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ORGINAL PAPER

The Capacity of *Mycobacterium tuberculosis* Complex Species and *M. bovis* BCG Substrains Specific Identification – Implications for Optimized PCR-Based Diagnostics in Adverse Events Following Vaccination Suspected Cases

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Abstract

The capacities of differentiation of *Mycobacterium bovis* BCG from other members of *M. tuberculosis* complex species using PCR-RFLP, multiplex PCR, and PCR-based genomic deletion analysis approaches were compared. In the study, mycobacteria isolated from patients suspected of adverse events following vaccination with BCG, primarily classified according presence of RD1 marker as virulent and avirulent mycobacteria, were used. The PCR-based genomic deletion analysis was found the best option for mycobacteria diagnostics improvement, as it was capable precisely differentiate virulent and avirulent mycobacteria or virulent species of *M. tuberculosis* complex. The routine confirmation of mycobacteria species in the cases of adverse events following BCG vaccination is highly expected, especially in clinical practice of patients with primary immunodeficiency.

Key words: *Mycobacterium bovis* BCG, mycobacteria specific identification, PCR-based diagnostics

Introduction

Although extensive studies have been performed to develop advanced vaccines against tuberculosis (TB), vaccination with attenuated *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) is the only commercially available vaccine against TB (WHO, 2011). The BCG vaccine is used worldwide (Brewer, 2000) and generally is regarded as being the safest in use, but the real incidence of disease evoked by BCG strains, *e.g.* BCG-itis or osteitis, is not known (Behr, 2002). Localized abscesses, regional lymphadenopathy, and disseminated disease in immunocompromised hosts are generally regarded as rare but well recognized complications following BCG vaccination (Bernatowska *et al.*, 2007). Abscesses at BCG injection sites and in places other than BCG injection sites have also been described in healthy hosts (Pankowska and Rożniecki, 1997; Gołębiowska *et al.*, 2008). In Poland, BCG vaccination has been a part of the National Immunization Program since 1951, and currently involves a single BCG dose given to neonates only. BCG AEFI (Adverse Events Following Immunization) cases are registered through passive surveillance and most of them are not routinely confirmed in the laboratory. The rate of AEFI in Poland is regarded as low (Szczuka, 2002), however, lack of simple and routine diagnostics does not allow to estimate its real incidence. Lack of routinely used species-specific diagnostics also complicates fast and reliable diagnosis of mycobacteria grown from BCG vaccinated individuals suffering from immunodeficiencies.

The *M. tuberculosis* complex consists of mycobacteria characterized by high DNA-DNA homology (from hybridization studies) and identical 16S rRNA sequences but differ in phenotypes and pathogenicity. This group has consisted of seven closely related species: *M. tuberculosis* – infecting human and primates; *M. africanum*, a heterogeneous group causing human tuberculosis in Africa; *M. microti*, found as a source of infection in voles and very rarely in immunocompromised humans; *M. bovis*, causing tuberculosis in cattle and a wide variety of other animals, including man; *M. bovis* Bacille Calmette-Guérin (BCG), an attenuated vaccine strain; *M. canetti*, a very rare, smooth variant of *M. tuberculosis*, usually isolated in Africa; and *M. caprae*, primarily causal agent of caprine TB with transmission to human and wild animals reported (Brosch *et al.*, 2002; Cousins *et al.*, 2003; Rodríguez

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et al., 2009). More recently, novel species has been described: *M. mungi,* pathogen inducing high mortality rates among banded mongooses living in close association with humans in Botswana (Alexander *et al.*, 2010); *M. pinnipedii,* previously known as seal bacillus primarily infecting pinnipeds but is also pathogenic humans and, possibly, cattle (Cousins *et al.*, 2003) and *M. orygis,* causative agent of tuberculosis in oryxes, gazelles, and waterbucks in Africa; cows and rhesus monkeys in South Asian; and humans (van Ingen *et al.*, 2012).

It has been estimated that as many as 49 BCG substrains might be used in the world (Corbel *et al.*, 2004) since the original Bacille Calmette-Guérin attenuated strain has been distributed worldwide. BCG daughter strains were found heterogenic as the result of the microevolution due to specific production processes, which can be currently specifically identified with molecular tools (Behr, 2002).

