A SIMPLE METHOD FOR INHIBITION FREE PCR AMPLIFICATION OF TARGET DNA DIRECTLY FROM CLINICAL SPECIMENS.

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Preparation of the clinical specimens for PCR amplification has become an important aspect of nucleic acid probe based diagnosis and the final sensitivity of the diagnostic method is influenced by the sample processing method as PCR requires target DNA free of any potential inhibitors. The number of organisms in the clinical specimens like sputum is highly variable and it depends on various factors like the stage of infection, site of infection, the age of the patient. Handling highly infectious material like TB samples preferably should be easy (involve minimum number of steps), simple, economical (particularly for developing countries where the sample size is enormous) and yield at the same time PCR amplifiable target DNA.

Several methods have been published for processing sputa and blood samples for PCR diagnosis of *Mycobacterium tuberculosis* (1-5) involving several steps and reagents and are tedious and also increase the chances of cross contamination and infection of the laboratory personnel. As PCR does not require intact DNA for amplification such elaborate and expensive procedures are not required. We report here a very simple, universal method for preparing the target DNA for PCR amplification from mycobacteria present in various clinical specimens and the method can easily be adopted for any sample.

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The sample processing is done in two stages, stage I and stage II. In the first stage the clinical specimen is thoroughly washed and in the second stage the DNA is released for PCR amplification. The number steps in the first stage depends on the type of clinical specimen. Any specimen contaminated with blood needs to be thoroughly washed free of last traces of blood. The only reagent that is required is the lysis buffer. The composition of the lysis buffer (TEX buffer) is Tris.Cl (pH 8.0) 10mM, EDTA 5mM and Triton X-100 1% (V/V in water).

All operations with clinical specimens are carried out in the biological safety hood of level 2 as the process generates aerosols. All precautions necessary for this category of biosafety must be followed.

Stage 1: Biopsy tissues: the sample is collected in normal saline containing 5mM EDTA. The tissue is minced on a clean porcelain tile with the flat surface of a clean scalpel, transferred quantitatively to a 1.5 ml eppendorff tube, mixed by vortexing with 1 ml of TEX buffer and centrifuged at 12000 rpm in a microfuge for 10 min. The washing with TEX buffer, vortexing and centrifugation is repeated until the supernatant is colourless. The supernatant is discarded.

Formalin fixed tissues: Tissues in formalin are washed once in normal saline and soaked overnight for rehydration in TEX buffer at 37°C. The tissue is minced and processed as described above for fresh biopsies.

Paraffin embedded tissues: The tissue mate-

rial is carefully released from the wax by slicing the wax block into 1.5 ml eppendorff tubes. One ml (or 2 volumes of xylene) of xylene is added to each tube, allowed to stand at room temperature for 2 h and centrifuged at 12,000 rpm for 15 min in a microfuge at room temperature and the supernatant discarded. One ml of xylene is added and allowed to stand at room temperature for 30 min and the tissue is pelleted at 12,000 rpm in microfuge for 15 min. One ml of TEX buffer is added to the pellet, vortexed, incubated at 37°C overnight and centrifuged as above. The supernatant is discarded.

Blood samples: Blood samples (0.5 ml) are collected in 5 mM EDTA as anticoagulant, centrifuged at 12,000 rpm for 10 min in a microfuge at room temperature. The pellet is vortexed briefly, mixed by vortexing (one min continuously) with one ml of TEX buffer and centrifuged as above. Washing of the pellet is repeated (3-4 times) as above till the supernatant is colourless. The supernatant is discarded.

Sputum samples: 0.5 ml of the sputum sample is processed as described for blood samples except that the pellet required washing only twice with the TEX buffer.

Stage 2: Common for all samples. The pellet obtained from stage 1 is thoroughly resuspended in 100 µl of TEX buffer and boiled for 20 min (varies with the type of micro organism, results in 95% lysis of mycobacteria as observed under microscopy) in a boiling water bath and 3-5 µl of the lysate is directly taken for every 100 µl of PCR mixture.

The lysate thus prepared is stable (for 6-12 months) at 4°C for PCR analysis. The TEX buffer has been successfully employed in our laboratory for amplification of specific sequences from various *E. coli* transformants (requires only 3-5 min direct boiling in TEX buffer), *Bacillus sphaericus*, *Bacillus thuringiensis* (requires about 10-15 min direct boiling in TEX buffer) etc.

For PCR amplification 1 µl of lysate is subjected to 30 cycles of amplification in 30 µl of PCR mix containing 1X buffer (10x buffer composition is Tris.Cl. pH 8.8, 20 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 1 mM DTT, 100 g ml⁻¹ BSA and 0.1% Triton X-100, 2mM dNTPs, 100 u moles of genus primers (6) or *M. tuberculosis* specific IS6110 (2) primers or TB400 (5) primers with 1 unit of Taq DNA polymerase.

Fig. 1 PCR amplification of the mycobacterium genus specific (lane 1) and lack of amplification of *M. tuberculosis* specific products (lane 2 with IS6110 primers and lane 3 with TB400 primers) from cervical lymph node biopsy stored in formalin. Primary isolation by culture showed the presence of non-tuberculous mycobacteria in this biopsy. **(B)** shows the amplification of genus specific (and absence of *M. tuberculosis*) PCR product (lane 1) from uterine endometrial biopsy and absence of *M. tuberculosis* with IS6110 primers (lane 2) and TB400 primers (lane 3). Culture was negative in this case.

Fig. 2 Amplification of *Mycobacterium* genus (lane 1) and *M. tuberculosis* specific (lane 2) DNA sequence from the formalin fixed wax block of intestinal (endoscopy) biopsy.

Fig. 3 (a) Presence of *M. tuberculosis* specific PCR products from blood samples of HIV positive patients amplified with IS6110 primers (lane 2, lane 1, ϕ x 174RF HaeIII marker) and (b) with TB400 primers.

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