OPTIMISATION OF THE DNA-EXTRACTION METHOD FROM FOAL FAECES FOR THE DIAGNOSIS OF RHODOCOCCUS EQUI INFECTIONS USING PCR

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Abstract

The aim of the study was to compare the effectiveness of selected DNA extraction methods for the detection of Rhodococcus equi in foal faeces using PCR. Four different nucleic acid extraction methods were compared, which were based mainly on the use of proteolitic enzymes. Moreover, the commercial kit for DNA isolation from faeces was used. In two of the methods additional components, including CTAB detergent and powdered glass, were introduced. The PCR results showed that traditional DNA extraction methods involving proteolitic enzymes and the commercial kit were ineffective in terms of preparing the DNA matrix amplified by PCR. The only method useful for preparing the pure DNA matrix free from inhibitors of the enzymatic reaction was that with the use of powdered glass.

Key words: Rhodococcus equi, faeces, DNA extraction, PCR.

Rhodococcosis, caused by Rhodococcus equi, is a bacterial disease of the respiratory tract occurring in foals aged 1 to 6 months with symptoms of suppurative pneumonia. Besides tropism to the respiratory tract, R. equi undergoes replication in the alimentary tract of young foals till the age of 3 months (8, 20, 22). The carrier state and proliferation of the organism in the alimentary tract of foals are connected with the animals’ contact with contaminated grass and soil (13). The presence and replication of the microorganism in the intestines result in a characteristic clinical picture and pathological lesions observed additionally in approx. 50% of sick foals with the symptoms of pneumonia (4). As a consequence of the replication of R. equi in the alimentary tract, it is present in faeces and spreads to the environment (11, 21). In practical terms this fact can be used for the antemortem diagnosis of R. equi infections in foals and for determining the level of environmental contamination by virulent and non-virulent strains of the microorganism (5, 11). The aim of the study was to compare the effectiveness of selected DNA extraction methods for the detection of R. equi in foal faeces using PCR.

Material and Methods

Samples. Faecal samples taken from foals aged 1 to 6 months from R. equi-free stud and the suspension of the reference strain of R. equi (ATCC 33701) in the LB liquid medium were used. The faeces were sampled into plastic tubes (Medlab, Poland) directly from the rectum or bedding. Initially, a pooled sample of a mixture of 10 various samples was prepared. Using microbiological culture and PCR, the presence of bacteria from the genus R. equi was excluded from this sample. The 10% suspension of the tested material in PBS underwent further procedures. Hundred microlitres of bacterial suspension of the titrated culture of the reference strain of R. equi, containing a known number of cells: 10^5, 10^4, 10^3, 5x10^2, 10^2, and 10 were added to the faecal samples of the volume of 1.0 ml. Mixed samples were centrifuged (1,400 g, 15 min), supernatant was removed and then the sediment was suspended in 1.0 ml of PBS.

DNA extraction methods. The following methods were used for the extraction of DNA from the faecal samples: a. enzymatic digestion using lysostaphin, lysozyme, and proteinase K; b. digestion using lysozyme and proteinase K with the addition of CTAB; c. enzymatic digestion with the additional use of powdered glass; and d. commercial kit for the isolation of DNA from faeces (QIAamp DNA Stool Mini Kit, Qiagen, Germany). The first method was performed with the use of phenol-chloroform according to the procedure developed in the Ecole Nationale Veterinaire Lyon, France (10). In the second method the additional step of extraction of the nucleic acid was introduced through the incubation of the sample in the presence of 10% cationic detergent CTAB (Hexadecyltri-
methy lammonium Bromide, Sigma, USA) in 0.7 M solution of NaCl (Sigma, USA) (17). The initial stages of DNA isolation with the method of enzymatic digestion and powdered glass (c) were performed using the procedure described in the second method. After double extraction with phenol-chloroform-isoamyl alcohol, 17.4 µl of the fine-ground-glass powder suspension (Glass Milk) from the commercial kit, Geneclean II (Q-Biogene, Canada) was added to the samples. Details of the procedure were described in earlier studies (6). As the fourth method, the commercial kit for the isolation of DNA from faeces based on silicon beads was used. The individual steps of the procedure were performed according to the manufacturer’s recommendations. For the DNA extraction from the liquid culture of the reference strain of R. equi, the previously-given methods were used, except the commercial kit for the isolation of DNA from faeces. The list of methods used for isolation of the nucleic acids and components used in extraction procedures is given in Tables 1 and 2, respectively.

