RAPID DETECTION OF BRACHYSPIRA HYODYSENTERIAE AND LAWSONIA INTRACELLULARIS IN SWINE FAECAL AND MUCOSAL SPECIMENS BY MULTIPLEX PCR

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Abstract

Multiplex polymerase chain reaction (M-PCR) was developed to determine its usefulness as a rapid and specific method for routine diagnosis of Brachyspira hyodysenteriae and Lawsonia intracellularis infections in pigs. The assay was based on simultaneous amplification of the tlyA-encoded haemolysin gene of B. hyodysenteriae and 16S rRNA sequences of L. intracellularis. The results of this study demonstrated that the M-PCR method is a useful tool for rapid diagnosis of the etiological agents only of acute forms of swine dysentery (SD) and proliferative enteropathy (PE). Screening of pooled diagnostic specimens from chronic form of PE was possible by using only our modified one tube nested PCR where detection limit was 1.1x10⁷ bacterial cells per 0.1 g of faeces. No positive results were obtained when M-PCR for the identification of L. intracellularis from chronic forms of this disease was used. On the other hand, detection of B. hyodysenteriae in faeces from acute and chronic cases was both sensitive and specific by either single or M-PCR techniques. The sensitivity of the single and M-PCRs for the detection of B. hyodysenteriae in 10-fold dilutions of bacterial culture was estimated on the level of 10⁻⁸. It was revealed that only two dilutions were countable: 10⁻⁶ and 10⁻⁷ corresponding to 203 and 14 CFU/ml, respectively.

Key words: pigs, Brachyspira hyodysenteriae, Lawsonia intracellularis, multiplex PCR.

Swine dysentery (SD) and proliferative enteropathy (PE) are two of the most serious enteric diseases affecting pigs after weaning and in all phases of finishing. Brachyspira hyodysenteriae is the etiological agent of SD, a disease characterized by mucohaemorrhagic diarrhoea with lesions confined to the large intestine (9, 21). The β-haemolytic activity of B. hyodysenteriae in vitro is strongly correlated with enteropathogenicity in pigs in vivo. A haemolysin produced during the acute phase of the disease is responsible for characteristic necrotic lesions in the intestine of affected animals. The haemolysin activity of the cytotoxin is encoded by three genes - tlyA, tlyB and tlyC which are present in single copies on the chromosome of all B. hyodysenteriae strains (24, 34). The well conserved protein encoded by the tlyA gene is known to be cytotoxic for the intestinal epithelial cells of infected pigs (17). On the other hand, nonpathogenic strains of B. innocens lack the tlyA marker, while these strains contain genes related to the tlyB and tlyC sequences. Several experiments have demonstrated that the proteins encoded by the B. hyodysenteriae tly genes have both haemolytic activity in vitro and are cytotoxic for epithelial cells in vivo. Many investigations indicated the involvement of the tlyA-encoded haemolysin in the pathogenesis of acute SD. Therefore, it has been suggested that this cytotoxin is the main virulence factor of B. hyodysenteriae and it is responsible for characteristic lesions of SD (31, 32). It was also observed that the tlyA region could be used for specific oligonucleotide primers selection for PCR (27).

Proliferative enteropathy, caused by the intracellular bacterium Lawsonia intracellularis, is also an enteric disease characterized by hyperplasia of the ileal and colonal mucosa of affected pigs. The infection has two clinical expressions: porcine intestinal adenomatosis (PIA) with chronic diarrhoea and slow growth of grow-finishing pigs, or proliferative haemorrhagic enteropathy (PHE) with mortality of replacement gilts and finishing pigs close to the market age showing signs of acute haemorrhagic diarrhoea (15, 21). Diagnosis of L. intracellularis is usually based on the PCR technique using nested primer sets created from pCL078 probe (8, 10, 11) which allows amplification of conserved regions of the 16S rRNA gene. The PCR technique was capable to detect 10⁷ L. intracellularis organisms or, in case of faeces, 10⁴ bacteria/g (11).

Recently, multiplex PCR (M-PCR) has been successfully applied in identifications of many bacteria (2, 4, 5, 7, 16, 25). By this genotypic-based technique more than one locus is simultaneously amplified during the same reaction. Therefore, M-PCR could be a suitable tool for the initial rapid screening of the tlyA-encoded haemolysin gene of B. hyodysenteriae and the 16S rRNA
gene of *L. intracellularis* in one reaction tube. For the diagnosis of SD and PE, this procedure might avoid several limitations concerning time consuming of standard conventional bacteriological methods such as isolation of *B. hyodysenteriae* on trypticase soy agar or identification of *L. intracellularis* in the rat enterocyte IEC-18 or the human foetal INT-407 cell lines (14, 28). Laboratory identification of these two pathogens is laborious and often takes several days or even weeks for the final results. To reduce the cost and time which are needed for conventional bacteriological procedures, M-PCR as a diagnostic method seems to be very promising.

