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Novel ESAT-1081 Multiplex PCR for Direct Detection and Identification of BCG Strains from Other Members of *M. tuberculosis* Complex

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Abstract: Bacille Calmette Guerin (BCG) was initially used as vaccine against tuberculosis, however, it uses for verity of clinical application. Detection of adverse effects and complications of BCG is essential for necessary timely quality control of the vaccine, administration techniques and its application Direct microscopy and culture are still the methods of choice for detection of BCG in most laboratories, but they are very difficult processes and time-consuming. An ESAT-IS1081 multiplex PCR amplification of targeted gene segments was optimized to detect and differentiate the BCG strains from other members of *M. tuberculosis* complex. The sensitivity of detection for H37Rv, *M. bovis* and BCG were dilution of 10^4,10^5 and 10^5 cells mL^{-1} of cell stock suspension, respectively. The results from this study showed that the ESAT-IS1081 multiplex PCR assay permits a specific, sensitive and reproducible system for the detection and differentiation of the BCG, thereby improving the clinical management of BCG complication to clinician.

Key words: Bacille calmette guerin, adverse effect, complication, multiplexe PCR

INTRODUCTION

Bacille Calmette Guerin (BCG), a live attenuated strain of *Mycobacterium bovis*, was initially used as vaccine against tuberculosis. Since 1976, BCG remains the anticancer agent of choice and the most effective available treatment option for non-muscle invasive urothelial carcinoma (Fanghong *et al*., 2005; Eleonora *et al*., 2005; Morales *et al*., 1976). Repeated doses of BCG have therapeutic effect on human asthma accompanied with allergic rhinitis (Jing *et al*., 2005). There is evidence that BCG has therapeutic and prophylactic effects in patients with severe oral aphthosis (Sharquie and Hayani, 2005). Serious side effects of BCG vaccination in immunocompromised patients have been reports from many countries (Lamm *et al*., 1992; Dhamija *et al*., 1998; Viallard *et al*., 2002; Naranjo *et al*., 1981; Lotte *et al*., 1984; Lotte *et al*., 1988; Milstien and Gibson, 1990; Sasmaz *et al*., 2001; Ali and Almoudaris, 2004). Adverse effects and complications of BCG vaccination occur in 1-10% of vaccinated person according to the CDC (1988). In recent years, a series of inherited disorders of the IL-12-IFN-c axis have been described as predisposing factor for disseminated BCG infection after vaccination (Mansouri *et al*., 2005). The severe and long-lasting complications that are seen after BCG immunotherapy are characterized by local or systemic manifestations. Although in all country that

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uses BCG, not all complications of BCG vaccination and immunotherapy have been established, however, each adverse effect should be reported, because it's monitoring enables quality control of the vaccine, administration techniques and its application. Detection and dentification of BCG complications in clinical laboratory is a very difficult process. Microscopic examination and culture are still the methods of choice for detection of BCG in most laboratories. Quick differentiation between BCG and other members of *M. tuberculosis* complex is difficult for most laboratories. The recently developed nucleic acid amplification (NAA) methods may provide us with very sensitive, specific and rapid tests for detection Mycobacteria. We describe the ESAT-IS1081 multiplex-PCR method using in mycobacterial laboratory for direct detection and identification of BCG strains from other members of *M. tuberculosis* complex.

MATERIALS AND METHODS

DNA extraction: Prepared cell stock suspension containing 2×10^4 -10^5 cells mL^{-1} of *M. tuberculosis* (H37Rv), *M. bovis* and BCG was diluted from 10 to10^7. DNA from a 250 µL aliquot of each of the cell stock was extracted for SDS/Lysozyme extraction method (Bahador *et al*., 2004).

Primer designing: Two sets of primers, each set of which was specific for esat-6 and IS-1081 genes of *M. tuberculosis* complex, were designed to develop ESAT-IS1081 multiplex PCR assay. The primers specific for ESAT-6(5`-ATGCAGAGCAGCAGTGGAA-3`,5`-TTTGCTGGACACCCTGGTA-3`) enclosed a 169 bp fragment in the *esat-6* gene, located at positions 13 to 181 of the sequence available under GenBank accession number AF420491 (Table 1). The primers specific for IS-1081(5`-CGAATCAGTTGTTGCCCAAT-3`,5`-GTTCTTCGGTGCTGGTCAGT-3`) enclosed a 344 bp fragment located at positions 85 to 428 of the IS-1081 gene (GenBank accession number MBBIS1081).

