Methods of mycobacterial DNA isolation from different biological material: a review

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ABSTRACT: Mycobacteria cause serious infections in animals and human beings. Huge economic losses on farms are caused by selected species of this wide family. A high risk of transmission of infection from animal to human exists. The knowledge of exact pathogen characteristics is an important factor which can improve quick and adequate healing. Cultivation and determination of phenotype is still the "gold standard", but has the disadvantage of taking a long time and also low detection limit. Biochemical characterisation of isolates is not exact, and it is expensive. A more popular method used is the amplification of specific loci by polymerase chain reaction (PCR). For this method, the isolation of sufficient amounts of purified DNA is necessary. In this paper the most frequently used method for DNA isolation from live mycobacterial cells, body fluids, tissues, histological samples and forensic materials are outlined. This paper assists only as guide for these methods, so we describe them briefly.

Keywords: Johne's disease; Crohn's disease; zoonoses

List of abbreviations: **BCG** = Bacille Calmette-Guérin; **CB18** = C18-carboxypropylbetain; **CFU** = colony forming units; **CPH** = N-cetylpyridinium chloride, cerebrospinal fluid; **CTAB** = cetyltrimethyl ammonium bromide; **DNA** = deoxyribonucleic acid; **DR** = direct repeat domain; **DTT** = dithiotreitol; **EDTA** = disodium ethylene diamine tetraacetate 2 H_2O; **FB** = freezing and boiling; ***HIV/AIDS*** = Human Immune-Deficiency Virus/Acquired Immune Deficiency Syndrome; **IgG** = class G immunoglobulin; **IMS** = immunomagnetic separation; **IS** = insertion sequence; **LCM** = laser capture micro-dissection; ***MAC*** = *Mycobacterium avium* complex; **MOTT** = mycobacteria other than tuberculosis; ***MTC*** = *Mycobacterium tuberculosis* complex; **NALC** = N-acetyl-L-cystein; **OTN-PCR** = one-tube nested polymerase chain reaction; **PBS** = phosphate-buffered saline; **PCI** = phenol-chloroform-isoamylalcohol; **PCR** = polymerase chain reaction; **PRA-PCR** = polymerase restriction analysis-polymerase chain reaction; **RFLP** = restriction fragment length polymorphism; **rRNA** = ribosomal ribonucleic acid; **SDS** = sodium dodecyl sulphate; **TE** = Tris-HCl + EDTA buffer; **TLC** = thin-layer chromatography; **TSA** = tuberculostearic acid: 10-methyloktadekan acid

Contents

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1. Introduction

The genus *Mycobacterium* comprises of over 100 species (Tortoli et al., 2001; www.bacterio.cict.fr; www.dsmz.de/bactnom/nam1932.htm); many of them are pathogenic for humans and animals. The best-known human and bovine mycobacterial pathogens being members of *Mycobacterium tuberculosis* complex (*MTC*). It is noteworthy that more than 30 million cases of tuberculosis have been registered across the world at the beginning of the 21st century. An annual occurrence of new cases and a mortality rate of over 8 million and 2 million, respectively (Savic et al., 1992; www.who.int/mediacentre/factsheets/who104/en/). Other well known mycobacteria are members of *M. avium* complex (*MAC*; include *M. a. avium* and *M. a. hominissuis*), and atypical (conditionally pathogenic) mycobacteria like *M. kansasii*, *M. xenopi*, and others (Wayne and Kubica, 1986; Murray et al., 2002).

Rapid detection of current infection is extremely important for early therapy, or for control measures in human and animal populations, respectively. For example, the diseases caused by members of *MAC*, causing avian tuberculosis and mycobacteriosis, became more important in recent years, particularly in association with the worldwide pandemic situation caused by *HIV/AIDS* (Pavlik et al., 2000b; Dvorska et al., 2001; Mijs et al., 2002). Besides *HIV*-positive patients, people with impaired immunity due to other factors (cytostatic or immunosuppressant treated patients, alcoholics, drug addicted individuals, and diabetics) rank among the most threatened groups. The major cause of detected mycobacterial infection is due to the members of the *MAC* (Garriga et al., 1999; Pavlik et al., 2000b). In such cases rapid and accurate diagnosis is essential, and adequate treatment may be initiated in order to prevent further transmission.

Early detection of the causal agent in farm animals is important for the elimination of infected animals from a herd, adopting adequate measures to localize outbreaks of infection and consequently to reduce the risk posed by animal products that might be sources of infection for humans. For example, *M. a. paratuberculosis*, the causal agent of paratuberculosis or Johne's disease in ruminants, is difficult to control after late detection in the herd (Pavlik et al., 2000a; Ikonomopoulos et al., 2004).