The aim of this study was to develop M-PCR as a diagnostic technique for simultaneous detection of two bacterial pathogens, *B. hyodysenteriae* and *L. intracellularis* in a single reaction, using DNA isolated from intestinal scrapings and faeces obtained from pigs with acute and chronic forms of SD and PE. Another purpose of this investigation was to determine usefulness of M-PCR in routine diagnostic procedure of the above mentioned diseases.

**Material and Methods**

**Bacterial strains and intestinal specimens.**

The reference positive material (3 filtrates from infected intestines containing 6x10^6, 6.5x10^6, and 1.1x10^7 bacteria/ml evaluated after immunostaining) of *L. intracellularis* was originated from Danish Veterinary Institute, Copenhagen, Denmark. The strains of *B. hyodysenteriae* B204 (ATCC 31212) and B-78 (ATCC 27164) were included as positive reference controls. Five fragments of porcine ileum were collected from naturally infected with *L. intracellularis* pigs showing the acute form of PE. This material kindly provided by the Federal Institute for Veterinary Research, Linz, Austria, and confirmed to be *Lawsonia*-positive, was frozen at -70°C until use. These intestinal tissues were processed as follows: the ileum was opened and the mucosa was scraped with a microscope slide and stored in small portions for DNA extraction.

**Clinical sample collection.** In the period from January 2000 to September 2003, total of 606 faecal samples were taken directly from the rectum of weaned and finished pigs, and from 92 intestines (containing faeces and mucosal scrapings). The material originated from total of 190 pig farms located predominantly at the western part of Poland. The faecal samples were collected from individual pigs showing typical signs of chronic form of PE and/or additionally pooled from different pens. In the same time, 406 faecal samples and 91 large intestines were taken mainly from fatteners from 165 farms which were suspected to be infected with *B. hyodysenteriae*. The material was immediately transported to the laboratory and frozen at -20°C for further examination.

From 91 intestinal specimens 14, consisting faeces and mucosal scrapings from the caecum and colon of infected pigs, were obtained from animals with acute form of SD characterized by the presence of blood in faeces.

**Bacteriological examination.** Immediately after arrival to the laboratory, the mentioned above 14 mucosal scrapings were streaked on trypticase soy agar (TSA) (Becton Dickinson, Detroid, Mich), supplemented with fresh defibrinated sheep blood (5-10%), L-cysteine (50 mg/l), spectinomycin (40 mg/l), vancomycin (7 mg/l) and yeast extract (500 mg/l) (30). The inoculated plates were then incubated at 37°C for 4 d in an anaerobic chamber (Oxoid, Hampshire, England). The presence of spirochetes was determined by the examination of the surface growth of the bacteria on the plate that spread from the site of primary inoculation and formed a zone of a strong β-haemolysis.

In order to obtain a pure culture of *B. hyodysenteriae*, a re-cultivation on the same medium was done. Confirmation of the bacterial growth taken from the surface of TSA agar plates and/or directly cut out of the agar at the periphery of the haemolysis zone was done with the dark-field examination according to Achacha and Messier (1).

**PCR analyses**

**Extraction of DNA from faeces and mucosal scrapings.** DNA was extracted from pig faeces and/or intestinal mucosa using a multi-spin column of a commercial kit (Genomic DNA Prep Plus, Helicinuous, A&A Biotechnology, Gdansk, Poland) according to the manufacturer’s protocol with slight modifications. Prior to extraction, 0.1 g of faeces or intestinal mucosa was thoroughly mixed with 900 µl of Milli-Q-water and incubated at room temperature for 15 min. After sedimentation, the upper homogeneous suspension was used for DNA extraction. Briefly, a 100 µl sample was mixed with 100 µl of 10 mM Tris-HCl buffer, 50 µl of lysis solution, 20 µl of Proteinase K (20 mg/ml) and incubated at 55°C for 1 h. After centrifugation at 13 000 g for 3 min the lysate supernatant was added to the multi-spin column and washed with 500 µl solution containing 70% of ethanol. Then, the column was centrifuged at 13 000 g for 1 min and after discarding the flow-through the washing step was repeated again. The final washing step was followed by centrifugation for 2 min at 13 000 g to dry the column. Bounded DNA was eluted by adding 100 µl of 10 mM Tris-HCl buffer at 75°C and incubating for 5 min at room temperature followed by centrifugation for 1 min at 13 000 g. Five microliters of eluted DNA was used as template for PCR.