Optimization of multiplex PCR: Samples were amplified by PCR in 25 µL reaction mixtures containing: 1 µL of 10 mM dNTP, 1 µL of 20 pM of each primer (Table 1), 0.25 µL of 500 U Taq polymerase (Fermentas), 3 µL of 25 mM $MgCl_2$, 2.5 µL of 10X PCR buffer and 5µL of DNA template. Samples were subjected to 40 PCR cycles, after 4 min of primary denaturation at 95°C, each cycle consisting of, 30 sec of denaturation at 95°C, 30 sec of annealing at 56°C and 45 sec of elongation at 72°C. After 5 min of elongation at 72°C, PCR products were then electrophoresed on 1.2% agarose gels and stained with ethidium bromide.

Table 1: Primers used in ESAT-IS1081 multiplex PCR

Primer name	Sequence	G C%	Length
F-IS1081	5`-CGAATCAGTTGTTGCCCAAT-3`	45	20
R-IS1081	5`-GTTCTTCGGTGCTGGTCAGT-3`	55	20
F-esat-6	5`- ATGACAGAGCAGCAGTGGAA-3`	50	20
R-esat-6	5`-TTTGCTGGACACCCTGGTA-3`	52	20

RESULTS

Multiplex PCR from cell stock suspension successfully allowed the detection of all three mycobacterial stains. Present experiments show that ESAT-IS1081 multiplex PCR can differentiate BCG from *M. bovis* and H37Rv. The molecular weights of the target genes, esat-6 and IS1081 were 0.169 and 0.344 kbp, respectively. Results for the tested strains are shown in the Fig. 1.

Specificity: All BCG, *M. bovis* and H37Rv strains tested were positive for IS1081. However, the BCG strain was negative for esat-6 gene, while *M. bovis* and H37Rv strains were positive for esat-6 (Table 2) .

Sensitivity: The ESAT-IS1081 multiplex PCR assay was used to detect H37Rv, M bovi and BCG in cell stock suspension with different concentrations of target bacteria. The diluted detection limit was found to be about 10^4, $10^5$and 10^5 CFU per mL^{-1} of H37Rv, M bovis and BCG suspension, respectively.

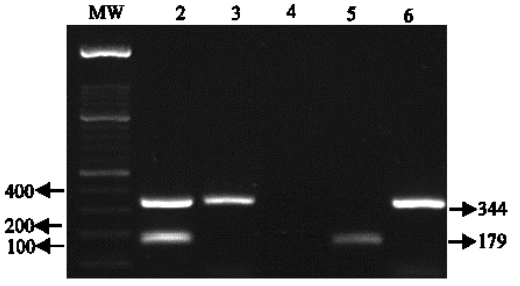

Fig. 1: 2% Ethidium bromide-stained gel of multiplex PCR products. Lane MW: 100-bp molecular size marker; lane 2: *M. bovis*; lane 3: BCG (vaccine strain); lane 4: negative control; lane 5: *M. tuberculosis* (single ESAT-PCR); lane 6: *M. tuberculosis* (single 1081-PCR)

Table 2: ESAT-IS1081 multiplex reaction with DNA of H37Rv, *M. bovis* and BCG

Strain	Multiplex PCR *esat-6*, IS1081	Single PCR *esat6*, 179 bp	Single PCR IS1081,344 bp
H37Rv	+/+	+	+
M. bovis	+/+	+	+
BCG	–/+	–	+

DISCUSSION

The side effects that are seen after BCG vaccination are divided into two groups, specific and nonspecific. Specific side effects are directly related to BCG and they contain lupus vulgaris, Koch phenomenon-like reaction, severe local adenitis, regional subcutaneous abscess, osteitis, distant tissue tuberculosis (Sasmaz *et al*., 2001) lethal disseminated infection in infants with severe combined immunodeficiency syndrome, Chronic Granulomatous Disorder (CGD) HIV infection and immunosuppressive therapy (Casanova *et al*., 1995; Jouanguy *et al*., 1966) generalized adenitis (Clic *et al*., 2004; Santos *et al*., 2004) bilateral panuveitis, optic neuritis (Hegde and Dean, 2005) granulomatous pneumonitis (Martin-Escudero *et al*., 2003) cutaneous mycobacterial infection (Ng *et al*., 2006) regional lymphadenopathy, local draining sinuses, purulent drainage at the puncture site (Daoud *et al*., 2003) and mycobacterial spindle cell pseudotum or of the auxiliary lymph nodes (Hong-Lin *et al*., 2004). Non-specific side effects are epithelial cyst, keloid formation eczematous changes, granulomas, generalized hemorrhagic rashes and erythema nodosum (Sasmaz *et al*., 2001).