The use of reliable molecular tools applicable for confirmation of species identity in AEFI suspected cases are expected to precisely identify all *M. tuberculosis* complex representatives together with a possibility to identify *M. bovis* BCG substrains. In order to evaluate the capacities of molecular diagnostic tools described previously as potentially applicable for *M. tuberculosis* complex identification, in this pilot study we have used reference mycobacteria species and randomly chosen mycobacteria isolates grown from AEFI suspected cases and collected at the National Tuberculosis and Lung Diseases Research Institute (NTLDRI). The mycobacteria isolated from patients and chosen for the purpose of the study have been primarily classified as virulent and avirulent mycobacteria, as they were found habour and not harbour RD1 marker, respectively. Three potentially applicable variants of PCR-based methods involving different species-specific or substrain-specific sequences published previously were introduced for testing and compared.

Experimental

Material and Methods

Strains. A total of 10 mycobacterial strains isolated from BCG AEFI suspected cases within 2004–2010, have been included in the study. At NTLDRI they were diagnosed as virulent (7575, 3221, 1339, 5995, 5379) and avirulent (60, 4138, 868, 1078, 2714) mycobacteria according PCR performed with primers specific for RD1 region (Talbot *et al.*, 1997). All strains were grown on solid Löwenstein-Jensen medium for about 20 days at 37°C, harvested, heat inactivated. The following reference strains were used: *M. bovis* BCG Moreau originating from BCG vaccine lot no. 00108, *M. bovis* BCG Danish 1331 (NIBSC, 07/270), *M. bovis* BCG Tokyo 172 (NIBSC, 07/272), *M. tuberculosis* H37Rv (ATCC 25618), *M. bovis* (ATCC 19211), *M. microti* (ATCC 19422), *M. africanum* (ATCC 25420), *M. pinnipedii* (ATCC BAA-688), and *M. canetti* (kindly obtained from Prof. Dr. Dick van Soolingen from Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment, The Netherlands).

DNA extraction. Chromosomal DNA was isolated from mycobacterial strains as described by van Soolingen *et al.* (1991).

PCR. In the study we have tested PCR-RFLP developed by Kasai *et al.* (2000), PCR-based genomic deletion analysis described by Warren *et al.* (2006) and multiplex PCR developed by Bedwell *et al.* (2001). System developed by Kasai *et al.* (2000) involved restriction of amplified product of the partial *gyr*B gene with *Rsa*I or *Taq*I enzymes. PCR-based genomic deletion analysis involved PCR method with use of primers specific for RD1, RD4, RD9, and RD12 regions (Warren *et al.*, 2006). In a multiplex PCR described by Bedwell *et al.* (2001) primers specific for RD1, RD2, RD8, RD16 and the *senX3-regX3* regions were primarily developed for purposes of identification of different substrains of *M. bovis* BCG.

Results

Among 10 mycobacteria isolates, 5 primarily classified at the NTLDRI as avirulent mycobacteria (60, 4138, 868, 1078, 2714), were easily identified as *M. bovis* BCG Moreau isolates (Fig. 1A) as they showed a multiplex-PCR profiles presence of 196 bp (ΔRD1), 252 bp (RD14), 276 bp (*senX3-regX3*), 315 bp (RD2) and the 472 bp (RD8) amplified fragments. Multiplex-PCR profiles of reference *M. bovis* BCG Danish and *M. bovis* BCG Tokyo substrains according of size of amplified fragments were easily differentiated from *M. bovis* BCG Moreau substrain. Other 5 mycobacteria isolates, classified at the NTLDRI as virulent (7575, 3221, 1339, 5995, 5379), presented profiles different from those of *M. bovis* BCG reference strains and samples of avirulent mycobacteria. In the profiles of mycobacteria samples, pre-classified as virulent, there were found products seen in the profiles of all *M. tuberculosis* complex reference strains. The amplification profiles of virulent mycobacteria samples were composed of fragments RD1 (146 bp), RD14 (252 bp), RD2 (315 bp), RD16 (401 bp), RD8 (472 bp) regions but did not contained the PCR product expected for the *senX3-regX3* region. As multiplex PCR profiles did not contained 352 bp and/or 276 bp products, single PCR reactions were performed with s*enX3-regX3* region primers revealing their presence and suggesting the inhibition

