RESEARCH LETTER

# Application of real-time PCR in Ghana, a Buruli ulcer-endemic country, confirms the presence of *Mycobacterium ulcerans* in the environment

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## Abstract

This study reports the first successful application of real-time PCR for the detection of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer (BU), in Ghana, a BU-endemic country. Environmental samples and organs of small mammals were analyzed. The real-time PCR assays confirmed the presence of *M. ulcerans* in a water sample collected in a BU-endemic village in the Ashanti Region.

## Introduction

*Mycobacterium ulcerans* is the causative agent of Buruli ulcer (BU), a severe disease of the skin (Portaels, 1995; Portaels *et al.*, 2009). The disease is mainly endemic in Central and West Africa, where it affects mostly poor rural communities (Portaels, 1995; Debacker *et al.*, 2004). Epidemiological evidence strongly associates BU with aquatic ecosystems and *M. ulcerans* is considered an environmental pathogen (Portaels, 1995; Stinear *et al.*, 2007). However, its reservoir and mode(s) of transmission are not yet determined (Duker *et al.*, 2006). Presently, detection of *M. ulcerans* in the environment is based on demonstrating by PCR the presence of IS*2404* (Ross *et al.*, 1997), an insertion sequence with $>200$ copies in *M. ulcerans* (Stinear *et al.*, 2007). IS*2404* has been identified in water, fish, aquatic insects, detritus, leeches, crustaceans, molluscs, and mosquitoes (Portaels *et al.*, 1999; Eddyani *et al.*, 2004; Kotlowski *et al.*, 2004; Johnson *et al.*, 2007), indicating that *M. ulcerans* is probably present in such samples. However, other closely related *Mycobacterium* species including *Mycobacterium liflandii*, *Mycobacterium Pseudoshottsii*, and mycolactone-producing *Mycobacterium marinum* strains (Stinear *et al.*, 1999; Stragier *et al.*, 2007) have been found to harbor IS*2404*. Thus, the conventional IS*2404* PCR assay cannot be relied upon for the specific detection of *M. ulcerans*.

In order to increase specificity, facilitate rapid analysis of specimens, and to interpret the results of both environmental and clinical specimens with certainty, Fyfe *et al.* (2007) developed two TaqMan Multiplex real-time PCR assays targeting three independent repeated sequences in the *M. ulcerans* genome, two multicopy insertion sequences (IS*2404*, IS*2606*), and a multicopy sequence encoding the ketoreductase B domain (KR-B). These real-time PCR assays quantify the copy number of the targets, allowing the





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differentiation of *M. ulcerans* from other IS*2404*-containing mycobacteria. Moreover, the assay allows for the control of PCR inhibitors such as humic and fulvic acids, commonly present in environmental samples.

In spite of its advantages for the analysis of clinical and environmental samples (high throughput, high sensitivity and specificity, less prone to contamination, and inhibition control), facilities for real-time PCR are available only in a few research laboratories in West-African BU-endemic countries, including Ghana. However, swift analysis of environmental samples could be crucial in the search for the *M. ulcerans* reservoir. Therefore, the current study describes the first application of real-time PCR for the detection of *M. ulcerans* in environmental samples at the Noguchi Memorial Institute for Medical Research (NMIMR) in Accra, Ghana. Both the acquisition of these technologies through international technology transfer and their diffusion will foster effective technological change as follow-on innovation and adaptation occurs.

## Materials and methods

The real-time PCR assays were carried out as described by Fyfe *et al.* (2007). Briefly, IS*2404*/internal positive control (IPC) mixtures contained 1 μL of template DNA, 0.9 μM of each primer, 0.25 μM of the probe, 1 × TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and TaqMan exogenous IPC reagents (Applied Biosystems) in a total volume of 25 μL. IS*2606*/KR assays were preformed on IS*2404*-positive samples in a similar multiplex way without IPC. Detection was performed on a 7300 real-time PCR System (Applied Biosystems) using the following thermal profile: one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Triplicate positive/negative PCR controls, positive/negative extraction controls, and fluorescence controls were included in each assay. A UNG enzyme step (50 °C for 2 min) ensured hydrolysis of all single-stranded and double-stranded contaminating PCR products. Cycle threshold ($C_T$) values $> 40$ cycles were considered negative.

The sensitivities of the IS*2404*/IPC and the IS*2606*/KR multiplex assays achieved in this setting were compared with the values described by Fyfe *et al.* (2007) by performing real-time PCR on serial dilutions of purified *M. ulcerans* DNA. Like Fyfe *et al.* (2007), our assays reliably detected two copies of IS*2404*, nine copies of IS*2606*, and 1.5 to three copies of KR. We studied the effects of postponing a run of a prepared reaction plate on assay sensitivities in a similar way by keeping prepared plates at 4–8 °C for a period of $> 12$ h before real-time PCR analysis was carried out, simulating the effects of a possible power cut before analysis could be started. This delay in analysis did not alter the sensitivities of the assays in any way.

## Results and discussion

Pooled organs of 62 small mammals (36 *Praomys* spp., 10 *Mastomys* spp., five *Lemniscomys* spp., three *Lophuromys* spp., four *Crocidura* spp. and four *Mus* spp.) caught in houses and around water bodies of a BU-endemic village (Ananekrom, in the Ashanti Region of Ghana; Fig. 1) as described before (Durnez *et al.*, 2008) were analyzed after DNA extraction using the modified Boom method (Boom *et al.*, 1990; Durnez *et al.*, 2009). Although none of the PCR reactions were inhibited, IS*2404* was not detected in any of the specimens.