Primers. Primers for PCR reaction were selected based on the literature data (2, 18). A pair of primers, Rq1 and Rq2, was used, which was complementary to the conservative gene-fragment encoding the 16S subunit of the ribosomal DNA of R. equi. The use of these primers enabled one to prove that the tested bacterial strains belong to the species R. equi. The names of the primers, their sequences, and the size of the amplified product are given in Table 3.

PCR reaction. The matrix for the PCR was the DNA isolated from the liquid culture of the reference strain of R. equi (ATCC 33701) on the LB medium and from faeces. The amplification was performed in a volume of 50 µl of mixture, using 5 µl of the DNA matrix. The DNA sample was added to the mixture containing 5 µl of 10x buffer for Taq DNA polymerase (MBI Fermentas, Lithuania), 5 µl of 2 mM solution of dNTPs (MBI Fermentas, Lithuania), 1 µl (25 pMol) of primers Rq1 and Rq2, 3 µl of 25 mM MgCl2 solution (MBI Fermentas, Lithuania), 0.3 µl (1.5 U) of Taq polymerase (MBI Fermentas, Lithuania), and 29.7 µl of water. The mixture was initially incubated at 95°C for 3 min and then subjected to 30 cycles of the following parameters: denaturation – 94°C, 30 s, annealing – 64°C, 30 s, primer extension – 74°C, 1 min. Final primer extension was performed at 74°C for 10 min. For each PCR reaction, the amplification of a negative control (with 5 µl of water instead of the DNA matrix) was performed in parallel under the same conditions. The PCR products were analysed electrophoretically in 1.5% agarose gel (Sigma) in TBE buffer in the presence of the molecular weight marker (100 bp DNA Ladder Plus, Fermentas, Lithuania). The electrophoretic conditions were as follows: current flow – 100-110V, 1A, duration – 1 h, buffer – 0.5 x TBE. After the completion of electrophoresis the gels were stained with ethidium bromide (Sigma, USA), viewed under UV light (Transilluminator Cole-Parmer, France), and photographed (Gel-Doc Vilber Lourmat, France).

Results

Three methods of extraction of the nucleic acids were assessed in the studies, based mostly on the effect of proteolytic enzymes, causing the disintegration of the bacterial cell wall. Additionally, a commercial kit was used for the isolation and purification of bacterial DNA from faecal samples. Besides the enzymatic agents used for the DNA extraction, additional components were used in two procedures, i.e. CTAB cationic detergent and powdered glass (Glass Milk), which were added to neutralise the non-specific substances inhibiting and/or blocking the enzymatic reaction. These methods were used to isolate nucleic acids from the bacterial suspension of the reference strain of R. equi (ATCC 33701) in liquid LB medium and faecal samples to which the determined number of R. equi cells was added. DNA isolated using the individual methods was amplified in a PCR reaction using primers complementary to the fragment of the gene encoding the 16S subunit of rRNA of the genome of R. equi. The PCR results and the sensitivity of the test are given in Tables 2 and 1, respectively. Data given in Table 2 indicate that in relation to the bacterial suspension of the reference strain, the choice of the DNA extraction method has no effect on the results of PCR. Each of the methods of the isolation of the nucleic acids guaranteed that the DNA matrix was amplified in PCR, i.e. it was free of contaminants and inhibitors. Data given in Table 1 show that in relation to the faecal samples the methods of enzymatic digestion using various enzymes and with addition of CTAB appeared to be of no use for obtaining DNA matrix free from substances inhibiting the enzymatic reaction. Regardless of the initial bacterial concentration, the specific PCR product was not obtained in any of the DNA samples isolated using these methods. The only method useful for elimination of the inhibitors of the reaction was that with the use of powdered glass as an additional component (Table 1, Fig. 2).