Isolation of mycobacteria and their identification based on phenotypical manifestations, particularly culture, has been constantly used as a "golden standard" (Taylor et al., 2001). Due to the fact that these methods are much more time consuming, their use is on the decline. They have been replaced by methods based on DNA detection.

Selection of an adequate isolation method of mycobacterial DNA is essential for being successful in the following detection steps. The optimum method is different for each sample type. Various comparative studies, e.g. by Wards et al. (1995), have been beneficial.

The purpose of the present study is to review the DNA isolation methods currently used, followed by PCR analysis, which has become one of the most critical diagnostic tools besides culture examination, usually used in the laboratory.

2. Isolation of mycobacterial DNA from living mycobacterial cells

In general, a variety of methods can be used for DNA isolation from different biological materials, from boiling the sample in distilled water, autoclaving, disruption by glass beads or sonication, to the use of different enzymes and surfactants. However, isolation of nucleic acids from mycobacteria is more difficult than from other microorganisms because of a thick peptidoglycan layer characteristic of the mycobacterial cell wall, which makes it resistant

to a number of lysis buffers (Wards et al., 1995; Cornejo et al., 1998; Murray et al., 2002).

2. 1. DNA isolation from mycobacterial isolates

2.1.1. Physical methods

The simplest way of DNA release from mycobacterial suspension is boiling for 10 to 15 min in distilled water (Tortoli et al., 2001; Svastova et al., 2002).

Other authors have used a combination of different techniques. For instance, Savic et al. (1992) used enzymes, silicon beads (<150 μm), and sonication (20 kHz) treatment of cells isolated from sputum simultaneously. Zirconium beads together with proteinase K and subsequent phenol-chloroform extraction were used for DNA extraction from bacterial cells isolated from ileal mucosa samples (Englund et al., 2001).

2.1.2. Chemical methods

In cases where a higher purity of mycobacterial DNA is needed, an isolation method using Cetyltrimethyl Ammonium Bromide (CTAB) is used. Centrifuged bacteria are re-suspended in TE buffer containing proteinase K (in some cases also SDS) to process the resistant cell wall. After 3 hrs of incubation, proteins are precipitated with 5M NaOH and CTAB/NaOH (CTAB in 0.7M NaOH). This step is conducted at 65°C for 20 min. Subsequently, DNA is purified by extraction with chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1) (PCI). After the last step of extraction is completed, DNA is precipitated with isopropanol at –70°C for 1 hour. After centrifugation, DNA is washed from the pellet with 70% ethanol and dissolved in distilled water (Hill et al., 1972; Pierre et al., 1991).

This method was also used (e.g. by Rodriguez et al., 1997) for DNA extraction from *MTC* obtained by culture of the sputum. Subsequently, the sensitivities of PCR (amplicon was detected by dot-blot with a specific probe) and nested PCR were compared. The levels of PCR detection with the probe and nested PCR were 50 fg and 10 fg, respectively, which was about two mycobacteria genomes equivalent. A similar isolation procedure is currently used as one of the standardized methods (for example RFLP) on *M. a. paratuberculosis* (Pavlik et al., 1999) or *M. a. avium* (Dvorska et al., 2003).

2.1.3. Combination of physical and chemical methods

Garriga et al. (1999) simplified and combined the aforementioned physical and chemical methods. A loop of bacteria scraped off the medium was re-suspended in an alkaline solution of 0.5M NaOH and 0.05M Na-citrate. After 10 min of incubation and centrifugation, the pellet was washed with 0.5M Tris-HCl (pH 8.0). The remaining cells were re-suspended in distilled water and heated to 95°C for 30 min.

In McGill University (Montreal, Canada) they first collected colonies into Tris-EDTA buffer (pH 8.0) and heated for 20 min at 80°C. Then they added lysozyme and incubated the mixture at 37°C for 2 hours. After the addition of SDS and proteinase K, and a short incubation (20 min at 65°C) they added a mixture of CTAB and NaCl. The precipitation of DNA was carried out using ethanol after chloroform-isoamylalcohol (24:1) extraction (Ausubel et al., 2002; Somerville et al., 2005).

2.2. Isolation of mycobacterial DNA from body fluids

Mycobacteria are present in various body fluids, secretions and excretions: milk, blood, sputum and bronchoalveolar lavages, cerebrospinal fluid and semen during pathological processes (Ayele et al., 2004, 2005).

