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Biosafety Recommendations for the Contained Use of *Mycobacterium tuberculosis* Complex Isolates in Industrialized Countries.

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Abstract

Staff working in microbiological diagnostic and research laboratories is likely to be exposed to infection risk with pathogens. Among human infectious diseases, tuberculosis is one of the most severe, killing 2 millions people worldwide every year. *M. tuberculosis* is essentially an airborne pathogen included in Risk Group 3 according to the international classification. It is transmitted via aerosols or less frequently by accidental inoculation. The definite diagnosis of tuberculosis relies on the isolation and identification of the *Mycobacterium tuberculosis* complex in clinical specimens. The incidence of this infectious disease among laboratory personnel involved in tuberculosis diagnosis is known to be three to nine times higher as compared to personnel manipulating other clinical specimens. This significant threat must be correlated with the resurgence of tuberculosis. As most developed countries, Belgium has adopted strict biosafety rules for the contained use of human pathogens in laboratories. However, due to the airborne transmission way of *M. tuberculosis*, specific biosafety recommendations have been established to better define the containment level, the required safety equipment and the work practices which should be adopted in diagnostic and in research laboratories where tubercle bacilli are manipulated. Diagnosis activities including primary cultures of clinical specimens potentially infected by bacilli of the *M. tuberculosis* complex should be carried out under Biosafety Level 2 (BSL-2) containment with BSL-3 safety equipment and work practices. Every further manipulation, involving opening of tubes or vessels containing *M. tuberculosis* positive cultures, for microscopic analysis, DNA or RNA extraction, biochemical tests and/or secondary cultures for diagnostic or research activities, requires a BSL-3 containment level, safety equipment and work practices.

Introduction

Tuberculosis is a severe infectious disease caused by species of the *Mycobacterium tuberculosis* complex. This complex includes *M. tuberculosis*, *M. africanum*, *M. bovis, M. pinnipedii* and *M. microti* (Wayne, 1984; Cousins *et al.,* 2003). The four first species are human pathogens, *M. microti* infecting voles, guinea-pigs, rabbits and sometimes bovines. *M. pinnipideii* is responsible for tuberculosis in seals (Cousins *et al.,* 2003). *M. bovis* is responsible for pulmonary disease in bovine and sometimes to mammary lesions with passage of tubercle bacilli in milk. Both *M. bovis* and *M. pinnipedeii* are responsible for zoonosis. *M. bovis* is responsible for extra-pulmonary infections in human following ingestion of contaminated milk or milk products, but also pulmonary infections by inhalation of infected droplets through direct contact with infected animals. *M. africanum* is responsible for 20 to 80 % of human tuberculosis in sub-Saharan Africa, but also for some tuberculosis cases diagnosed outside this continent. In most of the occidental countries, tuberculosis cases are mainly caused by *M. tuberculosis*. Some domestic animals, in contact with people suffering from tuberculosis, are able to develop tuberculosis and become themselves a source of infection (Cousins *et al.,* 2003; Grange, 1990).

According to World Health Organization (WHO), tuberculosis remains the second leading cause of death worldwide, killing nearly 2 million people each year (WHO, 2005). The global tuberculosis caseload appears to be growing slowly. If tuberculosis hits first the Third World (around 95% of the cases, mainly in sub-Saharan Africa and South East Asia), there has been a significant increase in countries of the former Soviet Union and in Eastern Europe these last years (WHO, 2005; Frieden *et al.,* 2003). Since the mid 80's tuberculosis is decreasing less rapidly than expected in the majority of other industrialized countries (Dolin *et al.,* 1994).
Different factors contribute to the resurgence of this infectious disease in industrialized countries: degradation of socio-economical conditions, increasing immigration from countries with high tuberculosis prevalence, dismantling of tuberculosis sanitary structures from the period where the disease was decreasing, and high sensitivity of people infected with HIV (Bloom, 1994; Porter & Mc Adam, 1994; Castro, 1995). This outburst is also correlated with the increasing development of species of the *M. tuberculosis* complex resistant to first line antituberculous drugs. Late diagnosis, inadequate patient's treatment, unadapted safety equipments and premises, contribute to the disease transmission.