The results of the sensitivity threshold for the detection of DNA extracted from the R. equi reference strain in the liquid culture medium and in faecal samples with the addition of bacteria, are given in Table 4, and Figs 1 and 2, respectively. The data in the Table 4 show that in case of DNA extracted from the faecal samples containing a different number of bacterial cells and the method using powdered glass, the positive PCR result was achieved with at least 1,000 cells in the tested material. If the cell count was lower, no specific products of the amplified DNA were observed (Fig. 2), and the result was considered as negative. When the DNA extracted from the liquid culture of the reference strain was used as a matrix for PCR, a positive result was observed in samples containing at least 100 bacterial cells (Fig. 1). The PCR sensitivity threshold determined in relation to this material was ten times higher than that determined for faecal samples with the addition of R. equi (Table 4).
### Table 1

DNA extraction methods from faeces, components used for DNA isolation, PCR results and sensitivity

<table>
<thead>
<tr>
<th>Method</th>
<th>Components used for DNA isolation</th>
<th>PCR result/sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Lisozyme +, Lysostaphin +, Proteinase K +, CTAB −, Powdered glass −</td>
<td>− − − − − −</td>
</tr>
<tr>
<td>II</td>
<td>− + − + + + + +</td>
<td>+ ≥1,000 bacterial cells</td>
</tr>
<tr>
<td>III</td>
<td>+ − − + + + + +</td>
<td>+ ≥500,000 bacterial cells</td>
</tr>
<tr>
<td>IV</td>
<td>Commercial kit (QIAamp DNA Stool Mini Kit)</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table 2

DNA extraction methods from liquid culture of *R. equi*, components used for DNA isolation, PCR results and sensitivity

<table>
<thead>
<tr>
<th>Method</th>
<th>Components used for DNA isolation</th>
<th>PCR result/sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Lisozyme +, Lysostaphin +, Proteinase K +, CTAB −, Powdered glass −</td>
<td>− − − − +</td>
</tr>
<tr>
<td>II</td>
<td>− + − + + + + +</td>
<td>+ ≥100 bacterial cells</td>
</tr>
<tr>
<td>III</td>
<td>+ − − + + + + +</td>
<td>+ ≥100 bacterial cells</td>
</tr>
</tbody>
</table>

### Table 3

Sequences of the primers of the PCR reaction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction (5’→3’)</th>
<th>Sequence (5’→3’)</th>
<th>Region</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rq1</td>
<td>→</td>
<td>GGTCTAAATACCGGATATGAGCTCCTGTC</td>
<td>16S rRNA</td>
<td>450 bp</td>
</tr>
<tr>
<td>Rq2</td>
<td>←</td>
<td>CGCAAGCTTGTTGGTTGAGCCCA</td>
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<td></td>
</tr>
</tbody>
</table>

### Table 4

Sensitivity of PCR in the *R. equi* DNA detection

<table>
<thead>
<tr>
<th>Biological material</th>
<th>Bacterial concentration</th>
<th>PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid culture</td>
<td>10^5 10^4 10^3 5x10^2 10^2 10</td>
<td>+ + + + + −</td>
</tr>
<tr>
<td>Faeces</td>
<td>+ + − − − −</td>
<td></td>
</tr>
</tbody>
</table>

+ - positive PCR result  
− - negative PCR result

Fig. 1. The PCR sensitivity threshold to detect *R. equi* in liquid culture medium.

M - molecular weight marker (100 bp DNA ladder); K(−) – negative control; 1 – 10^5 c.f.u.; 2 – 10^4 c.f.u.; 3 – 10^3 c.f.u.; 4 – 5x10^2 c.f.u.; 5 – 10^2 c.f.u.; 6 – 10 c.f.u.
Fig. 2. The PCR sensitivity threshold to detect R.equi in faecal samples.