2.2.1. Sputum, bronchoalveolar lavages, and bronchial and tracheal aspirates

A number of commercially available kits have been developed for the detection of *M. tuberculosis* in various samples from the respiratory tract. Many authors (Schirm et al., 1995; Vuorinen et al., 1995; Beavis et al., 1995) compared the sensitivity and specificity of the above methods with their own laboratory procedures. The commercially available test Amplicor was compared with the culture and microscopic examinations and with the "in-house" PCR for the detection of mycobacterial infection in sputum and bronchial samples (Schirm et al., 1995). DNA was isolated by centrifugation after

addition of guanidine thiocyanate and silicon particles. Amplification products were detected by dot-blot. "In-house" PCR had the highest sensitivity (92.0%); however, its specificity was lower (92.6%) than that of culture (100%) and direct microscopy (99.8%). Despite the low sensitivity of the Amplicor test (70.4%), the authors viewed this method as a useful diagnostic tool because it is rapid, easily performed and non-expensive.

A comparison of methods for extracting *M. tuberculosis* DNA from respiratory specimens (spiked sputum) made Aldous et al. (2005). They compared Tris-EDTA (TE) boil extraction, PrepMan ultra extraction (Applied Biosystems, Inc., Foster City, California, USA), Infectio Diagnostics, Inc. (IDI) lysis extraction (Infectio Diagnostics, Inc. Quebec, Canada), QIAGEN QIAmp DNA mini kit (QIAGEN, Inc., Valencia, California, USA), sodium dodecyl sulphate (SDS)-Triton X extraction and SDS-Triton X plus sonication and quantified the effectiveness of each extraction method by two quantitative real-time PCR assays. Column-purified DNA should theoretically be the cleanest, containing the fewest PCR-inhibitory substances. Their results showed that column purification is not necessary for the extraction of DNA from sputum samples to be tested for *M. tuberculosis* by PCR. IDI lyses tubes provided the greatest yield of DNA in both PCR assays. SDS-Triton X couldn't be recommended for extraction of mycobacterial DNA because of the presence of PCR inhibitors.

Most of the authors working in human medicine are aimed at the detection of mycobacterial DNA from sputum samples and bronchoalveolar lavages from pulmonary form of tuberculosis. Before *M. tuberculosis* detection in human sputum, the samples are decontaminated using the N-acetyl-L-cystein (NALC)-NaOH method (Kent and Kubica, 1985; Vuorinen et al., 1995). Most microorganisms, except for mycobacteria, are killed during this process. Samples may be further treated with proteinase K and SDS and concurrently heated to 65°C. DNA is then extracted with phenol-chloroform and precipitated with ethanol (Fukushima et al., 2003).

Savic et al. (1992) treated sputum with dithiothreitol (Sputolysin) before conducting subsequent procedures (culture, microscopy, gas chromatography, and PCR). They prepared samples for PCR by boiling for 10 min. DNA was extracted using silicon coated glass beads (<150 μm), proteinase K and sonication (20 kHz). In comparison with culture and microscopy examination, the highest sensitivity level was detected for PCR (95%); however, the highest specificity was found with direct microscopy (100%). The lowest sensitivity (55%) and specificity (87%) were found for TSA detection using gas chromatography.

Samples of bronchial lavages are commonly examined in human medicine. Precipitation of samples with 1M NaOH followed by centrifugation performed well for isolation of DNA from mycobacteria (Hidaka et al., 2000). The pellet is subsequently re-suspended in a lysis buffer, supplemented with RNase and proteinase K. After an overnight incubation, phenol-chloroform extraction and alcohol precipitation were conducted. Hidaka et al. (2000) compared sensitivity of PCR-RFLP examination of samples obtained by bronchoscopy (tissue collected with a brush was re-suspended in saline and processed as a lavage), bronchial lavages and combinations of the methods. They found out that the sensitivity of the reaction was the best for the combination of brushed and washed samples.

Besides sputum, Vuorinen et al. (1995) tested bronchoalveolar lavages and bronchial and tracheal aspirates, and compared Ziehl-Nielsen staining with Amplicor and Gene-Probe amplified *M. tuberculosis* direct tests. All three methods had comparable specificity (about 99%); however, a higher sensitivity was found for the commercial kits (about 84%).

2.2.2. Semen

Ahmed et al. (1999) recovered *M. bovis* BCG from experimentally inoculated bull semen. Samples were centrifuged; the obtained pellet was frozen in liquid nitrogen and subsequently heated. The cells were lysed with lysozyme and deproteinated with proteinase K, SDS, and CTAB. The suspension was then treated with a PCI method and DNA precipitated with isopropanol. This resulted in the detection of 10 to 100 CFU/ml per sample.