**Sensitivity of PCR for *B. hyodysenteriae* and *L. intracellularis* in porcine faeces.** The sensitivity of PCR for the detection of *B. hyodysenteriae* in porcine faeces was tested by serial 10-fold dilutions of spirochete cultures from TSA agar which were added to constant volumes of undiluted normal porcine faeces. The sensitivity of the PCR assay was estimated on the basis of the number of spirochetal cells present in the original culture. Bacteria harvested from 4 TSA agar plates were suspended in 2 ml of BHI broth (Becton Dickinson, USA) to a concentration between 1x10^6 and 5x10^7 CFU/ml. The inoculum was then serially 10-fold diluted in 4.5 ml of BHI. Fractions of 0.5 ml from each dilution were then processed for the determination of total number of *B. hyodysenteriae* organisms by the
plate counting method. For the viable cell count method, 0.5 ml of each dilution was placed onto freshly made TSA selective medium and the number of CFU per 0.5 ml was determined after incubation at 37°C in the GasPak Anaerobic System (Becton Dickinson) for 5 d. Briefly, sterile tubes containing 0.1 g of normal faeces were inoculated with 1 ml containing either BHI broth (negative control) or serial 10-fold dilutions of B. hyodysenteriae B204. The samples were vortexed and allowed to stand for 10 min. After sedimentation the supernatant (approximately 0.1 ml) was drawn off and processed for total DNA extraction by the Genomic DNA Prep Plus kit. Total DNA from each tube was used for PCR amplification followed by agarose gel electrophoresis. The sensitivity of the PCR test for L. intracellularis was determined by inoculating sterile tubes containing 0.1 g of faeces with 1 ml containing either Tris buffer (negative control) or serial 10-fold dilutions of filtrate containing of 1.1x10^7 L. intracellularis per milliliter. One ml fractions from each 10-fold dilutions were processed for total DNA extraction as described above. 

**Table 1**

Characteristics of the primers used in M-PCR for simultaneous detection of L. intracellularis and B. hyodysenteriae

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Sequence (5’→3’)</th>
<th>Target</th>
<th>Tm (°C)</th>
<th>Expected PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TATGGGCTGCTAAACACTCCG</td>
<td>16S rRNA sequences of L. intracellularis</td>
<td>55</td>
<td>319</td>
<td>11</td>
</tr>
<tr>
<td>B</td>
<td>TGAGGTTATTGATTTCTCC</td>
<td></td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₁</td>
<td>GAATATGTCATAGTGGAAGG</td>
<td>Haemolysin gene tlyA</td>
<td>60</td>
<td>658</td>
<td>27</td>
</tr>
<tr>
<td>H₂</td>
<td>GGAGAGATGGTCTATTAAAC</td>
<td></td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All PCR reactions were run in a thermal cycler (MJ Research, Watertown, Mass., USA) and the amplification products were examined by electrophoresis in 1.5% agarose gel and Tris-Borate-EDTA (TBE) buffer. Gels were stained for 30 min in distilled water containing 0.5 µg/ml of ethidium bromide. DNA fragments were visualized by UV transillumination and documented with the BioPhotonics and Mighty Bright system ( Hoefer Scientific Ins., San Francisco, Ca). The fragment sizes of 658 bp and 319 bp were verified as positive for L. intracellularis and B. hyodysenteriae, respectively. A 100-bp DNA ladder (Fermentas) was used as a molecular size standard.

