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# Serodiagnosis of environmental mycobacterial infections

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ABSTRACT

To demonstrate the usefulness of enzyme-linked immunosorbent assay for serodiagnosis of mycobacterioses due to environmental mycobacteria we utilized a panel of glycolipid antigens selective for *Mycobacterium avium–intracellulare*, *Mycobacterium kansasii*, *Mycobacterium xenopi*, *Mycobacterium scrofulaceum* and *Mycobacterium gordonae*. The levels of circulating antibodies were determined against the environmental mycobacteria, and *Mycobacterium tuberculosis* in human immunodeficiency virus-negative and -positive patient sera. The method used immunomagnetic separation of the antigens, with covalent immobilization of antibodies to superparamagnetic amine and carboxyl terminated particles in solutions of the specific antigens. Enzyme-linked immunosorbent assay was performed on 195 patient sera: 34 with infections due to environmental mycobacteria, 114 with tuberculosis, 47 with other respiratory diseases. There were 46 human immunodeficiency virus-1 infected individuals. Among the 34 infections due to environmental mycobacteria, 9 patients were singularly infected with an environmental mycobacterium, and 25 co-infected with both *M. tuberculosis* and an environmental mycobacterium. Sensitivity, specificity and false positivity ranges were determined for each of the volunteer groups: tuberculosis positive, human immunodeficiency virus negative; tuberculosis positive, human immunodeficiency virus positive; those with infections due to individual environmental mycobacteria (such as *M. scrofulaceum* and *M. kansasii*); and those with other respiratory diseases. We demonstrate that such multiple assays, can be useful for the early diagnosis of diverse environmental mycobacterial infections to allow the start of treatment earlier than henceforth.



## 1. Introduction

Environmental mycobacteria (EM) are emerging pathogens causing opportunistic infections in humans and animals (Primm et al., 2004) and include those *Mycobacterium* species that are not members of the *Mycobacterium tuberculosis (M. tuberculosis)* complex or *M. leprae*. Only some of these mycobacteria, natural inhabitants of soil and waters, are pathogenic, for the healthy immunocompetent adult (Falkinham, 1996; Phillips and von Reyn, 2001). Diagnosis of EM infections is difficult, particularly as mixed infections with *M. tuberculosis* but important due to different treatment regimens (Nagai et al., 2001). The gold diagnostic standard is culture, since EM and *M. tuberculosis* exhibit distinctly different morphological and biochemical features (Heifets and Desmond, 2005); however, culture

*Abbreviations:* EM, environmental mycobacteria; *M. tuberculosis*, *Mycobacterium tuberculosis*; TB, tuberculosis; AFB, acid-fast bacillus; ELISA, enzyme-linked immunosorbent assay; GPL, glycopeptidolipid; LOS, lipooligosaccharides; PGL, phenolic glycolipids; MAI, *M. avium/M.intracellulare*; ATS, American Thoracic Society; NTM, non-tuberculous mycobacteria; ORD, other respiratory diseases; GL, glycolipid; TLC, thin layer chromatography; PBS, phosphate buffered saline; IgG, immunoglobulins G; BSA, Bovine Serum Albumin; MES, 2-(N-morpholino)ethanesulfonic acid; MP, magnetic particles; C18, MPG CPG-C18, (magnetic porous glass particles, with a lateral chain of 18 Carbon atoms; BM, BioMag® superparamagnetic Iron Oxide; HRP- SpA, Horseradish peroxidase- Staphylococcal protein A; PPV, positive predictive value; NPV, negative predictive value; FNR, false negative rate; FPR, false positive rate; GL-NH2, GL-BioMag ®Plus Amine- *M. tuberculosis* H37Rv IgG; GL-COOH, GL-BioMag ®Plus Carboxyl- *M. tuberculosis* H37Rv IgG; OD, optical density; IU, immunoenzymatic units; HIV, human immunodeficiency virus.

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is a protracted process due to the notoriously slow growth rates of all species of *Mycobacterium*. Accordingly, rapid, sensitive and inexpensive assays to confirm EM infections, as distinct from tuberculosis (TB) are required. Molecular techniques such as PCR and the use of DNA probes, or mycolic acid analysis by high pressure liquid chromatography (Heifets, 2004) are unaffordable for the developing world or for small clinical settings (Tonjum et al., 1998). A variety of immunological, particularly serological, tests have been developed to identify those infected with *M. tuberculosis* (Amicosante et al., 1999; Andersen et al., 2000; Daniel, 1996; Garg et al., 2003; Hoff et al., 2007; Kitada et al., 2005; Stavri et al., 2005). The search for a convenient serological test for TB and EM diseases is challenging because it requires balancing sensitivity against specificity (Abebe et al., 2007; Julian et al., 2004). The results of *M. tuberculosis* serologic diagnosis showed a high variability due to the enzyme-linked immunosorbent assay protocols used (different antigens, such as culture filtrate antigens (Laal et al., 1997), purified extracts of glycolipid (Escamilla et al., 1996), mycobaterial sonicates (Rosen, 1990), or more defined mycobacterial antigens such as 38-kDa (Rv0934), MPT59 (Rv1886c), MPT51 (Rv3803c) and LAM (Garg et al., 2003; Daniel, 1987). The sensitivities and specificities of the 38 kDa antigen in ELISA-based serological tests vary with the population, from respectively 89%/93% for Chinese (Cole et al., 1996), 55%/98% for Polish (Demkow et al., 2004), to 29%/96% for South Africans (Lodes et al., 2001). These subjects had all pulmonary TB and smear/culture positive status. When glycolipid antigens were utilized in ELISA, the sensitivities and specificities, based on positive acid-fast bacillus and culture of *M. tuberculosis*, vary from 60 to 71%/100% (Julian et al., 2004), to 75.2%/91,8% (Niculescu et al., 1995).