In Lotte *et al*. (1984) classified complications associated with BCG vaccination based on clinical, bacteriological, histological and biological information in detail. Category 1 involves extensive regional suppurative lesions and lymphadenitis. Categories 2 are included non-fatal cases (localized or multiple changes). Fatal cases (generalized lesions usually associated with immunodeficiency) are in category 3. Category 4 includes side effects which occur upon BCG administration (Keloid formation), but not definitely confirmed either microbiology or histologically. Some risk factors of BCG vaccination are related to the vaccine itself (certain strains of vaccine), administration of vaccine (intradermal route, high doses, etc.) and age of children being vaccinated (Higashi *et al*., 2005). BCG immunotherapy is an effective and widely used treatment for superficial bladder cancer. Local complications (cystitis, hematuria, prostatitis, contracted bladder, epididymoorchitis, ureteral obstruction, renal abscesses, epididymitis, arthralgia and/or arthritis, sepsis and rash) are frequent, where-as systemic complications are rare, but life-threatening. Chorioretinitis (Guex-Crosier *et al*., 2003) pure squamous cell carcinoma (Yurdakul *et al*., 2005) granuloma (Eleonora *et al*., 2005) renal toxicity and progressive renal dysfunction, pneumonitis, hepatitis, psoas abscess, sepsis, central nervous system infection, sepsis-like syndrome with multi organ involvement (Elmer *et al*., 2004; Grange *et al*., 1998) and vertebral osteomyelitis (Eleonora *et al*., 2005) may occur with disseminated infection after intravesical BCG therapy in superficial bladder cancer (Casanova *et al*., 1995; Jouanguy *et al*., 1966; Modesto *et al*., 1991). Systemic BCG infection should be suspected in any patient who presents with persistent fever after BCG instillation for bladder cancer. Due to increased utilization of BCG and safety considerations, each adverse effect should be reported in countries that used BCG. This necessitates the development of high sensitive diagnostic methods for the detection of BCG complication. Acid fast bacilli (AFB) microscopic examination and culture are still the methods of choice and traditional diagnostic methods for diagnosis of BCG complication in most laboratories (Rosenzweig *et al*., 2006). Isolation of BCG from different sites not associated with vaccination may be confused with *M. tuberculosis*. The BCG strain cannot, with confidence, be differentiated from other members of *M. tuberculosis* complex on biochemical tests alone. A disadvantage of using the conventional method is the long incubation time (3 to 8 weeks). A characteristic of BCG strains is the deletion of the genomic region RD1, a 9,505 bp region, that present in all *M. tuberculosis* complexes except BCG strain (Mahairas *et al*., 1996). Detection of RD1 forms the basis of a multiplex PCR to detection BCG strains. The RD1 encompasses the genes Rv3871 to Rv3879c (Cole *et al*., 1998) which include esat-6, the genes for the 6 kDa early-secreted antigen (Berthet *et al*., 1998). The esat-6 gene is situated immediately and it's suitable for early diagnosis of tuberculosis (Van Pinxteren *et al*., 2000). Kearns *et al*. (1999) designed multiplex PCR to amplify the sequence of RD1. Their assay was based on three primers ET1, ET2 and ET3. Another PCR procedure based on multiplex PCR was reported to be able to distinguish *M. bovis* BCG Tokyo 172 from other *M. tuberculosis* complex strains (Okazaki *et al*., 2005). Our novel multiplex PCR contains primers for ESAT-6 and 1081 genes, for the purpose of direct detection and quick identification of the Pasture and Tokyo BCG strains. In summary, the analytical sensitivities derived from a 10-fold serial dilution of BCG, indicated that ESAT-IS1081 Multiplex PCR is a sensitive, rapid, simple and effective method for direct detection and identification of BCG strains from other members of *M. tuberculosis* complex.

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