COMPARISON OF THREE METHODS OF RELEASING DNA FROM MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS CELLS

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Received for publication January 29, 2003.

In the present study, three methods of releasing DNA from MAP cells were described. The first one consists in cell wall lysis with the use of sodium lauryl surface (SDS), NaOH and convertible application of low and high temperatures. In the second method, a mini bead-beater was used. In the third method, apart from using mini bead-beater DNA elution was performed additionally from the obtained dilution. The best results of DNA extraction from the MAP cells were obtained with the use of SDS and NaOH.

Key words: Mycobacterium avium subsp. paratuberculosis, cell wall lysis, template DNA releasing, DNA elution.

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) – *M. johnei* is an etiologic factor of paratuberculosis (Johne’s disease) – a chronic, infectious disease of the intestines, especially of jejunum and ileum, occurring in all domestic animals as well as wild ruminants (cattle, sheep, goats, camels, and deer) (3, 11, 14, 20, 23, 25). Diagnosis of the infection is difficult due to a long period of the disease incubation, the lack of characteristic clinical symptoms, problems connected with the germ isolation, and the cross-reactions occurring after the skin test with mycobactin J. The latter results from the similarity of MAP and *Mycobacterium avium* genomes. These genomes are consistent in 99% and ribosome rRNA sequence is the same for both microorganisms (24). For this reason until 1989, when insertion sequence IS 900 (8, 16) was found in the MAP genome, the application of polymerase chain reaction (PCR) for confirmation of its presence in the investigated material was impossible. IS 900 - specific only to this bacillus, occurring in 14-18 copies (8) allows identification of even the lowest number of MAP cells in the infected material with the use of the PCR.

Cell wall structure of a mycobacterium, including MAP, is very complicated, which makes the release of DNA (necessary for the PCR) extremely difficult (5, 10). It
is a bilayer structure with high lipid content reaching about 30-40% of the total wall content. Their considerable amount is composed of loosely floating lipids (extracted by the use of organic solvents). The others are the bound lipids that can be extracted only through saponification. They are esterified into galactine arabinose forming the cell wall structure (2, 13). The other elements of the cell membrane are as follows: arabinose, galactose, muramic acid, amine glucose, alanine, diaminepilimic acid and glutamic acid.

The pathogenic strains of mycobacteria, including MAP, contain additional capsular structure - CAP. CAP consists of sugars, lipoarabinate and lipopolysaccharides bound to the fatty acid radicals in the electron – translucent membrane of the cell wall. Such a structure provides stability of the cell wall as well as its poor permeability for the hydrophilic and hydrophobic compounds including many antibiotics (22). This structure inhibits DNA detection, therefore, the MAP cells must be preliminary prepared for the PRC. For other microorganisms, the methods of DNA releasing in the PCR are relatively easier (19) and the cells do not require initial processing as in the case of E. coli (12).

The objective of this study was to compare three methods of preparing genetic DNA material from MAP cells for determination of the best method of template DNA detection.

**Material and Methods**

The 2276 MAP strain multiplied on the HEYM medium (Herrold’s egg yolk medium) with the addition of mycobactin J for 16 weeks at 37°C was the experimental material (4). Following this, the slant from the HEYM medium was prepared and stored overnight at -20°C. After freeze-thaw at room temperature, it was used to prepare bacterial suspension (about 10^6 cells in 1 ml) and its decimal dilutions (from 10^6 to 10^1). The prepared dilutions were used for comparison of three methods of releasing DNA from MAP cells.

The first method:

50 µl dilutions containing 10^6 –10^1 of MAP cells suspensions were set in single, twist Eppendorf test-tubes, heated for 17 min a the water bath at 90°C. Next, the suspensions were frozen at −20°C and freeze-thaw for 4 h at room temperature (17). After complete thawing the suspensions were immediately set in ice prepared from deionised water. Each sample was supplemented with 50 µl mixture of 25 µl 0.25% sodium lauryl sulfate SDS and 25 µl 0.1 M NaOH prepared ad hoc. Next, the samples were heated again for 17 min in the water bath at 90°C and set into ice till the PCR.

The second method with the application of the mini bead-beater (7, 9, 15, 18, 21): 375 µl of prepared decimal dilutions (10^1-10^6) of MAP cells suspension and 375 µl of sterile redistilled water free from DN-ase were transferred to the earlier prepared single, sterile, twist test-tubes (2 ml). Prior to that, 750 µl of glass beads (diameter: 0.1 mm) were added to each of the tubes. The tubes were set in the mini bead-beater and shaken 3 cycles for 45 s and 6.5 M/s rotation. Next, they were left in the ice till the initiation of the PCR.