The use of purified, species specific antigens, particularly in simple ELISA formats, could fulfill the criteria of high sensitivity and specificity, and affordability for diagnosis of EM infections. However, to date, no rapid antibody detection systems for diagnosis of mycobacterial infections other than TB, have been reported. Yet, the wide diversity of mycobacteria within the environment is matched by an equally diverse range of species and serotype-specific glycolipid antigens, variously known as glycopeptidolipids (GPLs), lipooligosaccharides (LOSs) and phenolic glycolipids (PGLs) (Brennan et al., 1982). In this paper, we developed a diagnostic immunoenzymatic assay based on some of these specific antigens for individuals infected with the most widely encountered species i.e. *M. avium*/*M. intracellulare* (MAI) complex, *M. kansasii*, *M. xenopi*, *M. scrofulaceum*, and *M. gordonae*.

## 2. Materials and methods

### 2.1. Study groups

Serum samples were obtained from 195 patients (12–72 years old) from three centers in Bucharest: M. Nasta Pneumophtysiology Institute; Dr. Victor Babes Infectious and Tropical Diseases Hospital; and Carol Davila Emergency Military Clinical Hospital. Selection criteria included: (a) patients with suspected EM infection or EM/*M. tuberculosis* co-infection, based on clinical, radiological data supported by bacteriological tests for EM; (b) patients with EM infections (positive controls who met clinical, radiological, and bacteriological criteria for mycobacterioses and were classified according to the American Thoracic Society (ATS) guidelines for diagnosis of non-tuberculous (NTM) related diseases (American Journal of Respiratory and Critical Care Medicine, 1997)); (c) patients with TB diagnosed according to the World Health Organization guidelines (WHO, 2009); (d) patients with other respiratory diseases (ORD) or without any EM infections (negative controls) including: lung infections (acute respiratory tract infections, i.e. lower respiratory tract infections and pneumonia but not nosocomial forms); acute or chronic bronchitis; and asthma.

Bacteriological diagnosis (direct examination by microscopy, culture and resistance testing) was performed on all patients. All diagnoses of mycobacteriosis or/and TB were confirmed by identification of bacteria by culture according to standard methodology, using Lowenstein-Jensen medium. The acid fast smears were prepared from sputum, by the modified Petroff technique, described by Kent and Kubica (1985). Sputum specimens for Ziehl–Neelsen staining were obtained from some patients in the first three days after admission to hospital, usually before the beginning of specific treatment (Kubica, 1984), while others have been admitted to hospital for control, during and after their treatment. The sera were collected in the same way, either at the start of the treatment, for suspected infection with EM or/and *M. tuberculosis* or ORD, or when the patients have been observed, during or after therapy.

All samples were tested without knowledge of the clinical diagnosis of each patient, but the study groups were not randomly selected, as samples were intentionally collected for the development of the ELISA EM from patients strongly suspected of having mycobacteriosis or tuberculosis and from those with other respiratory diseases. In addition to positive AFB and culture of *M. tuberculosis*, radiographic evidence and clinical symptoms were used as the basis for diagnosis to estimate sensitivities and specificities.

The study was approved by local ethical committee (IRB #0002508) and the patients provided informed consents to participate in the study. All sera samples were blinded and stored at −70 °C. All of the subjects in this study had been vaccinated at birth with BCG according to the Romanian National Immunization Program. The subjects recruited from the three centers and their bacteriological diagnoses are presented in Table 1.

### 2.2. Growth of mycobacteria

The mycobacteria reference species used were obtained from: Pasteur Institute Paris, France (*M. tuberculosis* H37Rv, *M. kansasii,*

**Table 1**
Distribution of subjects according to culture results and HIV status.

| Culture results | No. of subjects | Hospital 1 | Hospital 2 | Hospital 3 |
|---|---|---|---|---|
| | | HIV status positive/negative | HIV status positive/negative | HIV status positive/negative |
| *M. tuberculosis* | 114 | 0/57 | 27/0 | 0/48 |
| EM | 34 | 0/27 | 3/0 | 0/4 |
| MAI | 12 | 0/8 | 2/0 | 0/2 |
| *M. kansasii* | 5 | 0/5 | 0/0 | 0/0 |
| *M. xenopi* | 11 | 0/9 | 0/0 | 0/2 |
| *M. gordonae* | 4 | 0/3 | 1/0 | 0/0 |
| *M. scrofulaceum* | 2 | 0/0 | 0/2 | 0/0 |
| Negative | 47 | 0/21 | 14/0 | 0/12 |
| Total | 195 | 0/103 | 44/0 | 0/48 |

Hospital 1 — "Marius Nasta" Pneumophtisiology Institute, Bucharest; Hospital 2 —"Victor Babes" Hospital for Tropical and Infectious Diseases; and Hospital 3 — "Carol Davila" Emergency Military Clinical Hospital.