There are an estimated 8.8 million new cases of tuberculosis each year of which 3.9 million cases are sputum-smear positive (WHO, 2004). In Belgium, the incidence of tuberculosis stopped decreasing since 1993 and was stabilised for ten years about 12/100.000 inhabitants. In 2003, the incidence was 10,9/100.000 (FARES, 2003). The resurgence of tuberculosis in Belgium, as in other Western Europe countries, might be intensified in the forthcoming years due to immigration from countries with high tuberculosis prevalence.

Considering the most recent epidemiological data for tuberculosis and the high risk of laboratory-acquired infection (LAI) for personnel manipulating samples potentially containing *M. tuberculosis*, and in order to ensure the highest level of protection for human health and environment, it seems necessary to specify the biosafety regulatory framework currently in force by providing substantial details about the containment measures, safety equipment and work practices to be applied in diagnostic and research laboratories working with mycobacteria of the *M. tuberculosis* complex.

In this work, hospital and laboratory-acquired tuberculosis cases, as well as data concerning the situation in Belgium are reviewed. Then, biosafety recommendations based on a thorough risk assessment of laboratory activities handling *M. tuberculosis* complex isolates are proposed.

Epidemiology of Hospital and Laboratory-Acquired Tuberculosis

If health care workers are particularly exposed to infection risk, acquired mycobacterial infection of people working in diagnostic and research laboratories is also well documented (Grist & Emslie, 1985; Miller *et al.,* 1987; Müller, 1988; Collins CH, 1988). Several surveys indicate that LAI with *M. tuberculosis* is 100 times greater than for the general population (Reid, 1957). At the end of the 70's already, tuberculosis was one of the most frequent LAI's (Pike, 1976; Pike, 1979). Yearly incidence of tuberculosis among laboratory staff ranges from 0.3 per 1000 people (Jacobson *et al.,* 1985). More recently it was confirmed that tuberculosis remained in the so-called "top-10" LAI with 223 overt cases from 1981 to 1985 in the USA (Harding & Brandt Byers, 2000). The incidence of tuberculosis among personnel manipulating samples potentially contaminated with *M. tuberculosis* is 3 to 9 times higher than that observed in people not working with such tubercle bacilli samples (Saint-Paul, 1972; Harrington & Shannon, 1976; Sepkowitz, 1995; Shinnick, 1995; Germanaud & Jamet, 1994). A survey carried-out in 26 mycobacteriology laboratories in Spain indicated that less than half of the employees received periodic information on the health hazards linked to their work. More than one third of the lab-workers mentioned lack of effective air-filtering systems in the mycobacteriology laboratories and half stated that negative pressure was not maintained in the work area (Vaquero *et al.,* 2003). A review also highlighted the risk of infection with *M. tuberculosis* species during autopsy of infected patients. It is reported that the risk of infection was independent from distance from the autopsy table, emphasizing the importance of airborne transmission of the bacilli (Nolte *et al.,* 2002).

A study showed that behavioural factors are important in the contribution of laboratory-acquired infections. It was showed that 80% of all accidents were due to human error and 20% to equipment problems (Phillips, 1965). Nowadays, even if equipment troubles were partially solved by the adoption of appropriate safety equipment in many diagnostic and research laboratories, behavioural factors may be a source of concern.
There are numerous records of laboratory-acquired tuberculosis infection through aerosols or skin puncture (Pike, 1978; Kubica, 1990; Sharma *et al.,* 1990; Menzies *et al.,* 1995). A survey of 56 state and territorial public health laboratories in the USA has examined the status of existing tuberculin skin testing (TST) and assessed the probable laboratory-acquired tuberculosis. Among 49 laboratories, 13 reported that 21 employees converted TST (period of 4 years). Seven of these 21 lab-workers were reported to have LAI. These authors showed that inadequate isolation procedures, the high volume of handled specimens and bad ventilation accounted for these LAI. Needle stick injuries have been uncommon causes of laboratory-acquired tuberculosis however, with use of the Bactec™ system for rapid culture, needle stick-associated tuberculosis cases have been reported (Kao *et al.,* 1997). In Canada it was observed that the average annual risk of TST conversion was 1.0% in lab workers (Menzies *et al.,* 2003). Recent reports of laboratory-acquired tuberculosis in European diagnostic and research laboratories emphasize the necessity of continuing the effort in biosafety measures and regulations compliance (Vaquero *et al.,* 2003).