For the isolation of DNA from naturally infected bull semen, Ayele et al. (2004) used a commercial kit (QIAamp DNA Mini Kit; QIAGEN, Germany). The IS*900* was employed to determine the presence of *M. a. paratuberculosis* by PCR. The modifications made were an extension of the amplification of 60 cycles, and the use of Hot-Start Taq PCR Master Mix Kit (QIAGEN, Germany). This modification enabled the recognition of 1 to 10 genome units in a single reaction.

2.2.3. Milk

Antognoli et al. (2001) tested milk for using a one-tube nested PCR (OTN-PCR) method. Milk was inoculated with two different dilution series of *M. bovis* cells. Five methods of preparation of samples were used in parallel as follows:

(i) Centrifugation alone; the pellet and remaining supernatant were used for further testing.
(ii) Treatment with C18-carboxypropylbetain (CB18), resin 1 and proteinase K (Cornejo et al., 1998).
(iii) Centrifugation with resin 1 and proteinase K treatment.
(iv) Centrifugation with resin 2 with proteinase K treatment.
(v) Centrifugation and immunomagnetic separation (IMS).

By subsequent testing with OTN-PCR, the authors were able to detect one mycobacterium per reaction. Significant differences in sensitivity of detection of mycobacteria between both dilution series were revealed. The authors ascribed this fact to the high affinity of the mycobacterial surface to lipids. This was because a different thickness of the cream layer was in each of the tested groups of milk. They suggested that methods of DNA IMS or DNA binding to CB18 and Chelex 100 perform best for *M. bovis* detection.

Efficiency of mycobacterial DNA isolation from milk can for instance be enhanced by IMS as described by Grant et al. (1998) and Antognoli et al. (2001). The advantage of this method is its ability to strongly concentrate mycobacteria in samples.

2.2.4. Blood

Ahmed et al. (1998) has tested the potential detection ability of *M. tuberculosis* DNA from the blood of patients with localised pulmonary tuberculosis. After osmotic lysis of erythrocytes, leucocytes were centrifuged, frozen in liquid nitrogen and heated to 100°C for 10 min. Cell lysis was induced with lysozyme, proteinase K, and hexadecyl-triammonium bromide. DNA was purified using chloroform extraction. The authors ascertained that they detected less than 10 copies of mycobacterial DNA per 5 ml of blood. However, the detection in blood can only be successfully performed in immunocompromised patients (*HIV*-positive, alcoholics, and diabetics) where mycobacteria massively penetrate into the blood (specifically lymphocytes).

Some laboratories use centrifugation alone for the preparation of samples, although a higher sensitivity is obtained by the application of specific DNA sequence detection methods. Barry et al. (1993) detected *M. bovis* in bovine blood by a combination of PCR and DNA probes. The authors developed a DNA probe derived from the spacer region between the 16S and 23S rRNA genes. PCR was followed by immobilization of samples on a nylon membrane and hybridization with oligonucleotide probe. They detected about 100 mycobacteria per 1 ml of blood with this combined DNA probe/PCR method. In some samples, only the cell pellet was positive, and in others, both the fractions-pellet and supernatant were positive. The advantage of this PCR method employed for blood sample testing consists of the fact that it can be used irrespectively of the immune status of the animal; in contrast, *M. bovis* can only be detected by a culture of blood in the advanced stages of the disease.

Naser et al. (2004) identified *M. a. paratuberculosis* from peripheral blood from patients with Crohn's disease. Genomic DNA was extracted from uncultured buffy coat cells. Authors used boiling of cells suspension in TE buffer at 100°C for 30 min. After centrifugation they extracted DNA by PCI. They used this method in order to search for the relationship between the presence *M. a. paratuberculosis* and Crohn's disease.

2.2.5. Cerebrospinal fluid

The PCR method described by Kaneko et al. (1990) for the detection of *M. tuberculosis* in cerebrospinal fluid is very effective. The authors only treated samples with SDS and proteinase K. After incubation at 65°C for 1 h, they extracted DNA with phenol-chloroform. Mycobacterial DNA was detected by PCR and subsequent hybridisation. The sensitivity of their experiment was 83.3%.

2.3. Isolation of mycobacterial DNA from tissues

Various methods can likewise be used for the isolation of mycobacterial DNA from animal and human tissues. With respect to the fact that these samples are more complex, it is much more difficult to obtain

a pure DNA extract. Moreover, they may contain various inhibitors of PCR amplification (Wards et al., 1995).

