DETECTION METHOD OF AIRBORNE MYCOBACTERIUM TUBERCULOSIS COMPLEX DNA IN ENVIRONMENT

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ABSTRACT
The incidence of tuberculosis among homeless people is 10 to 50 times higher than in the general population. To evaluate airborne environmental Mycobacterium tuberculosis in risk indoor areas, a non-invasive useful method was developed to study the spread of Mycobacterium tuberculosis particles in bioaerosols expelled from human respiratory tract. A semi-quantitative approach, based on a Polymerase Chain Reaction (PCR), was coupled with air sampling methods. The modified PCR method described allows to detect less than 95 CFU/m³ in impinger air samples and less than 140 colony forming unit (CFU)/cm² in sedimentation samples. As an “in use” example, environmental samples were collected during one month in bus carrying homeless people, but Mycobacterium tuberculosis was not detected in collected samples, probably because the method was tested with a slight number of people.

INDEX TERMS
Bioaerosols, Environments, Risk assessment, Mycobacterium tuberculosis, Detection.

INTRODUCTION
In many developed countries, incidence of Mycobacterium tuberculosis infections in big cities remains high. For example, in Paris (France) the 46,3 cases per 100,000 habitants correspond to 4 times the French national average (Decludt and Campese, 2001). This high score is mainly attributed to the worsening of the social and economic conditions for a part of the population excluded of health care system (Gutierrez et al., 1998). Epidemiological studies from several regions have shown that incidence of tuberculosis among homeless peoples is 10 to 50 times higher than in the general population (Nolan et al., 1991). Biological and social factors are suggested to be the reason of this high incidence (Diez et al., 1991). It was shown that homeless patients represent a major part in the spread of M. tuberculosis in the community and that poor socio-economic conditions are the main risk factors, associated with active transmission (Gutierrez et al., 1998).

M. tuberculosis bacilli are carried through airborne droplet nuclei produced from coughing, talking, or even singing (Loundon et al, 1967, 1968, and 1969). Studies on patients with chronic pulmonary tuberculosis have shown that bacilli concentration in sputum were typically in the range of 10⁶ to 10⁷ organisms per milliliter (Yeager et al.,1967), while it was evaluated that smear-positive persons expectorate 10⁸ to 10¹⁰ bacilli daily (Pottenger, 1948). But airborne bacteria can be stressed by environmental factors, as desiccation, physical force, and osmotic shock compromising there viability (Griffiths and DeCosemo, 1994). Detection and analysis of such biological particles involve, in general, collection, culture and enumeration of resulting colonies. The most important available aerosol sampling techniques are gravitational sedimentation, inertial impaction and filtration. Although investigators have

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successfully aerosolized and sampled mycobacteria in laboratory conditions (Lounden and Bumgarner, 1969; Riley and Knight 1976), no one has successfully sampled M. tuberculosis airborne particles, or droplet nuclei, expelled from human respiratory tract by culture. Moreover, efforts to culture the mycobacteria after collection by cascade impactors, for example, resulted in no growth or fungal overgrowth (Macher et al., 1992). In fact, the low concentration of airborne tubercle bacilli cannot be detected simply using standard bioaerosol detection culture methods (Schafer et al., 1998).

Previous studies have successfully combined air sampling by filtration and Polymerase Chain Reaction (PCR) to detect aerosolized M. tuberculosis in laboratory conditions (Schafer et al., 1998; 1999) and in hospitalized patients rooms (Mastorides et al., 1999). Our aim was to improved a detection method to study airborne spread of M. tuberculosis expelled from human respiratory tract even if the environmental contamination is several days old. So, a sensitive M. tuberculosis DNA analytical method designed for clinical specimen, without microbial culture, was modified and coupled with original air sampling methods.

METHODS

Detection method by Mycobacterium tuberculosis test (Roche AMPLICOR)
The Roche Amplicor M. tuberculosis test (Roche Diagnostic Systems Inc) routinely employed to detect M. tuberculosis complex in clinical specimens was used according to the manufacturer’s instructions. The test consists of a PCR detection: Biotinylated genus-specific primers are used to amplify a 584-base pair sequence. M. tuberculosis complex is detected by the hybridation of an oligonucleotide species-specific probe with the amplified sequence formed during the amplification process. The results are read at 450 nm and considered positive if the absorbance is greater than or equal to 0.35. The major obstacle in using PCR on environmental samples is the presence of substances collected during normal air sampling protocols, that inhibit the detection. As PCR inhibition induce false-negative reactions, we had incorporated internal controls in each reaction tube. To relieve PCR inhibitors from environmental samples and to obtain a sensitive method we added two-steps to the original test: a filtration (4 mm Millex, Millipore) and an addition of Bovine Serum Albumin at 8µg/µl (BSA, Sigma) in PCR mixture before amplification.