Underreporting of LAI's appears to be the rule, rather than the exception. Taking into account the recent resurgence of tuberculosis, coupled with growing number of samples to be tested and the development of MDR strains, it seems evident that workers of medical laboratories are susceptible to be exposed to *M. tuberculosis* infection when working without efficient primary and secondary protective barriers and without appropriate training. It is reasonable to think that staff working in research laboratories is exposed to the same biological risk than diagnostic laboratory workers when manipulating *M. tuberculosis*.

In Belgium, about 160 diagnostic and 5 research laboratories are likely to receive and culture clinical or experimental specimens susceptible to contain mycobacteria of the *M. tuberculosis* complex. About twenty to twenty five of the diagnostic laboratories perform further identification tests,

antibiotic susceptibility testing or secondary cultures for research and development purposes. Although very few data are available concerning the incidence of laboratory-associated tuberculosis infections in microbiological laboratories in Belgium, an inquiry performed in 1995 showed that the incidence of tuberculosis among health-care workers in hospitals was 2.5 times higher than that observed in the normal population, and even 5.4 times higher for people working in the laboratory (Ronveaux *et al.,* 1997).

Biological Risk Assessment of Laboratory Activities Using Species of the *Mycobacterium tuberculosis* Complex

When performing a case-by-case assessment of an activity with a given pathogen, it is important to first take into account the classification of this agent into one of the four classes of biological risk also called "risk groups". The type of activity (direct examination, culture, identification, antibiotic susceptibility testing) is also an important factor to consider during the risk assessment process. The following recommendations were established in that perspective, as to determine the biosafety measures appropriate for the handling of tubercle bacilli in diagnostic and research laboratories. The same activities involving human pathogens and recombinant relatives also enter the scope of Directive 2000/54/EC repealing Directive 90/679/EEC and regulating the protection of workers exposed to biological agents at work (Official Journal of the EC, 2000).

According to the Directive 2000/54/EC, the group of biological agents belonging to risk group 3 can cause severe human disease and represent a serious hazard to workers. They may present a risk of spreading to the community but usually there is effective prophylaxis or treatment available. The airborne route of transmission of the tubercle bacilli greatly contributed to the final classification of this pathogen into risk group 3. Moreover, the increase of MDR strains identified in diagnostic laboratories supports the current classification of this biological agent. **Table 1** summarises the main known *M. tuberculosis* properties that lead to classify this human pathogen in class of risk 3 (Wayne, 1984; Grange, 1990; Riley, 1961; Kunz & Gundermann, 1982). These intrinsic properties and the laboratory techniques that are likely to generate infectious aerosols are detailed in the risk assessment below. It should be noted that the H37Ra *M. tuberculosis* strain (ATCC 25177) used in experimental settings as well as the bacillus Calmette-Guérin (BCG) are classified as class of risk 2 pathogens. However, the H37Rv strain (ATCC 2618, ATCC 27294) belongs to the class of risk 3.

- Characteristics of the mycobacteria belonging to *Mycobacterium tuberculosis* complex

Even though only 1% of clinical specimens submitted to test for *M. tuberculosis* species really contain pathogenic mycobacteria, the risk assessment must essentially take into account two facts. First, the infectious dose in humans is very low (ID_{50} 1-10 bacilli), whereas a sputum of an infected patient can contain several millions of bacilli per millilitre (Riley, 1957; Riley, 1961). Second, infection predominantly occurs by inhalation of airborne bacilli and manipulation of liquid clinical specimens likely involves generation of infectious aerosols. Mycobacteria can be isolated from about any type of human specimens. Indeed, tubercle bacilli may be present in sputum, gastric wash fluids, cerebrospinal fluids, lymph nodes and in tissues harvested from a variety of lesions (Bloom, 1994).

Exposure to laboratory-generated infectious aerosols has been shown to represent the most serious hazard encountered in the laboratory although percutaneous injury or infection by secondary transmission (contaminated gloves or surfaces) may also result in infection (Miller *et al.,* 1987; Müller,1988).