A comparison of methods for DNA isolation from tissues is useful for their application in practical conditions (Wards et al., 1995). The investigators compared various methods of direct *M. bovis* DNA isolation from tissues (lymph nodes, lungs, and liver) that were simultaneously examined by culture and Ziehl-Neelsen staining. Besides testing real samples, they also compared the sensitivity of respective isolation methods. Various methods of DNA isolation were evaluated, and the method that performed best, was used for examination of 110 tissue samples. Each piece of tissue was homogenized in PBS (0.14M NaCl, 4mM KCl, 8mM Na_2HPO_4, 2mM KH_2PO_4, pH 6.5 buffer according to Wards et al., 1995) and stored frozen at –20°C before further extraction. The following methods of isolation were used:

(i) Magnetic bead capture: used Dynabeads magnetic beads coated with mouse IgG against *M. bovis* protein MPB70 (Dynal a.s., The Netherlands).
(ii) Zirconium bead lysis: DNA extraction by PCI, tRNA, and zirconium beads (0.5 mm).
(iii) Lysis by boiling: boiling mycobacteria with 1% Triton X-100.
(iv) Lysis in the presence of Chelex: boiling with 0.1% SDS and 10% Chelex-100.
(v) DNA recovery using glass powder: used glass powder (Geneclean, Qbiogene, USA) and alcohol precipitation.

However, despite proteinase K digestion together with DNA recovery, there was a relatively large amount of tissue material co-pelleted that could be inhibitory to the PCR reaction. The method of lysis, including the step of incubation with proteinase K and 1% SDS at 60°C for 1.5 to 2 hrs was most effective and practical. Although the zirconium bead lysis method proved to be equally as good as the above method, the authors viewed it as less practical as it involved additional transfer of material into another tube, which increased the risk of contamination. It was possible to recover amplifiable DNA using the boiling method, but only with numbers of CFU higher than 10^4 per reaction.

The method using Chelex was more sensitive. As few as 500 to 1 000 CFU could be detected; however, low effectivity of this method was found on tissue homogenates. Extraction with PCI was essential for successful amplification. Other positive samples were revealed using the CTAB extraction step. Although the method using glass powder (Geneclean, Qbiogene, USA) was likewise very effective, it was not markedly better than CTAB alone, and moreover, it was laborious and time consuming.

The procedure finally adopted to isolate mycobacterial DNA is described below. Homogenates were digested at 60°C with proteinase K for 1.5 to 2 hours. After digestion, PBS with 0.05% Tween 80 was added. Following centrifugation, the pellet was treated with proteinase K and 20% SDS for 1 h at 60°C. The suspension was mixed with CTAB in NaCl and incubated at 65°C for 10 min. After cooling, DNA was extracted with PCI and precipitated with tRNA and ethanol.

The combination of lysis buffer (1% Triton X-100, 10mM EDTA, 3mM dithiotreitol, 10mM Tris HCl, pH 7.5) and treatment of supernatant obtained by centrifugation, using Subtilisine, lysozyme and pronase with SDS (Sanderson et al., 1992) can be used for DNA recovery from tissues. This method was used for the detection of *M. a. paratuberculosis* from human intestinal tissue. DNA can be further extracted from tissue homogenates of the lymph nodes with zirconium beads, sonication, and proteinase K as proven by Taylor et al. (2001). DNA isolation from the cell lysate was subsequently performed with biotin labelled oligonucleotides complementary to the DR domain of *M. tuberculosis* genome. They separated labelled denaturised DNA using streptavidine bound magnetic beads.

Isolation of genomic DNA of *M. a. paratuberculosis* from intestinal mucosa and the cortical portion of mesenteric lymph nodes was described by Sivakumar et al. (2005). At first they homogenised samples in TE buffer and took up the supernatant after it settled. Lysozyme was added, and mixture was incubated at 37°C for 2 hours. Then, 10% SDS and proteinase K were added and incubated at 56°C overnight (16 to 20 hours). Digested samples were mixed with 5 M potassium acetate and centrifuged. The DNA was purified from the supernatant by PCI extraction. The DNA was precipitated from the aqueous phase by addition of 1/10 volume of cold 3 M sodium acetate and 2.5 volumes of absolute ethanol.

2.4. Isolation of mycobacterial DNA from faeces

It is problematic to isolate mycobacterial DNA from faecal samples because separation of all bal-

last materials is difficult. For example Garrido et al. (2000) processed sheep faecal samples by means of different isolation procedures and measured the PCR sensitivity. They selected *M. a. paratuberculosis* and its insertion element IS*900* as a model. Samples were homogenized in different concentrations of SDS or N-cetylpyridinium chloride (CPH). After sedimentation, the upper phase was collected and washed three times with PBS. The following methods were compared for their efficiency of cell disintegration: enzymatic (lysozyme, proteinase K), physical (freezing and boiling = FB), chemical (guanidine, Triton X-100), and their combinations. Testing different concentrations of SDS and CPH by the FB method, 5% SDS showed the best results.

