Mycobacteriology

Specific amplification of gene encoding N-terminal region of catalase–peroxidase protein (KatG-N) for diagnosis of disseminated MAC disease in HIV patients

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ABSTRACT

Disseminated Mycobacterium avium-intracellulare complex (MAC) infection is considered as severe complication of advanced HIV/AIDS disease. Currently available various laboratory investigations have not only limited ability to discriminate between MAC infection and tuberculosis but are also laborious and time consuming. The aim of this study was, therefore, to design a molecular-based strategy for specific detection of MAC and its differentiation from Mycobacterium tuberculosis (M. tb) isolated from the blood specimens of HIV patients. A simple PCR was developed based on the amplification of 120-bp katG-N gene corresponding to the first 40 amino acids of N-terminal catalase–peroxidase (KatG) protein of Mycobacterium avium that shows only ~13% sequence homology by clustal W alignment to N-terminal region of M. tb KatG protein. This assay allowed the accurate and rapid detection of MAC bacteremia, distinguishing it from M. tb in a single PCR reaction without any need for sequencing or hybridization protocol to be performed thereafter. This study produced enough evidence that a significant proportion of Indian HIV patients have disseminated MAC bacteremia, suggesting the utility of M. avium katG-N gene PCR for early detection of MAC disease in HIV patients.

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1. Background

Among the various environmental mycobacteria, opportunistic Mycobacterium avium-intracellulare complex (MAC) organisms are the most common nontuberculous mycobacteria (NTM) causing disseminated infections in HIV/AIDS patients (Guthertz et al., 1989; Horsburgh, 1991) with an annual frequency reported to be 10–20% worldwide. Clinically, Mycobacterium avium member of MAC group is the most prominent cause of infection in HIV/AIDS population contributing to morbidity and mortality in 40–57% of these patients (Griffith et al., 2007). Although the manifestations of MAC disease can be confused with signs and symptoms of tuberculosis caused by Mycobacterium tuberculosis (M. tb), yet, there is a significant difference in terms of control measures adopted (Salfinger, 2006) that demands for their accurate differential diagnosis. The 2007 American Thoracic Society (ATS) guidelines also recommend that

prior to treatment, the clinically significant mycobacteria should be identified to the species level (Griffith et al., 2007). Conventional diagnostic methods based on radiological pictures, acid fast staining of direct smear, tuberculin skin test, and cultures do not distinguish between MAC and M. tb disease (Swaminathan et al., 2010). The biochemical testing–based phenotypic characterization is, although species specific, is quite laborious and requires an additional 2- to 4-week period after 6–8 weeks of initial isolation.

The more promising field of molecular biology, which targets various conserved DNA or RNA sequences for amplification, provides the more sensitive and specific diagnosis in a time period of as short as 1 day and can be applied even in the direct clinical specimens. The various target genes described for identification and typing of clinically relevant MAC majorly include IS1245, mig, rpoB, etc. However, even after the vast improvement in the field of mycobacterial diagnostics, no single target sequence has been so far able to replace the conventional diagnostic procedures due to their limitations. IS1245 does not hybridize with 25% of human isolates as per the study of Ritacco et al., 1998; mig gene, although specific for MAC, has been reported to have low sensitivity (Koivula et al., 2004), and rpoB sometimes results in the misidentification of some NTM strains as M. tb complex (MTC) (Simmon et al., 2010). A commercial hybridization

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assay (AccuProbe, San Diego, CA, USA) using DNA probes, which is specific for the MTC, MAC, *Mycobacterium kansasii*, and *Mycobacterium gordonae*, has been developed. This assay has dramatically reduced the time to identify and differentiate the mycobacterial infection directly from the solid/liquid growth cultures (Alcaide et al., 2000), but it suffers from the limitations of cost and requires multiple reactions (Chimara et al., 2008). On the other hand, the high level of inter-species polymorphism, intra-species conservation, and the good reproducibility of 16S-23S internally transcribed spacer (ITS) region make it suitable for the rapid and definitive identification of clinically relevant mycobacterial isolates at the species level (Park et al., 2000; Roth et al., 2000; Xiong et al., 2006); however, specialized equipment limits its routine implementation in diagnostic laboratories. Thus, there is an urgent need to develop a PCR method based on the alternative genomic targets that could provide an efficient, inexpensive, and sensitive diagnosis of MAC directly in the clinical specimens of HIV patients.

Previously in our lab, 82-kDa immunodominant *M. avium* KatG protein (MAV_2753), a catalase–peroxidase enzyme, was identified in native culture filtrate having >90% homology with *M. avium* subspecies and *M. intracellulare*, but only 65% homology was depicted starting from the 41st amino acid with *M. tb*. When tested for its diagnostic potential in a small cohort of HIV$^+$MAC$^+$ patients, *M. avium* KatG–based antibody detection assay demonstrated ~90% sensitivity for MAC (Gupta et al., 2009). Upon Clustal W alignment, sequence of first N-terminal 40 amino acids of *M. avium* KatG protein (KatG-N) was found to be only ~13% similar to first N-terminal 40 amino acids of *M. tb* KatG. As KatG-N sequence is almost similar in the members of MAC, which do not require differentiation due to their similar line of treatment, the present study focused on the development of PCR assay based on *M. avium*–specific 120-bp nucleotide region of *katG* gene (MAV_2753) encoding the first 40 amino acids of the KatG protein for rapid identification of MAC bacteremia and its accurate differentiation from *M. tb* in HIV patients.