Table 1: *Mycobacterium tuberculosis* main known properties and pathogenicity

Factors	***Mycobacterium tuberculosis* complex species**
Characteristics	Gram positive rods, non-spore forming, non motile, acid-fast staining, aerobic, slow-growing
Host range	Humans, cattle, primates, rodents, seals
Pathogenicity	long incubation period, may progress to pulmonary or extrapulmonary disease
Infectious dose	10 bacilli by inhalation
Mode of transmission	Preferentially airborne and secondary ingestion or dermal inoculation
Communicability	as long as bacilli are in sputum (may be years)
Zoonosis	yes, by inhalation or direct contact with infected animals or tissues from infected animals, Milk
Reservoir	Humans, cattle, badgers, swine and other mammals (*M.bovis*)
Vectors	None
Survival outside the host	Sputum (cool and dark location) : 6 to 8 months, clothing : 45 days, paper - book : 105 days
Treatment	Antibiotic therapy
Immunization	Attenuate live vaccine (BCG) not routinely carried out (offers limited protection)
Geographical localization	Worldwide

Many microbiological techniques generate minute droplets of liquid called aerosols (Sewell, 1995; CDC, 1999). Each droplet may contain one or more micro-organisms. The fineness of division of the discharged particles determines their ultimate fate (**Table 2,** adapted from Wells, 1955). Smaller droplets settle very slowly and dry rapidly, they are transformed within hundredths of a second into a dehydrated mass (droplet nuclei) containing the previously dissolved solutes of the discharged solution and any particles that were carried within the droplet. These droplet nuclei float in the air of a room and are spread by very small air currents. Once inhaled their small size (≤ 5 µm in diameter) allows them to penetrate to the deeper regions of the lung, where some deposition in the alveolar spaces might occur by gravity. These small size particles containing *M. tuberculosis* can remain airborne for minutes to hours. Larger droplets could not dry and rapidly contaminate laboratory surfaces and fingers with as a consequence a secondary contamination of mouth and nasal cavities. When inhaled some bacilli are caught from the upper respiratory tract by the "filtering mechanisms" (Collins, 1988; Wells, 1941). It must also be emphasized that *M. tuberculosis* can survive for several days on inanimate surfaces (Kunz, 1982). Survival of *M. tuberculosis* outside the host can be particularly long with, for example: 90 to 120 days on dust, 45 days on manure, 105 days on paper, 6 to 8 months in sputum (cool, dark location) and 45 days on clothing (Rubin, 1991). Hence a work surface, that has not been properly disinfected, represents an additional source of moderate risk of transmission.

Table 2 : Size classification of aerosols

Particle type	Size range (µm diameter)	Setting velocity (cm/min)
Droplet	100 - 400	1800 - 15,200
Dust	10 - 100	18 – 1800
Droplet nuclei	1 - 10	0.2 – 18
	0.1 - 1	0.005 - 0.2

Among the laboratory techniques used for the identification and characterization of mycobacteria, the following ones are likely to increase the risk of contamination or to generate infectious aerosols producing droplet nuclei (Collins, 1988; Clinical Microbiology Procedures Handbook, 1992).

- **handling of containers with clinical specimens**: even if this situation is unlikely to generate aerosols, it is the initial step where laboratory personnel is potentially exposed to the tubercle bacilli. It was shown that the outside of containers used for collecting clinical specimens is frequently contaminated by *M. tuberculosis* (6.5%) or by other airborne pathogens (15%) (Allen & Darell, 1983).

- **centrifugations**: fluid may spill from centrifuge tubes or tubes may break, releasing a large amount of aerosols;

- **pipetting**: pipettes and Pasteur pipettes in particular are likely to generate bubbles which burst and form aerosols;

- **mechanical homogenizing** (vortexing, grinding, blending);

- **sonication, heating or boiling** of samples (for instance for the extraction of nucleic acids);
- **work with bacteriological loops**: when loops charged with infectious material are placed in an ordinary Bunsen burner, the material may be dispersed before it is burned and contaminate surfaces or the operator;

- **preparation and manipulation of frozen sections** (histology): when frozen material is cut, infected ice and tissue particles may be dispersed and contaminate the operator and material (even formalin-fixed tissues may still contain viable bacilli);

Generally, special care should also be taken for the following manipulations:

- **acid-fast staining** (AFB smear): smear fixation on slides (by heat or methanol) can generate aerosols. Although fixed smear may still contain viable organisms, they are not easily aerosolised (Allen, 1981);

- **manipulation of solid and liquid cultures:** Unlike sporulating fungi or bacteria, the opening of a Petri dish or a tube lid containing mycobacteria is not thought to pose a real risk. However, manipulation of the colony mass increases the likelihood of dispersal of the tubercle bacilli into the air, especially when organisms are incinerated from the bacteriological needle or loop. In case of accidental breakage involving culture tubes, a culture of *M. tuberculosis* grown on a solid medium is rated as producing a "minimal" aerosol requiring local disinfection (Kent & Kubica, 1985; Fleming, 1995). A special attention should be given to the manipulation of fluids, particularly those in which the mycobacteria has been amplified by culturing and water-based suspensions realized in dispersing agents such as Tween 80. Liquid cultures are readily subdivided into droplets when subjected to physical forces. The droplets aerosolized by the manipulation of fluids become droplet nuclei if they dry before landing on a horizontal surface.