M - molecular weight marker (100 bp DNA ladder); K(–) – negative control; 1 – 10⁵ c.f.u.; 2 – 10⁴ c.f.u.; 3 – 10³ c.f.u.; 4 – 5x10² c.f.u.; 5 – 10² c.f.u.; 6 – 10 c.f.u.

Discussion

The usefulness of PCR in microbiological diagnostics is partially limited by the presence of substances acting as enzymatic inhibitors in the samples of the biological material, like body fluids, tissues, secretions, and excretions (1, 7). It was shown that these substances can effectively limit or block the amplification of nucleic acids during PCR (10). They can also influence the efficiency of the reaction, especially the sensitivity of the detection of the genetic material (7, 9, 12, 23, 25). The strength of the inhibiting effect of the inhibitors may be different. Some of them have a specific inhibitory effect even in small concentrations (14). To prevent the effect of inhibiting or blocking PCR, the specific preparation of the sample and reaction mixture prior the performance of the reaction is essential (6, 7, 9, 10, 14, 15). In the case of microorganisms showing tropism to the alimentary tract like R.equi, the faecal samples are useful for the antemortem diagnostics. The nature of the inhibitors present in faeces and the mechanisms of their action on the enzymatic reactions are poorly known (7, 9, 14, 19).

To eliminate non-specific inhibitors present in faecal samples, several methods of extraction of the nucleic acids from the biological material were developed or modified (10, 16, 18, 24, 26). According to some authors, the nucleic acid extraction technique using the CTAB cationic detergent is effective in the removal of inhibitors present in faecal samples (7, 9, 16, 18). The method of the isolation of DNA of R.equi using this detergent, besides lysozyme and proteinase K, was described by Sellon et al. (17, 18). Vivrette et al. (24) modified Sellon’s method by introducing the additional step of incubation of the mixture in the presence of Nonidet P-40. The use of the additional detergent assured a higher level of purification of the DNA matrix of R.equi isolated from the biological material.

However, there is little data in the current literature on the use of PCR for the detection of R.equi in foal faecal samples. This is probably due to the technical difficulties in obtaining a qualitatively-accurate matrix of the DNA isolated from faeces without contaminations inhibiting the enzymatic reaction. We have used in the present studies the modification of the enzymatic digestion of the sample through the introduction of an additional step of DNA purification by using powdered glass. The comparison of the effectiveness of the DNA-extraction methods used in the study shows that traditional methods of faecal samples digestion with the use of proteolytic enzymes were ineffective. However, it was shown that the modified method of enzymatic digestion using powdered glass was useful. Using this procedure of DNA extraction for faecal samples, the PCR sensitivity threshold of 1,000 bacterial cells was achieved. This ought to be considered as satisfactory, especially as, according to Takai et al. (21), R.equi infection in foals may be diagnosed on the basis of bacteria concentration in faeces at the level of at least 10⁶ cells/g.

The positive effect of using powdered glass as a fixed carrier adsorbing the nucleic acid particles in the extraction method was described in relation to certain viruses in biological material (27). The procedure of extraction of nucleic acids using “glass milk” was earlier used by Grażdki (6) and Winiarczyk et al. (26) to detect porcine coronaviral and rotaviral infections using PCR.

In the present studies, the limited usefulness of the commercial kit for the isolation of DNA from faeces for the preparation of the DNA matrix for PCR was also demonstrated. However, in our additional studies, specific amplification of DNA was obtained but only in the presence of a minimum of 500,000 bacterial cells. The low sensitivity of PCR eliminates the possibility of using this type of kit for the initial preparation of faecal samples before amplification.

Based on the results obtained during our studies we can conclude that the most useful method of the nucleic acid extraction to prepare the DNA matrix from faecal samples in term of diagnosis of R.equi infections by PCR is that with the use of powdered glass as an additional component.
References