Detection limit of M. tuberculosis in environmental samples
An avirulent mycobacterial model, M. bovis BCG strain nearly genetically identical to M. tuberculosis was used to assess the minimum number of Mycobacterium tuberculosis Colony Forming Unit (CFU) detectable in environmental airsamples by the modified PCR test. A suspension of M. bovis BCG, at decreasing concentrations from 3-7 CFU/ml to 3.10^7-7.10^7 CFU/ml, was deposited in airborne samples. Control cultures of mycobacteria suspension were implemented and detection method was applied.

Settle Plate method
Settle plates (50 cm²; 5 cm x 10 cm), with an adhesive-coated upper surface (agarose gel 0.2%), were placed in triplicate on an horizontal surface during 6 hours (a journey time) or during 12 days. After sedimentation of particles, gels of the triplicate were pooled, liquefied by heating (90°C, 5 min), centrifuged (14 000 g, 15 min) and pellet containing sedimented particles was stored at - 20°C. Moreover settle plates of the 6 hours collection were pooled, 2 by 2: (a)+(d) and (b) + (c) in accordance with the height sampling, before treatment.
Impingement method
Airborne particles were sampled with an AGI-30 Impinger and operated at a flow rate of 12.5 liters/min (Tecora Bravo M2 pump). The flow rate was calibrated with a flow-meter (Brooks), and the initial volume of liquid in the Impinger was always 30 ml of sterile phosphate buffered saline solution (PBS, Sigma). During a trip, air was sampled for 60 min (2 x 30 min). The glass tube inlet was rinsed immediately after sampling, with 10 ml of sterile PBS, to collect particles impacted on glass inner side. Each sample was transferred into a capped sterile tube. In addition, the inner neck of the impinger was rinsed with 5 ml of sterile PBS. After homogenization, each sample was centrifuged (14 000 g, 15 min) and pellet containing airborne particles was stored at -20°C.

RESULTS
Validation in laboratory: Limit of M. tuberculosis detection in environmental samples
The minimum number of CFU that could be reproducibly detected, varied from 300-700 to 3000-7000 cultivable CFU by settle plates, that is to give finally 6 CFU/cm² to 140 CFU/cm². On the other hand for impingement sampling, the limit was 3-7 to 30-70 cultivable CFU by air sample, that is to give finally 4 CFU/m³ to 95 CFU/m³ of air. Airborne environmental samples have never induced PCR inhibitory when we have used BSA before amplification.

Sample collection in "in use" conditions
We have assessed the feasibility of our detection method in "in use" conditions. Environmental particles were collected inside a city bus carrying homeless people from Paris to a health care center during winter period (figure 1). Samples were collected during five journeys in march 1999: three in south-east area of Paris, one in south-west and one in north-east. Three to five homeless passengers by journey were driven during experiment. Thirty-nine sedimentation specimens, five impingement specimens have been sampled during one month. Mycobacterium tuberculosis was not detected in collected samples.
DISCUSSION AND CONCLUSION

In the study sampling conditions, *M. tuberculosis* was not detected in environmental samples in spite of high sensitivity of the technique. Two hypothesis can be discussed. The first one is that negative result may be explained by the very low number of homeless people with tuberculosis present during experimentation (only one of the 26 transported homeless people was suspected of tuberculosis). Moreover, it is likely that the presence of tubercular but not contagious people limits the number of positive results, particularly after chemotherapy which induced a rapid logarithmic decline in the number of bacilli excreted (Yeager et al., 1967).

It could be objected that some limitations of the study may explain negative results. In spite of settle plates interest (this sampling technique can be useful to collect samples without interruption during several days and to realize a map-making of contamination), the sedimentation collection (i) can't allow to know the air volume analyzed and (ii) airborne particles sedimentation depends of their densities, diameter (Salomon, 1984) and air turbulence. On the other hand, Riley and Nardell (1989) have shown that infectious droplet nuclei could be dilute in a large air volume, therefore volume of collected air (< 1 cubic meter) with the impinger sample was maybe to small to capture *M. tuberculosis* droplet nuclei potentially present in this airborne environment.

We must improve sampling technique and collect airborne particles in highly contaminated environment in hospitalized tubercular patient room or in bronchoscopy room (Nardell, 1990). The detection method is sensitive and specific in laboratory condition. It seems possible to relieve PCR inhibition induced by environmental samples relieved by BSA adding. Despite the fact that the preliminary assays in "in use" conditions failed, the selective detection of airborne *M. tuberculosis* bacilli carried on airborne droplet nuclei expelled from human respiratory tract is validated. We are actually testing a single-stage multiple-hole gel impactor which is able to collect greater air volume (100 liter/minute) than impingement technique.

The main application of these methods should be found when tubercular patient are hospitalized, for example in a bronchoscopy room. This method would also allow to realize epidemiological and infection control studies, and to document the transmissibility of *M. tuberculosis* complex within noninstitutional settings where groups of hazard people are involved.

REFERENCES