Other factors must be taken into account in a comprehensive risk assessment in case of more specific activities planned with *M. tuberculosis* complex species (*e.g.* manipulations implying flow cytometry or animal models).

- flow cytometry: applications of flow cytometry in clinical microbiology and research laboratories are numerous with direct detection of infected cells or isolated mycobacteria, serological tests, monitoring of infections, antimicrobial therapies and cell-sorting (Norden *et al.,* 1995; Kirk *et al.,* 1998; Moore *et al.,* 1999; Alvarez-Barrientos, 2000). In experimental settings, flow cytometry has also been used to assess sputum decontamination methods improvement (Burdz *et al.,* 2003). Flow cytometry analysis and/or sorting procedures can generate aerosols containing viable *M. tuberculosis*.

- **animal studies**: major risks are self-inoculation and exposure to aerosols. Non-human primates infected with *M. tuberculosis* are a proven source of infection, for human the annual tuberculosis infection rate among people working with infected primates is 70/10.000 against less than 3/10.000 in the population (Kaufmann & Anderson, 1978). Most of these infections result from the inhalation of aerosols produced by primates. The risk is lower with infected rodents, because the likelihood of producing infectious aerosols by coughing is relatively low. However, the litter of any infected animal can be contaminated and thus become a potential source of contamination.

Biosafety Recommendations for the Contained Use of *M. tuberculosis*

The WHO and the CDC classify *M. tuberculosis* among the pathogens that require a biosafety level 3 (CDC, 1999; WHO, Laboratory Safety Manual, 2004). For the manipulation of this human pathogen, greater emphasis is placed on the use of primary and secondary barriers to protect laboratory employees in direct contact with the micro-organism, and the community and environment from exposure to potentially spreading of infectious particles.
Based on the risk assessment and according to technical characteristics, safety equipment and work practices, the following recommendations for the contained use of *M. tuberculosis* are proposed:

1. Laboratory work with clinical specimens susceptible to contain species of the *M. tuberculosis* complex

The outside of containers used for collecting clinical specimens could be contaminated with tubercle bacilli, therefore the containers and packaging containing clinical specimens, primary or secondary culture samples or any other material known to contain *M. tuberculosis* should be opened in a class I or II biosafety cabinet (BSC). Personnel wearing gloves should disinfect the outside of the container.
For the laboratory involved in the diagnosis of tuberculosis, direct smear examination and primary culture of specimens require to work in BSL-2 facilities (**Table 3**, available on the Belgian Biosafety Server, 2006) with BSL-3 work practices (**Table 4**, available on the Belgian Biosafety Server, 2006). Primary cultures only concern cultures obtained directly from clinical specimens in solid or liquid culture medium. They should be performed in "unbreakable" vials. These laboratories should send the positive primary culture, without any subsequent examination, to a BSL-3 laboratory for further analysis.

2. Laboratory work with *M. tuberculosis* cultures

When the diagnostic or research laboratory is involved into subsequent characterisation of the tubercle bacilli by means of secondary cultures, antimicrobial susceptibility testing, and any other test performed on primary or secondary living cultures, BSL-3 facilities, equipment and work practices should be used (**Table 4**; **5** and **6**, available on the Belgian Biosafety Server, 2006). Needle sampling through vial's septum (e.g. for smear examination, nucleic acid amplification or any other biological test) should not be performed.

The following additional measures for facilities and work practices are recommended:
Rotors, buckets and tubes should be opened in a class I or class II BSC. Mechanical homogenizing (vortexing, grinding, blending) of samples should be performed in a class I or class II BSC.