The other extraction methods did not yield a detectable amount of DNA. Phenol-chloroform extraction was excluded because it was time consuming and toxic. Despite lower amounts of DNA obtained by the FB method in comparison with xylene extraction (Challans et al., 1994), which was used as reference method, the number of positive results obtained by the FB method was higher. Comparison of various types of chemical treatment combined with physical methods, most DNA with the highest purity was obtained using FB method and by treatment of samples with TE/Triton X-100. These results were also confirmed by obtaining higher yields using PCR. Sensitivity of isolation of mycobacteria was detected by adding a known amount of mycobacteria to faeces where the lowest detectable amount was 50 bacteria per gram of material. Sensitivity and specificity of FB protocol was 94.1% and 92.3%, respectively. Identical specificity (92.3%) was obtained using xylene, but sensitivity rapidly decreased to 41.2%. The authors viewed the FB PCR method as too expensive for routine use in the eradication programmes and primarily recommend it for research conditions.

3. Isolation of mycobacterial DNA from dead mycobacterial cells

3.1.1. Formalin-fixed tissues for anatomical dissection

Bartos et al. (2006) have studied the presence of mycobacteria in tissue samples from four cadavers fixed with formalin and tissue samples from a recently deceased unpreserved individual who had a history of human tuberculosis infection. The tissue samples were taken from four embalmed cadavers and one cadaver unpreserved undergoing a *post mortem* (cause of death not related to tuberculosis). All of which were examined for the presence of tuberculous lesions and the specific presence of *MTC* and *MAC* members by microscopy, culture, and PCR analysis of four genomic elements (IS*6110*, *mtp*40, IS*901*, and IS*1245*). Although microscopy and culture examination was negative in all tissue samples from the four embalmed cadavers, the PCR analysis of IS*6110* and *mtp*40 was positive in tissue samples of tuberculous lesions from the lungs of two embalmed cadavers and from intact kidney tissue of one of these cadavers. Microscopy and culture examination of liver and spleen tissue from the unpreserved cadaver were positive for mycobacteria. IS*901* and IS*1245* PCR analyses, specific for *M. a. avium*, were positive in both tissue samples with and without tuberculous lesions.

3.1.2. Formalin-fixed and paraffin-embedded tissues in blocks

Before various histopathological examinations, tissues samples were fixed with 10% formalin and stored in paraffin blocks (Marchetti et al., 1998; Whittington et al., 1999). During formalin fixation DNA fragmentation occurs, and covalent bonds by DNA-proteins are formed. The time of fixation is very important. For example, 270 bp fragments can be amplified after a 30-day formalin fixation, whilst 1 300 bp cannot be amplified after a fixation period longer than 24 hours. The time of sample storage is also an important factor. Short fragments (270 bp) of human β-globin gene can be amplified even after 15-year storage.

In contrast, the success rate of PCR of fragments longer than 500 bp rapidly decreases after 1 to 2 years of storage. Many inhibitors and ballast DNA are usually present in the isolates after fixation and extraction (Whittington et al., 1999). They can be removed by dilution, which is, however, accompanied by a reduced amount of target DNA. Generally, the success rate of PCR depends on the fixation and storage time, isolation procedure, length, and the amount of target DNA matrix (Marchetti et al., 1998; Whittington et al., 1999).

Some authors compared the efficiency of DNA isolation and detection methods. Whittington et al. (1999) compared three different procedures of

DNA isolation and their impact on possible IS*900* amplification in the *M. a. paratuberculosis* genome (431 bp and 229 bp). They used 1 to 6 year old formalin-fixed and paraffin-embedded tissues samples of intestinal tissue from sheep, cattle and goats:

Method A. Thin slices of paraffin-embedded tissue blocks were first centrifuged in sterile water containing 0.5% Tween 20. The sample was then boiled twice and rapidly frozen in liquid nitrogen. Finally, the sample was centrifuged and supernatant used for further investigation.

Method B. Paraffin was removed from a group of samples by centrifugation with xylene. Subsequently, the pellet was washed twice with 100% ethanol and after drying, re-suspended in TE buffer with NaCl, proteinase K, and SDS. DNA purification was performed with a commercially available kit.

