the percentage of unreadable slides was higher with DS stained by auramine than that of GS stained by auramine ($P = 0.005$). There were more fatty substances in DS, which might explain the difficulty in reading the slides. This did not occur with DS stained by the ZN method, where heat and washing might result in the clearing or removal of fats. This phenomenon was also not observed in GS, irrespective of the staining method as the fatty substances were in suspension. Thus care must be taken in making DS free of fatty substances, especially for auramine staining.

IS*2404*-PCR was used as reference method to calculate sensitivity and specificity of the combinations. Of the PCR-negative samples, seven were positive with at least one combination: three samples were only positive for one combination, three for two combinations and one sample was positive for the four (Tables 1–4). No significant difference was found in sensitivity and specificity between smear preparation methods irrespective of the staining method used. Similarly there was no significant difference

Table 1 Results of ground smears stained by auramine

	PCR		
Auramine	Positive	Negative	Total
Positive	63	3	66
Negative	48	16	64
Total	111	19	130

Sensitivity = 56.7% (48.2–65.3); Specificity = 84.2% (77.9–90.4).

Table 2 Results of GS stained by ZN

	PCR		
ZN	Positive	Negative	Total
Positive	58	3	61
Negative	54	16	70
Total	112	19	131

Sensitivity = 51.7% (43.2–60.3); Specificity = 84.2% (78–90.5).
GS, ground smears; ZN, Ziehl–Neelsen.

Table 3 Results of direct smears stained by auramine

	PCR		
Auramine	Positive	Negative	Total
Positive	51	3	54
Negative	52	13	65
Total	103	16	119

Sensitivity = 49.5% (40.5–58.5); Specificity = 81.3% (74.2–88.2).

Table 4 Results of DS stained by ZN

	PCR		
Auramine	Positive	Negative	Total
Positive	66	4	70
Negative	45	13	58
Total	111	17	128

Sensitivity = 59.4% (51.0–68.0); Specificity = 76% (69.1–83.1). DS, direct smears; ZN, Ziehl Neelsen.

between the staining methods with either DS or GS (Tables 5 and 6).

Although fluorescence microscopy is reportedly more sensitive than conventional microscopy, we did not find any significant difference between the two methods (Kent & Kubica 1985; American Thoracic Society Diagnostic 2000; Shinnick *et al.* 2005). Fluorescence microscopy is fast and less time-consuming than classic microscopy, and it is a more appropriate technique for laboratories that routinely process a high number of specimens (Steingart *et al.* 2006).

Recently, Bretzel *et al.* (2007) proposed a stepwise approach to improve the laboratory diagnosis of BU. In their study, the role of microscopy was further emphasized as the first line of laboratory diagnosis. This highlights the necessity of improving microscopy diagnosis in the peripheral laboratories. Given our findings that grinding tissue specimens had no effect in improving detection sensitivity for both straining methods, this time consuming and labour intensive step could be avoided.

Table 5 Comparison of sensitivity and specificity between two smear preparation methods

	Auramine			ZN		
	GS	DS	*P*	GS	DS	*P*
Sensitivity	56.7%	49.5%	0.25	51.7%	59.4%	0.22
Specificity	84.2%	81.2%	0.63	84.2%	76%	0.10

ZN, Ziehl–Neelsen; GS, ground smears; DS, direct smears.

Table 6 Comparison of sensitivity and specificity between staining methods

	GS			DS		
	Auramine	ZN	*P*	Auramine	ZN	*P*
Sensitivity	56.7%	51.7%	0.90	49.5%	59.4%	0.12
Specificity	84.2%	84.2%	0.98	81.3%	76%	0.27

The main limitation of the present study is the choice of IS *2404*-PCR as the reference method. To date, there is no reference method for the laboratory diagnosis of BU (van der Werf *et al.* 2005), but IS *2440*-PCR is very sensitive and specific (Bretzel *et al.* 2007). The insertion sequence IS *2440* is present in 249 copies per *M. ulcerans* genome, making the PCR highly sensitive (Stinear *et al.* 2007). Moreover, the IS *2404*-PCR is specific because the host range of IS *2404* is restricted to a few mycobacteria and among these, only *M. ulcerans* and exceptionally *Mycobacterium marinum* are known to infect humans (Chemlal *et al.* 2002; Yip *et al.* 2007).

Comparing two smear preparation methods in combination with two staining procedures for the detection of *M. ulcerans* in tissue specimens, we observed no difference in sensitivity and specificity between the methods. We therefore recommend the use ZN stained direct smears of unground tissue in peripheral treatment centres, because this method is suitable to field conditions.

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