2. Methods

2.1. Mycobacterial cultures

Various reference species used for the study included *M. tb* H37Rv (National Collection of Type Cultures [NCTC] 7416, London), *Mycobacterium bovis* (NCTC 10772, London), *M. avium* 104 (microbial type cultures collection [MTCC] 1723, NCTC 8551), *Mycobacterium smegmatis* (MTCC 6, NCTC 8152), *Mycobacterium fortuitum* (MTCC 1902, NCTC 10394), and *Mycobacterium phlei* (MTCC 1724, NCTC 8151). These organisms originally obtained from the NCTC, London, were procured from MTCC, IMTECH Sector-39, Chandigarh, India, and grown in liquid Youman's media (Subrahmanyam, 1964).

2.2. Categories of the subjects

This was a 2-year case-control prospective study in which peripheral blood samples were collected from HIV patients attending the ART center in the Department of Internal Medicine of PostGraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. HIV patients with mycobacterial diseases (n = 150) were positive for rapid HIV screening tests, i.e., Coombs AIDS, Tri Dot, and HIV ELISA (as per the guidelines of National AIDS Control Organization [NACO], India) with more than 95% of the patients having CD4$^+$ T-cell counts in the range of 0–400 cells/μL. These were selected on the basis of smear positivity in one of the body fluids (sputum, bronchoalveolar lavage [BAL], or fine needle aspiration cytology [FNAC]). Their chest radiographs showed abnormal infiltrate shadows, nodular cavitary lesions, or bronchiectasis and/or multiple small nodules with clinical manifestations such as fever, cough with or without expectoration, peripheral lymphadenopathy, weight loss, loss of appetite, vomiting, headache, and few of them with raised ALP (alkaline phosphatase)

levels (Rolla et al., 1999). HIV patients without any signs and symptoms of mycobacterial disease (n = 100) were positive for rapid HIV screening tests with more than 90% of the patients having CD4$^+$ T-cell counts up to 800 cells/μL. These had no radiological or clinical evidence of tuberculosis; body fluids being smear negative and no known contact with tuberculosis (TB) patients or their samples. HIV-negative healthy Bacillus Calmette–Guerin–vaccinated or non-vaccinated asymptomatic individuals (n = 100) had no signs and symptoms of HIV or mycobacterial disease. These healthy volunteers had no clinical evidence of tuberculosis and had no known contact with TB patients or their samples or with HIV patients' body fluids. Clinical information including sex, age, previous complications, history, and other parameters was obtained from each patient at the time of sample collection. The present study was approved by the Institute Ethics Committee, PGIMER, Chandigarh. Five milliliters of venous blood was withdrawn from the subjects under aseptic conditions after taking the informed written consent (2 mL in heparin for culturing the mycobacterial organisms and 3 mL in acid citrate dextrose for isolating DNA for PCR amplification).

2.3. Culture of mycobacteria from blood

Two milliliters of heparinized blood (100 IU of heparin per milliliter of blood) was cultured by the method of lysis centrifugation (Salfinger et al., 1998). Briefly, the blood was mixed with 0.3% sodium desoxy-cholate (Himedia, India) in the ratio of 1:6 for red blood cell (RBC) lysis. After 10-min incubation at room temperature, centrifugation was done at 3500 × g for 25 min. The pellet was mixed with 100 μL of 0.2% bovine serum albumin (BSA), plated on Löwenstein–Jensen (LJ) slants and 7H11 agar plates, and incubated for 6–8 weeks at 37 °C. The plates were examined once a week, and the colonies were tested for Ziehl–Neelsen (ZN) staining for acid-fast bacilli (AFB).

2.4. Phenotypic and biochemical identification

AFB-positive blood culture isolates were identified to the species level by following the conventional method of phenotypic identification based on colony morphology, pigment production, and a battery of biochemical testing, namely, niacin production, nitrate reduction, urease production, heat stable catalase production, tellurite reduction, tween 80 hydrolysis, and NaCl tolerance test (Kent and Kubica, 1985). Biochemical tests were performed twice or thrice, and 2/2 or 2/3 positivity was taken as the criteria for identification of mycobacterial species.

2.5. DNA extraction

The culture of the reference mycobacterial species as well as AFB-positive clinical isolates obtained from blood were subjected to DNA isolation (Deshpande et al., 2007; Hill et al., 1972). Briefly, 1 loop-full of mycobacterial culture was suspended in 500 μL of 1× Tris-EDTA (TE) (10 mmol/L Tris–HCl, 1 mmol/L EDTA) buffer and incubated at 37 °C for 2 hours with intermittent shaking. The suspension was lysed with 0.5% sodium dodecyl sulphate (SDS), and proteins were digested with 0.5 mg/mL proteinase-K at 55 °C for 15 min. Eighty microliters of 10% cetyltrimethylammonium bromide in 0.7 mol/L NaCl and 100 μL of 5 mol/L NaCl were added and incubated at 65 °C for 10 min. Aqueous layer was extracted by adding an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v), and DNA was precipitated overnight with 0.7 volumes of isopropanol at +4 °C. The DNA was washed with 70% ethanol and suspended in 20-μL TE buffer at 65 °C overnight.

For isolating mycobacterial DNA directly from the 3-mL blood collected in anticoagulant acid citrate dextrose (ACD: 0.25 g citric acid, 0.65 g sodium citrate, and 0.75 g glucose in 50 mL double distilled water [DDW]), an additional step of RBC lysis was performed in which RBC lysis buffer (320 mmol/L sucrose, 5 mmol/L MgCl$_2$, 10 mmol/L