Method C. Samples were first treated with xylene at 37°C for 20 min. Purification with 95% ethanol and 5% methanol followed, samples were then dried and re-suspended in 50mM Tris-HCl, $CaCl_2$, lysis buffer and frozen three times in liquid nitrogen, and thawed at 60°C. Proteinase K was inactivated at 95°C for 10 min. After addition of EDTA, the samples were stored.

Identical results were obtained by Method A using both the PCR techniques, whereas Methods B and C showed false negative results in some cases. The effect of dilution showed to be positive for Method B, whereas no effect was observed for Methods A and C. PCR assay of DNA obtained by Method A showed 12% and 31% false negative results for 229 bp and 413 bp region, respectively. Based on the results obtained, the above investigators began to routinely use the isolation Method A with dilution 1:10 and 229 bp PCR for detection of *M. a. paratuberculosis* from paraffin-embedded tissue blocks in their laboratory.

In contrast, Marchetti et al. (1998) compared various PCR protocols (for amplification of *mtp*40, gene for 65-kDa antigen, and IS*6110*) for the detection of *MTC* isolates. Paraffin was removed from a sample with xylene and cells were lysed with SDS and proteinase K. DNA was extracted using the phenol-chloroform method. The highest sensitivity was reached for amplification of multicopy insertion element IS*6110* (106 bp and 123 bp). Only 13 to 33% of false negative results obtained were in relation to the DNA amount and primers used. Popper et al. (1994) used a similar method for the detection of a gene fragment for the 65-kDa surface protein of *M. tuberculosis* (234 bp), however, without phenol chloroform extraction and supplementation with 0.5% Tween 20. Using this method the former authors were able to identify 20 mycobacterial genomes per reaction.

3.1.3. Formalin-fixed and paraffin-embedded histological sections

Laser micro-dissection (LCM) for the isolation of subepithelial granuloma from tissue sections from patients suffering from Crohn's disease was used by Ryan et al. (2002). The first control section was stained with haematoxylin and eosin and granulomas were localized. Other sections were treated with xylene and dehydrated with alcohol. DNA was isolated from identified granulomas with a commercially available kit (QIAGEN, Germany); and using nested-PCR, the authors detected 155 bp fragments from IS*900* region of *M. a. paratuberculosis*. Authors identified 40% of cases using this method. On the contrary, only 20% of cases were diagnosed by DNA isolation from whole sections of paraffin-embedded tissue blocks.

3.2. Isolation of mycobacterial DNA from archaeological and forensic samples

The detection of DNA from archaeological and forensic samples opened a new research area with many implications. Ancient DNA (aDNA) has been isolated from fossils, subfossil remains, artefacts, traces from biological sources, bones, and museum specimens. The comprehensive information about methods and principles of aDNA analysis has been carried out by Sensabaugh (1994). The first successful amplification of aDNA from animal tissue was reported by Higuchi et al. (1984). Paabo (1985) cloned aDNA from Egyptian mummies.

Hagelberg (1994) described the method in which pulverized bones were washed several times and decalcified in EDTA. Lysis buffer (0.5 M EDTA, proteinase K and 0.5% N-lauroylsarkosine K) was added to the sedimented pellet and DNA was extracted with phenol-chloroform.

The most standard method for DNA extraction from ancient and forensic material consists of surface decontamination by cleaning with 0.5% sodium hypochlorite solution and subsequent mechanical removal of the outer surface under strictly DNA-free conditions (Zink et al., 2005). Samples are then taken

