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Comparison of filtering methods, filter processing and DNA extraction kits for detection of mycobacteria in water

Marija Kaevska[1], Iva Slana[1]

[1] *Veterinary Research Institute, Brno, Czech Republic*

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Abstract

Introduction and objective. Mycobacteria have been isolated from almost all types of natural waters, as well as from man-made water distribution systems. Detection of mycobacteria using PCR has been described in different types of water; however, currently, there is no standardised protocol for the processing of large volumes of water.

Material and Methods. In the present study, different filtering methods are tested and optimised for tap or river water filtration up to 10 L, as well as filter processing and DNA isolation using four commercially available kits.

Results. The PowerWater DNA isolation kit (MoBio, USA), together with a kit used for soil and other environmental samples (PowerSoil DNA isolation kit, MoBio), had the highest efficiency. Filtration of 10 L of water and elution of the filter in PBS with the addition of 0.05% of Tween 80 is suggested.

Conclusions. The described protocol for filter elution is recommended, and the use of the PowerWater DNA isolation kit for the highest mycobacterial DNA yield from water samples. The described protocol is suitable for parallel detection of mycobacteria using cultivation.

Key words

DNA extraction, *Mycobacterium avium* subsp. *paratuberculosis*, IS*900*, qPCR, water filtration

INTRODUCTION

Mycobacteria are ubiquitous in the environment and are encountered naturally in soil, water, and in the interfaces between soil and air and water and air. Non-tuberculous mycobacteria (NTM) can cause disease in the elderly, or in people with impaired immune systems. Due to the growth of these populations in the western world, there is increasing focus on infections caused by NTM [1, 2].

Water is one of the main reservoirs of non-tuberculous mycobacteria, where they persist and may pose a threat to human and animal health [3, 4]. The role of potable water in disease transmission has been reviewed previously [5]. The link between isolates obtained from patients and household water has been established using genotyping methods [5, 6].

Mycobacterium avium subsp. *paratuberculosis* (*M. a. paratuberculosis*) is the causative agent of paratuberculosis, which is widely distributed in cattle herds across Europe and North America [7]. The contamination of the environment, especially pastures and water run-offs, is thus inevitable, in the UK a wide distribution of *M. a paratuberculosis* has been described [8].

The long-term persistence of mycobacteria in hostile environments stems from two key characteristics of these bacteria: 1) their ability to reduce growth rates and oxygen demand is important for their growth in water distribution systems where nutrient availability is very low, and 2) their highly hydrophobic and impermeable cell wall protects them from disinfectants and contributes to surface attachment and biofilm formation [3].

Address for correspondence: Marija Kaevska, Veterinary Research Institute, Hudcova 70, 62100 Brno, Czech Republic
E-mail: kaevska@vri.cz

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Mycobacteria have been isolated from almost all types of natural waters, as well as from man-made water distribution systems. However, their presence and quantity in water has been underestimated due to the serious drawbacks of mycobacterial culture. Isolation is hindered by the slow growth rate of mycobacteria as well as the presence of other, faster growing organisms. Recently, several methods have been developed for culture-independent detection of mycobacteria, of which PCR is the most widely used technique because of its robustness, specificity and sensitivity. Detection of mycobacteria using PCR has been described in different types of water [9, 10, 11]. The occurrence of mycobacteria in water and different detection methods has been reviewed previously [12]. When choosing an appropriate method, it needs to be considered that the sample treatment and DNA extraction have a large impact on the outcome. As a template for DNA isolation, water has the general problem that the concentration of microorganisms is very low. The volumes of water used for mycobacterial DNA isolation vary between studies, although volumes of more than one litre have very rarely been used.

Although mycobacteria are often detected in water, information about the efficiency of their isolation and comparison of methods or recommended protocols for samples of larger volume is missing.

OBJECTIVE

The aims of the present study were to optimise a filtration method and to assess different commercially available kits for DNA extraction, in order to improve the detection of mycobacterial DNA in water.

MATERIALS AND METHOD

Artificial contamination of the water samples. Tap and river water samples (each 10 L) were artificially contaminated with *M. a. paratuberculosis,* using field isolate from infected cattle, first subculture. Tap water samples were collected from the laboratory in the Veterinary Research Institute. River water was collected from a stream on the outskirts of the city of Brno in the Czech Republic. Water was collected in sterile 10 L plastic barrels. During each experiment, one water sample was analysed without artificial contamination, in order to ensure that the water did not contain *M. a. paratuberculosis*. The culture used for artificial contamination was prepared as follows; one millilitre of bacterial suspension grown on Middlebrook M7H9 medium with the addition of mycobactin J was centrifuged for 5 min at 6,000 g. The pellet was washed twice in phosphate buffer saline (PBS) and then re-suspended in one ml PBS. The suspension was then diluted to approx 10^4 cell equivalents/µl (measured using real time PCR). Each dilution was vortexed at maximum speed for one minute to avoid cell clumping. Fifty microliters of the spiking suspension were used for artificial contamination of each sample of water (10 L). For each experiment, three samples were processed in an identical manner.