The third method: the samples were prepared in the same way as in the second method. However, DNA elution with chemical compounds was additionally applied (18). One hundred µl guanidine thiocyanate and 500 µl phenol were added to each 500
µl sample (such volume remained after preparation in the mini bead-beater. Then, the samples were mixed and centrifuged (14 000 x g for 5 min). Supernatant containing DNA was transferred into the sterile tubes and supplemented with 500 µl of chloroform to remove the phenol residues. They were mixed and centrifuged again. The liquid from over sediment was transferred into the sterile tubes and 50 µl of sodium acetate and 1000 µl of 100% ethanol were added. Subsequently, it was centrifuged (for the third time) for 10 min. The obtained sediment was rinsed with 200 µl 80% ethanol and centrifuged once again for 5 min/14 000 g. The separated liquid fraction was removed, the sediment was left to dry in the open air. Sixty seven µl sterile, redistilled, free of DN-ase water was added to each prepared sample. The samples were mixed and left in ice till the PCR began.

The effectiveness of the initial procedure of the three described methods was examined by the PCR. For this purpose, 20 µl of reaction mixture was added to 5 µl sample. Each sample contained: 7.25 µl of sterile, redistilled, free of DN-ase water, 2.5 µl Cetebus buffer - Tris-HCl (10mM), 2.5 µl dNTP’s 2 mM, 1.5 µl MgCl₂, 2.5 µl Tween 20 5%, 2.5 µl gelatin 1%, 0.5 µl primer p90+ (100 pmol) 0.5 µl primer p91+ (100 pmol), 0.25 µl Amplitaq polymerase (7, 17). The next step was the amplification conducted in the thermocycler with the Inno 58, 60 s program, 30 cycles. For detection of MAP in the material derived from potentially infected animals, the 40 cycles program used for template DNA amplification. In the conducted experiment, the number of cycles could have been reduced to 30 cycles due to the use of a well-known 2276 MAP strain. The number of cycles was increased due to the fact that the number of cells sought in the investigated material can be very small. Unfortunately, an increased number of cycles increases the risk of contamination. Consequently, electrophoresis in agarose gel was conducted to reveal the amplification products that were evaluated after staining with ethidium bromide. To verify the PCR reliability, two control samples were always prepared: negative – only with the PCR 20 µl of reacting mixture, positive – with the PCR 20 µl of reacting mixture and 1 ml of pure template DNA of MPA in 10³ dilution.

The research was conducted in three repetitions for each of the three described methods.

Results

Table 1 shows the obtained results. While comparing the three methods of releasing the genetic material from MAP cells, considerable differences can be observed in the obtained results. The first method enabled the detection of DNA in the samples prepared from the 10⁵ dilution of the MAP cells suspension. The number of positive results confirming the presence of genetic material under the PCR was also the highest. Alternate freezing (at -20°C) and heating (at 92°C) of the bacteria suspension applied in the presence of chemical compounds which enabled the cell wall lysis, yielded stronger signal and was found to be the most effective and simplest method. The second method within which the bacteria suspension of various concentration was only once subjected to low and high temperature and shaking in the mini bead-beater resulted in the lowest number of positive results on the PCR. The use of the mini bead-beater caused additional losses in the solution, even with the precise, multiple checking of the device and tubes tightness, what could negatively influence the final results. The
third method resulted in a higher number of positive results compared to the second method. It could be the effect of guanidine thiocyanate and phenol activity used for the DNA elution. However, the results obtained in the third method were found worse than those obtained in the first method.

Table 1
Comparison of the results of DNA extraction using three methods

<table>
<thead>
<tr>
<th>Type of DNA extraction</th>
<th>MAP cells concentration in 1ml</th>
<th>(10^0)</th>
<th>(10^1)</th>
<th>(10^2)</th>
<th>(10^3)</th>
<th>(10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>NaOH and SDS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Method 2</td>
<td>mini bead-beater</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Method 3</td>
<td>mini bead-beater with DNA elution</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Discussion

In view of difficulties in Johne’s disease diagnosis as well as in MAP detection in food of animal origin (26), many scientific centers work on the optimisation of obtaining of the genetic material from the MAP cells (1, 7). The mini bead-beating method was successfully applied for detection of DNA in bacteria with a difficult to destroy cell wall (21). Similar tests were conducted to obtain DNA of *Salmonella enterica*, which was then used to determine these bacteria resistance to tetracycline (9). The mini bead-beating method was also used for obtaining plasmids from mycobacteria recombined by *E. coli* (16). The research group of the Guelph University (18) compared some methods of template DNA releasing from milk samples artificially infected with MAP. The results obtained from the combination of: samples heating, shaking in the mini bead-beater or samples heating, shaking in the mini bead-beater and extraction with isopropanol, made it possible to detect from 100 to 1000 cfu/ml, while applying commercial kits provides the detection limit of \(10^5\) cfu/ml. The best results were obtained with the combination of bead-beating method, heating with buffer and elution with isopropanol what provided detection of 10 cfu MAP in 1 ml of
milk. The authors of this experiment do not mention, however, losses in the material occurring during the mini bead-beater work that were observed in the two methods applied by our team. The results of the model experiments do not confirm any increase in the sensitivity of MAP detection with the bead beating method applied in the initial processing.

References