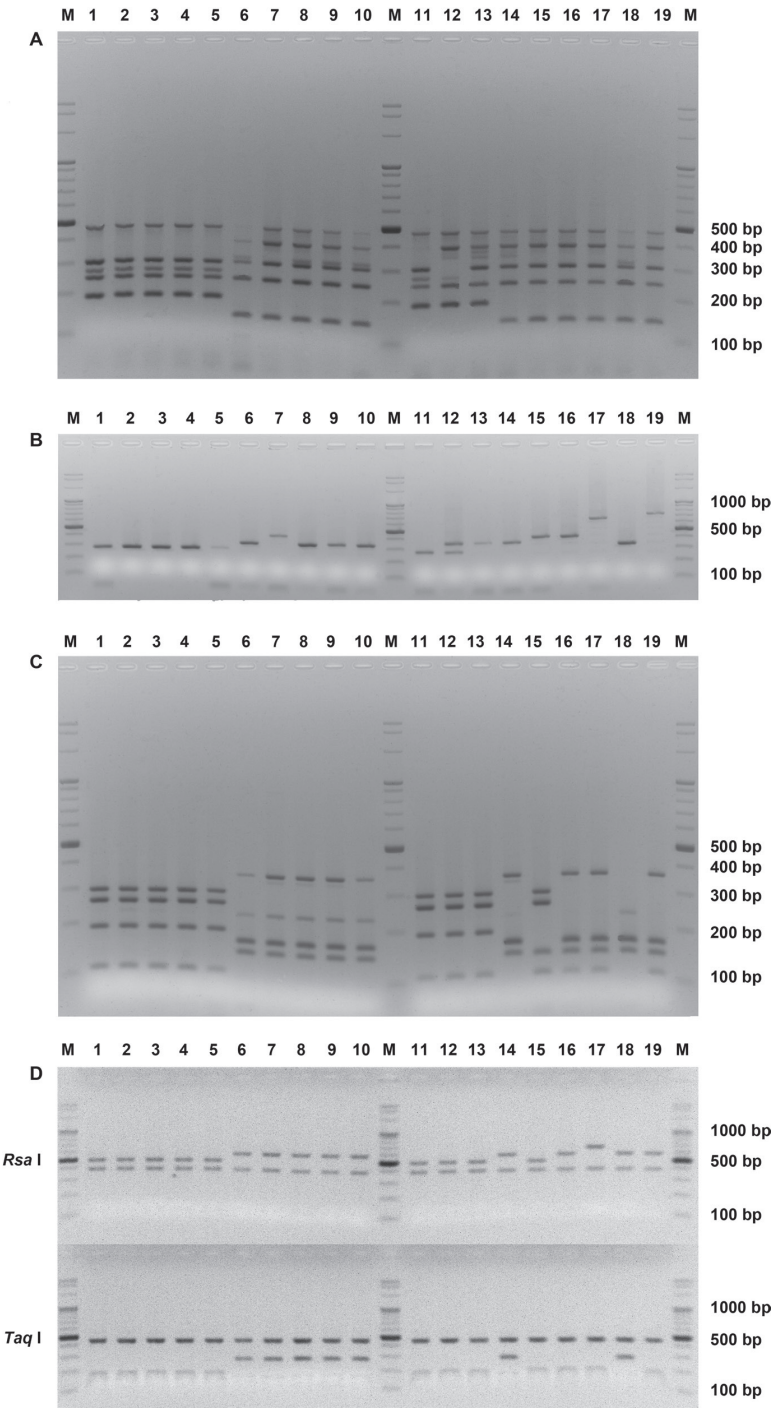

Fig 1. A, multiplex PCR; B, single reaction for *senX3-regX3*; C, PCR-based genomic deletion analysis; D, PCR-RFLP *gyr*B: lane 1, 60; lane 2, 4138; lane 3, 868; lane 4, 1078; lane 5, 2714; lane 6, 7575; lane 7, 3221; lane 8, 1339; lane 9, 5995; lane 10, 5379; lane 11, *M. bovis* BCG Moreau; lane 12, *M. bovis* BCG Danish; lane 13, *M. bovis* BCG Tokyo; lane 14, *M. tuberculosis* H37Rv; lane 15, *M. bovis*; lane 16, *M. africanum*; lane 17, *M. microti*; lane 18, *M. canetti*; lane 19, *M. pinnipedii*; M, molecular marker 100 bp.

of the PCR reaction in cases of the virulent mycobacteria isolates (Fig. 1B).

During the PCR-based genomic deletion analysis, 5 isolates classified at the NTLDRI as avirulent mycobacteria (60, 4138, 868, 1078, 2714), were easily identified as *M. bovis* BCG (Fig. 1C) as they showed a profile of deletion regions RD9, RD1, RD4 and RD12 (with amplicon sizes: 108 bp, 196 bp, 268 bp and 306 bp, respectively). Theirs profiles were identical with profiles obtained for *M. bovis* BCG Moreau, *M. bovis* BCG Danish and *M. bovis* BCG Tokyo substrains. Other 5 mycobacteria isolates, classified at the NTLDRI as virulent ones (7575, 3221, 1339, 5995, 5379), presented profiles specific for *M. tuberculosis* with presence of amplified fragments

from RD1, RD4, RD9, RD12 regions (146 bp, 172 bp, 235 bp, 369 bp products, respectively).