Preparation of positive control. A lysate was prepared from the same suspension for calculation of the input amounts of *M. a. paratuberculosis* DNA (IS*900* specific target). The qPCR method used in all of the experiments has been described previously [13]. Briefly, the reaction mixture contained 1× DyNAmo Probe qPCR Kit (Finnzyme, Espoo, Finland), 10 pmol of the primers (forward primer 5′-GATGGCCGAAGGAGATTG-3′, reverse primer 5′-CACAACCACCTCCGTAACC-3), 1 pmol of the probe (6FAM–ATTGGATCGCTGTGTAAGGACACGT–BHQ), 4 pmol of the Internal Amplification Control probe (Cy5–GGCTCTTCTATGTTCTGACCTTGTTGGA–BHQ), 0.2 U of Uracil DNA Glycosylase (Sigma, St. Louis, MO, USA), 5×10^1 copies of Internal Amplification Control plasmid (construct from potato DNA with flanking primer sequences for IS*900*) and 5 µl of the DNA template, in a total reaction volume of 20 µl. Amplification was performed under the following conditions: 37 °C for 10 min, followed by initial denaturation at 95 °C for 15 min and 47 cycles at 95 °C for 5 s and at 60 °C for 40 s. Subsequent analysis was carried out using the 'Fit point analysis'option of the LightCycler 480 software.

First experiment. In the first experiment, two different filtration methods were used for both tap and river water. The first was based on a nitrocellulose filter (Merck, Millipore), 90 mm diameter, in a stainless steel holder (Millipore). For river water, it was necessary to include pre-filters with variable pore size. Subsequently, one quarter of the filter was transferred to a 2 ml tube containing four 3.2 mm chrome steel beads and one millilitre of TE buffer, and homogenised in a MagNALyser (Roche). DNA isolation was performed from 250 µl of the supernatant using the PowerSoil DNA isolation kit (MoBio USA).

The second method was based on a protocol used for viruses in river water [14]. Briefly, the water was adjusted to pH 3.5 using HCl, and subsequently filtered through a glass wool column adjusted to pH 7.0. Incubation of the column in 200 ml of Beef extract (pH 9.5) for 30 min was followed by elution. Subsequently, the pH of the elution solution was adjusted to 3.5 (flocculation appeared) and the sample was centrifuged at 7,000 g/30 min. The pellet was re-suspended in 8 ml of PBS. DNA was isolated from 1 ml of the suspension by adding 350 mg of 0.1 mm zirconia silica beads (BioSpec), followed by cell disruption using a MagNALyser (Roche) at 6,400 rpm/60 s.

The preliminary data obtained with these experiments showed that better recovery of mycobacterial DNA was obtained using filtration through the nitrocellulose filter and glass fibre pre-filters.

Second experiment. In the second experiment, after determining which filter was most suitable, different elution methods for the filter were tested, and subsequently, four different DNA isolation kits.

Filtration was performed through a 0.22 µm nitrocellulose filter with a diameter of 90 mm (Millipore, Merck) in a stainless steel filter holder. Subsequently, the whole filter was cut into small pieces and transferred to a 50 ml tube containing 10 ml of one of four types of elution buffer and beads, which was mixed continuously for 30 min at 1,400 rpm. In elution method No. 4, the sample was vortexed at max. speed for 5 min. The different elution protocols were as follows:

1. Elution in 10 ml PBS with glass beads (diameter 3.5 mm, BioSpec).
2. Elution in 10 ml PBS with addition of 0.2% Tween 80.
3. Elution in 10 ml PBS with addition of 0.2% Tween 80 and glass beads (diameter 3.5 mm, BioSpec).
4. Elution in 10 ml PBS with addition of 0.05% Tween 80 and glass beads (vortexed).

Subsequently, the elution mixture was transferred to a new tube and centrifuged at 7,000 g for 10 min. The pellet was then re-suspended in 1 ml of the supernatant. The extraction of DNA was performed using four commercially available kits, three of them for water DNA isolation (PowerWater DNA Isolation kit, MoBio, USA; SurePrep Water DNA Isolation kit, Fisher Scientific; Water RNA/DNA Norgen Biotek Corp., Canada), and one developed for soil DNA isolation (PowerSoil DNA isolation kit, MoBio, USA). In all cases, the manufacturers' instructions were followed, with the exception of the cell lysis step where a MagNaLyser was used in all cases, using the beads provided by the manufacturer. All the samples were processed independently in triplicate.

Additionally, filter homogenisation was performed using one-quarter of the filter in a MagNALyser at 6,400 rpm/60 s, with the addition of TE Buffer and four 3.2 mm chrome steel beads (BioSpec.).