A total of 148 environmental samples (13 water samples, 45 detritus samples, 45 trunk biofilm, and 45 plant biofilm samples) collected from water bodies near five BU endemic villages ($n = 117$) and two BU nonendemic villages ($n = 31$)

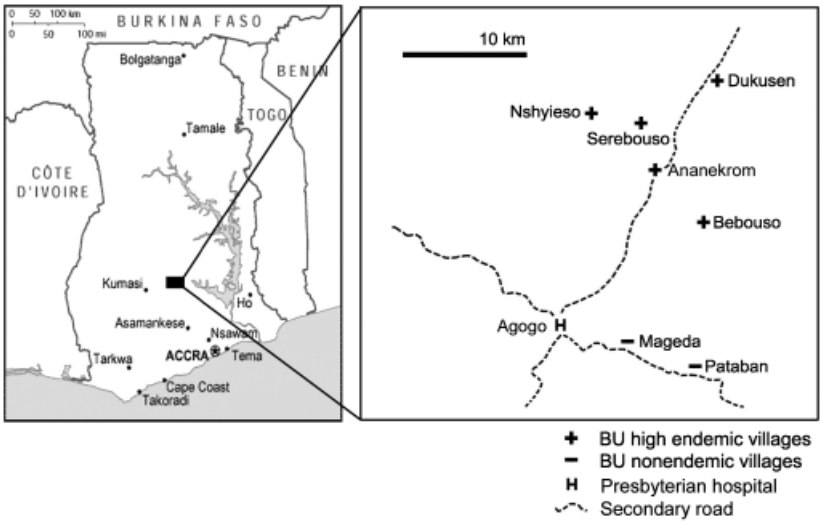


**Fig. 1.** The collection sites in the Ashanti Region, Ghana. Dukusen, Nshyieso, Serebouso, Ananekrom, and Bebouso are BU high endemic villages; Mageda and Pataban are BU nonendemic villages. The BU hospital is located in Agogo.

**Table 1.** $C_T$ values of environmental samples with positive real-time PCR results

| Locality | BU endemicity | Sample | $C_T$ (IS*2404*) | $C_T$ (IS*2606*) | $C_T$ (KR-B) | $\Delta C_T$ (IS*2606*-IS*2404*) |
|---|---|---|---|---|---|---|
| Nshyieso | High | Water | 36.31 | 38.27 | 37.6 | 1.96 |
| Serebouso | High | Trunk biofilm | 38.45 | ND | ND | NA |
| Pataban | Low | Plant biofilm | 37.95 | ND | ND | NA |

ND, not detected; NA, not applicable.

(Fig. 1) were also analyzed. Although the DNA extraction procedure included a purification step using diatomaceous earth, reactions in 50 of the 148 environmental specimens were inhibited as they had $C_T$ values of the IPC three cycles higher than the nontemplate controls. These inhibited samples were successfully reanalyzed with a newly developed environmental master mix adapted for real-time PCR-based detection in the presence of high levels of common environmental inhibitors (Applied Biosystems, TaqMan® Environmental Master Mix 2.0, ref. 4396838). Three samples (2.0%) were positive for IS*2404*, with $C_T$ values of 36.31, 38.45, and 37.95, respectively (Table 1). Of the three positive samples, only the water sample from Nshyieso also tested positive for IS*2606* and KR, with a $\Delta C_T$ (IS*2606*-IS*2404*) value of 1.96 (Table 1), suggesting that *M. ulcerans* DNA was detected and not DNA from other IS*2404*-containing mycobacteria that are known to have higher $\Delta C_T$ values (Fyfe *et al.*, 2007). The $C_T$ (IS*2404*) values of the other two IS*2404*-positive samples were higher than the sample that did contain IS*2606* and KR, suggesting that the failure to detect KR and IS*2606* was caused by a low DNA concentration, which is consistent with known differences in copy number per cell.

As such, we were only successful in showing that one sample (1/148; 0.6%) contained enough DNA to detect *M. ulcerans*. Our detection rate of *M. ulcerans* DNA differs considerably from the higher proportions described in a recent environmental study (Williamson *et al.*, 2008) performed in Ghana. Possible reasons for these discrepant results are: differing collection sites, collection during dissimilar seasons, and the analysis of different specimen types. Besides these reasons, the possibility of cross-contamination should not be disregarded.

The development of a suite of assays targeting multiple regions in the *M. ulcerans* genome enables a more sensitive and specific detection of this pathogen. Furthermore, the use of real-time PCR assays in BU-endemic countries for the detection of *M. ulcerans* could potentially increase chances of cultivating this pathogen from the environment, which has been shown to be very difficult (Portaels *et al.*, 2008), as PCR-positive samples can be cultured locally, without a loss in the viability of the organism because of transport to the country where analysis is performed. Additionally, environmental specimens can now be analyzed in a high-throughput approach with much greater confidence and with a reduced risk of false positives due to contamination. Furthermore, following the recent decline of real-time PCR consumable prices, the cost of real-time PCR analysis is comparable with that of conventional gel-based PCR.

However, the availability of basic laboratory facilities and a real-time thermocycler still remain prerequisites before application is feasible. Moreover, when applying this assay (as with all PCR-based assays), special care needs to be taken to avoid contamination, such as physical separation of pre- and post-PCR laboratories and extensive training of the laboratory staff.

In conclusion, the fluorescence-based real-time PCR assays for the detection of *M. ulcerans* were successfully adapted and applied at NMIMR. Although the reagents as well as the thermocycler used in the present study differed from those used by Fyfe *et al.* (2007), both studies achieved comparable sensitivities, even after a delay in the analysis of a prepared plate. The study also confirmed the presence of *M. ulcerans* in a water body in a BU-endemic area in the Ashanti region. The application of these real-time PCR assays in BU-endemic countries will thus contribute to improved studies on the environmental reservoir of *M. ulcerans*.

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