In the first step of PCR-RFLP *gyr*B analysis unique sequence for *M. tuberculosis* complex was amplified. Fragment of 1 020 bp was present in all mycobacteria strains used in the study (data not shown) and confirmed affiliation to the *M. tuberculosis* complex. At the second step, the amplified DNA fragments were digested by *Rsa*I or *Taq*I and enabled four species of *M. tuberculosis, M. africanum, M. bovis* and *M. microti* to be differentiated. As shown in the Fig. 1D, *M. bovis* and *M. microti* could be differentiated from the other species by the presence of *Rsa*I-digested fragments of 500 bp and 700 bp, respectively. *Taq*I digestion of the partial *gyr*B gene generated a 300 bp-fragment specific for *M. tuberculosis* as described Kasai *et al.* (2000) but also for *M. canetti*.

Discussion

The adverse events following vaccination with BCG, although registered by national surveillance after clinical recognition, are confirmed in the laboratory rarely. Moreover, the differentiation of *M. bovis* BCG from other members of the *M. tuberculosis* complex has previously been regarded difficult (Augustynowicz-Kopeć *et al.*, 2006). Some variants of NAT (Nucleic Acid Amplification Techniques) have been described in order to improve *M. tuberculosis* complex or *M. bovis* BCG substrains diagnostics possibilities (Jagielski *et al.*, 2010a, 2010b). In our study we have compared diagnostics capacity of *gyr*B-based PCR-RFLP, multiplex PCR, and PCR-based genomic deletion analysis using a set of reference mycobacteria strains and ten mycobacteria isolates originating from BCG AEFI suspected cases collected at the NTLDRI.

The first one – the *gyr*B-based PCR-RFLP method, previously shown to be useful for differentiation of closely related strains of bacteria such as *Vibrio* (Venkateswaran *et al.*, 1998) and *Bacillus* spp. (Yamada *et al.*, 1999), was adopted by Kasai *et al.* (2000) to identify four *M. tuberculosis* complex members (*M. tuberculosis, M. bovis*, *M. africanum* and *M. microti*). In our study, although proper restriction profiles for all above mentioned four species were observed, the system failed to differentiate *M. bovis* BCG substrains, *M. bovis* BCG from virulent *M. bovis,* and *M. tuberculosis* from *M. canetti* species.

The multiplex PCR described by Bedwell *et al.* (2001) easily distinguished *M. bovis* BCG substrains, however failed to differentiate *M. tuberculosis*, *M. africanum*, *M. canetti,* and *M. pinnipedii* species. Thus, *M. bovis* BCG Moreau substrain was easily identified in all isolates, determined at the NTLDRI as avirulent ones, however without the value for species identification in virulent isolates.

PCR-based genomic deletion analysis (Warren *et al.*, 2006), described originally as accurate tool for identification of *M. canetti*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae* or *M. bovis* BCG species, was found the most potent diagnostics option. Regions of differences distinguished *M. bovis*, *M. bovis* BCG, *M. tuberculosis*, *M. canetti*, *M. microti* and *M. africanum* species were described previously (Brosch *et al.*, 2002). Differentiation of *M. microti*/*africanum*/*pinnipedii* species was possible with a second PCR round with additionally designed primers. Although, PCR-RFLP of the partial *gyr*B gene (Kansai *et al.*, 2000) and multiplex PCR (Bedwell *et al.*, 2001) methods declared improvement of diagnostics potential, only PCR-based genomic deletion analysis developed by Warren *et al.* (2006), was found capable to precisely and simultaneously differentiate virulent and avirulent mycobacteria or virulent species of *M. tuberculosis* complex. Multiplex PCR developed by Bedwell *et al.* (2001) was found a valuable diagnostic option in situations when information on the *M. bovis* BCG substrain might be needed, *eg.* in foreigners infected with *M. bovis* BCG.

The criteria for the diagnosis of disseminated BCG infection in people with primary immunodeficiency, including definitive cases confirmed as *M. bovis* BCG substrain infection by culture and/or standard PCR (Bernatowska *et al.*, 2007) were previously proposed. However, the diagnostic details were not evaluated and discussed. As many different molecular tools were described, their evaluation is expected to improve AEFI surveillance measures. PCR-based genomic deletion analysis might be thus chosen for purposes of rapid identification of mycobacteria carriers, appropriate treatment of patients, epidemiological studies purposes, transmission studies of *M. bovis* between animals and humans, reliable confirmation of *M. bovis* BCG recovered from immunocompromised patients and healthy individuals suspected of AEFI after BCG vaccination.

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