After filtration and DNA isolation, qPCR was performed according to Slana *et al.* [13]. A positive control was also included, and PCR efficiency was calculated according to this positive control. Negative controls for isolation and PCR were included in each run.

Each sample was processed three times in replicates, so that in total six Cp values were obtained. From these values, the DNA isolation efficiency was calculated according to the protocols used, and expressed as a percentage (Fig. 1). DNA yield and purity were also measured using a NanoDrop instrument (Thermo Scientific).

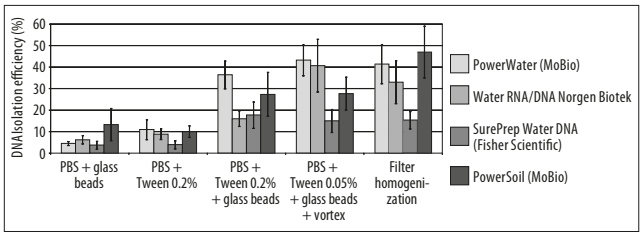

Figure 1. DNA Isolation efficiency of *M. a. paratuberculosis* from water using different elution methods and isolation kits

RESULTS

The first experiment evaluated two different types of filtration: 1) through a filter using a peristaltic pump and 2) through a glass wool column. Better efficiency of DNA recovery was obtained using the filter (55% efficiency compared to 15% using the glass wool column). For processing river water it was necessary to use one or two pre-filters (glass fibre, diameter 90 mm; pore sizes 0.8–8 μm) depending on the turbidity of the water.

The results of the second experiment are shown in Figure 1. DNA recovery rates varied with respect to the different elution methods, as well as with regard to the different DNA isolation kits. The addition of glass beads and use of a vortex instead of a mixing platform increased the efficiency in all samples. Also, the addition of Tween 80 to the PBS buffer resulted in improved recovery.

The commercially available kits for DNA isolation from water were chosen based on their availability on the market and on their utilisation of a simple protocol based on DNA extraction on a spin column. They performed with varying efficiencies (Fig. 1). The highest isolation efficiency was accomplished using the PowerWater DNA isolation kit and PowerSoil DNA isolation kit, which gave similar results. The SurePrep DNA/RNA water extraction kit from Fisher Scientific had the poorest performance in mycobacterial DNA isolation efficiency.

DISCUSSION

The presented study optimised a method for water filtration up to 10 L, and also determined optimal protocols for filter processing and DNA isolation using commercially available kits. 90 mm nitrocellulose filters were used to enable the filtering of large volumes of water.

The volume of water used for DNA isolation of mycobacteria varies between studies. In some cases, as little as 200 ml of water was tested [15]. Many authors have tested up to one litre of water and reported clogging of the filter, especially when surface water was used. On rare occasions, large volumes of water have also been tested (up to 100 L, Pickup et al. [11]). In order to obtain more concentrated samples for analysis of the presence of mycobacteria in water, either filtration or centrifugation has been used. After centrifugation of water, extraction of DNA from the pellet can be easily performed using a simple boiling method [15]. However, the volume of water which can be centrifuged is often limited, and in the majority of studies, filtration has been the preferred method. In the presented study, an attempt was made to extend the standard filtration volume to ten litres.

Results from the first experiment in the current study showed that filtration was more successful when using a filter, compared to glass wool columns. In the case of river water, it was necessary to use pre-filters: 90 mm glass fibre pre-filters with variable size pores – 0.8–8 μm).

DNA isolation from filtered water is usually performed from a fragment of the filter using a protocol suitable for environmental samples [10, 16, 17]. Elution was used to enable isolation of mycobacteria from the whole surface of the filter. The addition of detergents was reported to have an impact on elution efficiency [18]. In the present study, different elution methods resulted in varying isolation efficiencies (Fig. 1). The addition of Tween 80, glass beads, and the use of a vortex instead of a mixing platform, increased efficiency in all samples. Although the top performing elution method had comparable efficiency to filter homogenisation, elution is recommend because the eluate can then also be used for culture.

The highest isolation efficiency was accomplished using the PowerWater DNA isolation kit and PowerSoil DNA isolation kit, which gave similar efficiency results. Most of the isolation protocols reported in the literature are based on kits for DNA isolation from soil [11, 17]. Therefore, the PowerSoil DNA isolation kit was used as a reference. This kit had the best performance, together with the water DNA extraction kit from the same company.

CONCLUSIONS

Different types of filtration and filter processing were evaluated for the analysis of large volumes of tap and river water. According to the results obtained, filter elution with the addition of Tween 80 (0.05%) and glass beads is recommended. In addition to giving the best DNA isolation efficiency, this method is also suitable for microbiological culture, so that one sample can be analysed by both methods in parallel. When used together, these methods could provide greater insight into the contamination levels of water samples.

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