Quantification of *Mycobacterium tuberculosis* DNA using PCR and a simple dot blot-based DNA enzyme assay (DEA)

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The aim of this study was to develop a reliable and simple method for quantification of PCR-amplified DNA in order to determine accurately the *M. tuberculosis* (MTB) bacterial load in 7H12 broth.

Methods and results. Crude DNA was extracted from serial dilutions of $H37_{Rv}$ MTB McFarland 0.5 suspensions by sonication with glass beads and proteinase K digestion. For comparison, colony counts on solid media were also determined. The crude DNA extracted from bacterial suspensions and standard purified genomic MTB DNA (serial dilutions 160 fg–500 pg) were amplified using a strictly standardized PCR protocol (27 cycles) with primers specific for the IS6110 fragment[3]. The amplified 123 bp products and various amounts of standard purified 102 bp dsDNA (partially internal to the 123 bp fragment), ranging from 23–3000 fmol, were dot-blotted onto Nybond-N+ nylon membrane (Amersham) using the hybridot Manifold apparatus (Gibco, 6 × 6 mm/dot). Hybridization with a 102 bp probe directly labelled with peroxidase was performed according to methods described previously (Gene Detection System, Amersham)[2]. After high stringency washing the membrane was replaced in the dot blot apparatus and o-phenylendiamine dihydrochloride (OPD) added to each slot as substrate. After stopping the reaction by addition of H_2SO_4 to a final concentration of 1.5 N the soluble end products were transformed to a 96-well microtiter plate and read spectrophotometrically at 490 nm (Dynatech MR 7000). After stripping, repeated reprobing of the blot was possible. Results of the OPD-DEA were compared to Southern blot hybridization using the same probe and detection by enhanced chemiluminescence.

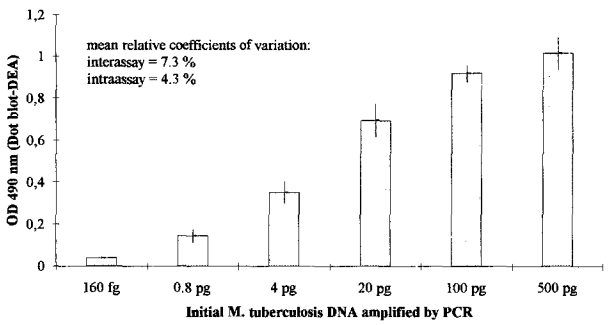

Figure 1. Dot blot-DEA quantification of *M. tuberculosis* DNA after PCR-based amplification. Results are mean ±SD for replicate determinations (n = 9).

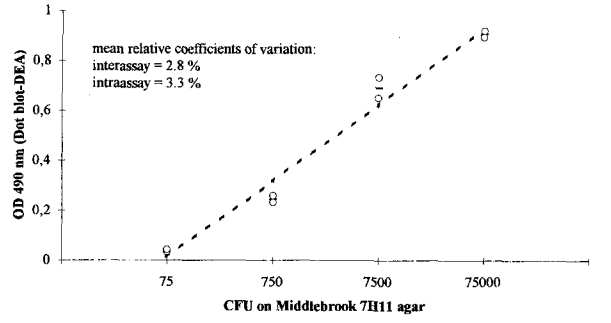

Figure 2. Determination of bacterial load using PCR and dot blot-DEA: correlation between CFU of *M. tuberculosis* and quantification of PCR amplicon. Results are mean ±SD for replicate determinations (n = 6). The dotted line represents the regression curve ($r^2 = 0.97$).

Sensitivity and reproducibility of the dot blot-DEA. The standard curve is linear for 102 bp dsDNA in the range of 30–1500 fmol. The assay curve is saturated with more than 2000 fmol due to spatial limitation of the dot surface. Sensitivity was comparable to conventional Southern blotting (detection limit 3–10 fmol). The intra- and interassay coefficients of variation were 4% and 11.4%, respectively (n = 10 in three independent experiments).

Quantification of MTB DNA. The ability of PCR dot blot-DEA to quantify MTB DNA is shown in figure 1. Although only 27 cycles were performed in order to obtain linear amplification, 160 fg of genomic DNA were still detectable (equivalent to approx. 80 bacteria). As shown in figure 2, determination of MTB bacterial load by DNA extraction and PCR-based dot blot-DEA correlated well with CFU.

Conclusions. Use of PCR for quantification of microorganisms has certain limitations. Methods currently under investigation, such as covalent binding of modified DNA onto the surface of microwells or coupling to magnetic beads with the aid of biotin-avidin, are hampered by problems concerning immobilization of DNA on a solid phase. Moreover, these strategies are laborious, including several washing steps and complicated detection systems (sandwich hybridization)[1].

We have developed an alternative method, exploiting the reliability of covalent DNA binding to positively charged nylon membranes enabling easy to handle direct hybridization. The method is adaptable for routine use in clinical laboratories. We have shown results of a quantitative assay to measure bacterial load of MTB. Quantification of MTB may have value in: (i) monitoring patients under anti-mycobacterial therapy, and (ii) early in vitro drug susceptibility testing.

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3 Eisenach, K. D., Cave, M. D., Bates, J. H., and Crawford, J. T., J. infect. Dis. *161* (1990) 977.