Contaminated pipettes should be discarded horizontally in a container immediately after use. This container must be dry in order to avoid aerosol production pipette laying down. Disposable plastic bacteriological loops are preferable; if wire loops are used, they must be sterilized in an electrically operated "micro-incinerator". Alternatively, they may be submerged into a flask filled with sand and 90% alcohol, before they are flamed. Needles and syringes or other sharps should be restricted in the laboratory and only used when there is no alternative: only disposable syringe-needle units (i.e., needle are sealed to the syringe) should be used for injection or aspiration of infectious material. Contaminated syringes should be carefully discarded after use in special puncture-resistant containers used for sharps disposal. Appropriate systems of respiratory protection with HEPA filtration (N/R/P/95/99/100 or FFP2, FFP3 filter level) should be worn when aerosols cannot be safely contained or for the handling of positive cultures in the BSC. FFP2 filter level mask should be worn (CEN, 2001; NIOSH, 1999). The slides used for AFB smear identification should be handled with care to prevent contamination of hands and discarded after use as potentially contaminated waste. Smears, which may contain *M. tuberculosis*, should be stored in a closed box as it was shown that viable tubercle bacilli could be excreted by cockroaches following ingestion from heat-fixed smears (Allen, 1987). Biosafety measures and work practices can always be improved. For example, an improvement of laboratory safety was published consisting in a new method for inactivating and fixing unstained smear preparations of *M. tuberculosis* (Chedore, 2002). Flow cytometry applications involving *M. tuberculosis* should take into account recent publications and specific biosafety guidelines (Schmid *et al.,* 1997; Schmid *et al.,* 2003). Biosafety measures to apply to main aerosol producing activities are shown in **Table 7**.

Table 7 : Biosafety measures to apply to aerosol producing activities

Activity	Biosafety measures
Falling droplets Blowing of pipets Acid-fast staining (AFB smear) Opening of primary and secondary culture Opening of wet caps	- all these manipulations should be performed in a class I or class II BSC
Work with inoculation loops	- performed in a class I or class II BSC - use of disposable plastic loops is preferable
Centrifugation of open buckets	- use aerosols-free buckets "safety cups" during centrifugation - opening rotors, buckets or tubes under class I or class II BSC after centrifugation
Flow cytometry (sorting procedures)	- use of "droplet containment module"
Handling of infected animals and animal litter	- animals should be maintained in isolators - cages should be opened in a class I or class II BSC

3. Infected Animals with *M. tuberculosis* complex species

Non-human primates infected with strains of the *M. tuberculosis* complex should be handled using standard precautions in BSL-3 animal facilities, equipment and work practices (**Table 8**; **9** and **10**, available on the Belgian Biosafety Server, 2006). Harvested samples from infected animals should be handled using standard precautions in BSL-2 facilities (**Table 3**, available on the Belgian Biosafety Server, 2006) and BSL-3 work practices (**Table 4**, available on the Belgian Biosafety Server, 2006).
Infected rodents (mice, rats, rabbits) can be housed in a BSL-2 containment since they are maintained in isolators. However, BSL-3 work practices should be adopted. Manipulations involving opening of cages should be realised in class I or class II BSC. Cages, litters and carcasses should be

autoclaved and/or incinerated before disposal or reuse. If a SCID, "nude" or any other immunodeficient mouse is used, it must be taken into account that in these animals the infection spreads more compared to immunocompetent animals and that high titers of bacilli can be found in certain organs. Consequently, it is recommended to wear a mask with HEPA filtration during the manipulation of infected immunodeficient animals.

4. Disinfection, inactivation of *M. tuberculosis* and waste management

The high lipid content of the cell wall confers to the mycobacteria a great resistance to classical disinfectants. The bacilli are generally more resistant to chemical disinfection than other vegetative bacteria. Their resistance to disinfectants is considered intermediate between other non-sporulating bacteria and spores (Kunz & Gundermann, 1982). The acquired multidrug resistance does not seem to modify the resistance to disinfectants (Sattar *et al.,* 1995). Quaternary ammoniums inhibit tubercle bacilli but do not kill them. *M. tuberculosis* is also resistant to acids and alkali. Mercurial compounds are considered to be ineffective against the mycobacteria. Efficient disinfectants are 5% phenol, 5% formaldehyde during at least ten minutes, 2% glutaraldehyde during 30 minutes exposure or sodium hypochlorite (5%) during one minute. Ethyl and isopropyl alcohols in high concentrations are generally accepted to be excellent mycobactericidal agents. 70% ethyl alcohol can be used as surface disinfectant. Formaldehyde vapours can be used to disinfect BSC's and facilities. Iodine and ionophores are considered to be effective against mycobacteria and are generally used in combination with ethyl alcohol (Rubin, 1991).