Table 1. Summary of described methods

Material	Methods of isolation	PCR sensitivity or detection limit	References
Mycobacterial isolates	Boiling	n/a	Tortoli et al. (2001), Svastova et al. (2002)
	Enzymes, silicon beads, sonication	95%	Savic et al. (1992)
	Proteinase K (+SDS), NaOH + CTAB, PCI extraction	100% if >100 CFU/ml	Hill et al. (1972), Pierre et al. (1991)
	NaOH + heating 95°C	cumulative detection limit was 100%	Garriga et al. (1999)
	Heating, lysozyme + SDS + proteinase K, chloroform-isoamyl alcohol precipitation	n/a	Ausubel et al. (2002), Somerville et al. (2005)
Sputum	Proteinase K+ SDS, heating 65°C, PCI extraction	n/a	Fukushima et al. (2003)
Bronchial lavages	Sample precipitation with 1N NaOH, RNase + proteinase K, PCI precipitation	70–91%	Hidaka et al. (2000)
Semen-experimentally infected	FB, lysozyme + proteinase K, SDS + CTAB, PCI precipitation	10–100 CFU/ml	Ahmed et al. (1999)
Semen-naturally infected	Commercial kit	n/a	Ayele et al. (2004)
Milk	Immunomagnetic DNA separation	0.1–1.0 cell	Antognoli et al. (2001)
Blood	FB, lysozyme + proteinase K + hexadecyltriammonium bromide, chloroform extraction	n/a	Ahmed et al. (1998)
	TE-boiling, PCI extraction	n/a	Naser et al. (2004)
Cerebrospinal fluid	SDS + proteinase K, phenol-chloroform extraction	83.3%	Kaneko et al. (1990)
Tissues-fresh	Proteinase K, Tween 80, SDS, CTAB, PCI extraction	0.2–2.0 genomes	Wards et al. (1995)
	Triton X-100 + EDTA + DTT, Suptilisin + lysozyme + pronase + SDS	10 fg of *M. avium* subsp. *paratuberculosis* DNA	Sanderson et al. (1992)
	Zirconium beads, sonication, proteinase K, immunomagnetic separation of DNA	93% (conventional PCR), 71% LightCycler PCR	Taylor et al. (2001)
	Lysozyme + SDS + proteinase K, PCI extraction	230 cells/g of spiked tissue	Sivakumar et al. (2005)
Tissues-formalin-fixed	Enzymes, silicon beads	n/a	Bartos et al. (2006)
Tissues-paraffin blocks	0.5% Tween 20, FB	88.2%	Whittington et al. (1999)
	Xylene, SDS + proteinase K, phenol-chloroform extraction	90%	Marchetti et al. (1998)
Tissue-histological sections	Laser micro-dissection, isolation of DNA by commercial kit	100%	Ryan et al. (2002)
Bones	EDTA, proteinase K + 0,5% *N*-lauroyl-sarkosine K, phenol-chloroform extraction	n/a	Hagelberg (1994)
	EDTA, proteinase K + guanidium thiocyanate, silica beads	n/a	Zink et al. (2005)
Faeces	FB, TE/Triton X-100	50 cells/g	Garrido et al. (2000)

SDS = sodium dodecyl sulphate, CTAB = cetyltrimethyl ammonium bromide, PCI = phenol-chloroform-isoamylalcohol, FB = freezing and boiling, EDTA = disodium ethylene diamine tetraacetate 2 H_2O, DTT = dithiotreitol, TE = Tris-HCl + EDTA buffer; n/a = not available

from the inner part of the bones. Thorough homogenisation of tissue is performed by grid mill, lyses powder by enzymes and by guanidium thiocyanate, and capturing DNA on silica beads (Morell, 1994; Spigelman, 1994; Taylor et al., 1996; Brosch et al., 2002; Donoghue et al., 2005; Zink et al., 2005).

DNA extraction and PCR are performed with standard precautions, including the use of ultraviolet-irradiated safety cabinets, ultra-pure reagents, and sterile disposables under full isolation procedures (Rothschild et al., 2001). Control samples that contain no bone material and bone material from non-lesional regions and from unaffected species are used to assess contamination during extraction and amplification processes (Salo et al., 1994).

This method of DNA isolation is suitable for spoligotyping (Sola et al., 2000), which was used to improved the study of epidemiology, for example in the characterization of *MTC* DNAs from Egyptian mummies (Zink et al., 2003) or to phylogenetic reconstruction within *M. tuberculosis* Beijing genotype in North-western Russia (Mokrousov et al., 2002). Besides DNA detection, the mycolic acid analysis is seldom used, e.g. for confirmation of an osteological diagnosis of tuberculosis (Gernaey et al., 2001).

4. Conclusions

Methods for the detection of mycobacteria are continuously being developed. Scientists from all over the world try to propose a rapid, accurate and low-cost test. However, we can generally say that a universal isolation and detection method does not exist. Most of the methods can only be used for a limited spectrum of applications, or they are technologically demanding and time consuming.

The majority of workplaces have developed their own isolation procedures during long-years of experience. Accordingly, it was not possible to encompass all of them into the present study. Nevertheless, commercially available isolation kits are more and more in use. It is possible to impartially compare the results obtained by those kits in different laboratories.

The presence of mycobacterial DNA is most commonly detected by PCR technique. The character of the produced amplicons is subsequently tested on agarose gel. For further typing, hybridisation with specific probes, spoligotyping, RFLP or direct sequencing is used. However, certain inaccuracies also exist with these procedures, given by natural polymorphism or the inability of the methods to identify dead microorganisms.

Advances in molecular biology offer the most promising methods for the future. Novel methods being developed are more and more rapid and accurate. Such methods will make it possible to reveal the dangerous mycobacterial diseases much more effectively and to initiate early, adequate and effective treatment of the diseases.

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