It is recommended to test killing methods used on *M. tuberculosis* suspension before removal from Biosafety Level 3 laboratory. A study compared the efficacy of several disinfectant mixtures on class of risk 3 *M. tuberculosis* Erdman strain. It was observed that fixatives containing low concentration of glutaraldehyde alone are not efficient to kill *M. tuberculosis*. The use of a combination of 2% paraformaldehyde and 2% glutaraldehyde or a solution of 5% formalin is recommended for *M. tuberculosis* inactivation (Schwebach *et al.,* 2001). Another experimental study has demonstrated that all tubercle bacilli killing methods should be validated by individual laboratories before removing material derived from *M. tuberculosis* to the outside of the BSL-3 laboratory (Blackwood *et al.,* 2005).

Work surfaces should be decontaminated at least once a day with an appropriate disinfectant and immediately after any accidental contamination with infectious materials. Laboratory workers should disinfect their hands after manipulations with an appropriate disinfectant, after removing gloves, and before leaving the laboratory. Worn gloves and protecting clothes should be autoclaved before leaving the laboratory.

Attention should be focused given to waste inactivation. Decontamination by autoclaving or incineration is essential. Ideally, an autoclave for the sterilisation of contaminated materials should be available in or adjacent to the laboratory. If the inactivation takes place outside the laboratory (autoclave or incinerator), wastes should be placed in a leak proof bag or an unbreakable and leak proof container (for liquid wastes), sealed and disinfected on the outside before removal from the laboratory. In addition to the international Biohazard symbol, bags or containers should be adequately labelled to prevent opening before decontamination. Removal of bags and containers should be performed according to written procedures.

5. Transport inside and outside the installation

Transport of samples should follow definite procedures. Transfer of positive cultures outside the BSL-3 area should be performed using primary leak proof receptacles. These should be packed in secondary leak proof containers in such a way that, under normal conditions of transport, they cannot break, be punctured or spill their contents into the secondary packaging.

For transportation outside the facility, the samples of the *M. tuberculosis* complex should be packed in a triple packaging. For clinical specimens transfer (*e.g.* from BSL-2 diagnostic laboratory to a

reference centre), the samples should be placed in a watertight primary screw-capped container which should be placed in a watertight secondary container (*e.g.* sealable plastic bag). It is important that the primary container is wrapped in absorbent material to completely soak-up the liquid in the clinical sample in case of run out of the container. Finally, the secondary container should be placed into a robust outer container properly labelled with the address and the nature of the clinical specimen.

M. tuberculosis cultures and other known materials that are positive for *M. tuberculosis* complex require identical packing measures, except that the secondary watertight container should be sturdy (*e.g.* aluminium can with a sealable cap) and labelled as "infectious substance".

Triple packaging should be realised according to the International Air Transport Association (IATA) Dangerous Goods Regulations and WHO recommendations (IATA, 2005; WHO, CDSR, 2004).

And finally, mistakes and accidents, which result in overt exposure to infectious materials, should be immediately reported to the head of the laboratory and eventually to the local biosafety officer. Written records of such events should be kept. Personnel concerned by the mycobacteria activity should be experienced and dedicated workers. Personnel should receive regular updates and appropriate additional training, under the supervision of the head of the laboratory.

Conclusions

The increase of tuberculosis in industrialized countries and concomitant emergence of antibiotic multidrug resistance have highlighted the necessity to elaborate specific biosafety measures for manipulation of mycobacteria belonging to the *M. tuberculosis* complex, in diagnostic and research laboratories. These recommendations are based on a thorough risk assessment taking into account the type of activity.

The adoption of a BSL-2 containment with BSL-3 work practices are recommended for medical laboratories limiting their analysis to *M. tuberculosis* primo-isolation from clinical specimens (i.e. primary culture, microscope examination of smears from clinical specimen, nucleic acids amplification, histological examination). The work on biological material susceptible to generate infectious aerosols must be performed in a class I or class II BSC placed in a specific area to be separated from the other bacteriological activities. The use of a centrifuge equipped with "safety cups" is highly recommended. BSL-3 containment, safety equipment and work practices are necessary for laboratories manipulating positive cultures of the *M. tuberculosis* complex at ends of diagnosis or research work (e.g. biochemical tests, susceptibility testing, subcultures for research work) until validated inactivation of mycobacteria.

The respect of these biosafety recommendations associated with appropriate measures of prevention and/or medical follow-up for laboratory staff should contribute to minimize risks of being infected by *M. tuberculosis* at work and protect environment.

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