**Nest PCR.** Closed one-tube nested PCR was performed only for the detection of L. intracellularis in faeces collected from different Polish farms. The sequences of two sets of PCR primers for the first step of PCR were the same as for M-PCR (Table 1). Nested oligonucleotide primers C and D (5'-TTACAGGTTAATGTTGG-3' and 5'-CTTTCTCATGTCCCATGCATGC-3'), respectively, were published previously by Gebhart et al. (8) and were used to amplify the fragment of 270 bp of p78 region of L. intracellularis. All steps of the procedure were carried out in a single, closed during all steps of the nested PCR, reaction tube. Into the lid of the tube 8.25 µl of the following mixture was added: 5 µl of 22% trichloroacetic acid (w/v), 0.4 µM of each of the internal C/D primers, 50 µM of each of dNTP, 1 U Taq DNA polymerase (Fermentas), and 1 x PCR buffer, and 5 µl of the extracted DNA. After initial denaturation for 5 min at 95°C, the PCR reactions were subjected to 35 cycles comprising 40 s at 94°C, 60 s at 55°C, and 26 µl of water. Mineral oil was included as a barrier between the PCR reaction and the dried reagents within the lid. The tubes were left unclosed at room temperature until the solution in the lid dried out. In the bottom of the same reaction tube the following reagents were placed: 3 µl of the tested DNA, 1 x PCR buffer, 100 µM of each dNTP, and 1.25 U of Taq DNA polymerase. The tubes were then subjected to 35 cycles comprising 40 s at 94°C, 40 s at 55°C, and 40 s at 72°C, with final extension at 72°C for 7 min.

**Single PCR.** For amplification of the conserved genes of B. hyodysenteriae the PCR primers reported by Rzewuska et al. (27) were used (Table 1). The 50-µl reaction mixture consisted of 0.8 µM of each primer H₁/H₂, 2.5 mM MgCl₂, 200 µM each of dNTP, 1 U of Taq DNA polymerase (Fermentas, Lithuania), 1 x PCR buffer, and 5 µl of the extracted DNA. Amplification was carried out using a program consisting of: initial denaturation for 60 s at 94°C, following 30 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 56°C, extension for 90 s at 72°C, and final extension for 7 min at 72°C.

The oligonucleotide primers described by Jones et al. (11) were used to amplify the 319 bp fragment from the L. intracellularis conserved region of the conserved rRNA gene (Table 1). The PCR amplification was performed in 50 µl of reaction mixture containing 0.8 µM of each of the primers A/B, 2.5 mM MgCl₂, 200 µM each of dNTP, 1 U Taq DNA (Fermentas), 1 x PCR buffer, and 5 µl of the extracted DNA. After initial denaturation for 5 min at 95°C, the PCR reactions were subjected to 35 cycles comprising 40 s at 94°C, 40 s at 55°C, and 40 s at 72°C, with final extension at 72°C for 7 min.
Multiplex PCR. The characteristics of two sets of PCR primers for specific amplification of *L. intracellularis* and *B. hyodysenteriae* genes are presented in Table 1. The primer sequences and their specificity were described previously (11, 27). These oligonucleotides were selected to have similar melting temperatures and resulted in products of different sizes that could be easily distinguished in agarose gel.

Each PCR test was carried out in a 50 µl volume containing: 1 x PCR buffer, 200 µM of each dNTP, 2.5 mM of MgCl₂, 0.5 µM of H₁/H₂ and 0.1 µM of A/B primers, 2.5 U of Taq DNA polymerase (Fermentas) and 5 µl of each DNA obtained from pigs suffering from SD and PE. The PCR was performed in the PTC-100 thermal cycler (MJ Research) using the following conditions: the initial DNA denaturation step at 94ºC for 5 min followed by 30 cycles beginning with 1 min of denaturation at 94ºC, 2 min of extension at 72ºC, and 1 min of annealing at 60ºC. The final extension step was run at 72ºC for 5 min. The amplified PCR products (10 µl aliquots) were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under a UV transiluminator as described above.

**Results**

Optimization of the PCR conditions. To standardize the amplification conditions of the M-PCR test, concentrations of each of 2 sets of primers (Table 1), optimal annealing temperature and Mg²⁺ content were determined using DNA obtained from bacteria responsible for acute cases of PE and SD. Initially, the same concentrations (0.5 µM) of each of primers were used. However, this approach resulted in some cases in the lack of amplified products. We found that decreasing the annealing temperature by 2-5ºC for these primers yielded several non-specific bands. To overcome this problem, the optimal annealing temperature (60ºC) and concentrations of Mg²⁺ were adjusted. The optimal Mg²⁺ concentrations for M-PCR were determined by adding 1.0, 1.5, 2.0, 2.5, and 3.0 mM of MgCl₂ to the PCR reaction. It was found that 2.5 mM of MgCl₂ gave specific and strong PCR amplicons corresponding to both target genes tested. The best concentrations of PCR primers were 0.5 µM for H₁/H₂ and 0.1 µM for A/B, respectively. All the primers were prepared as 20 µM solutions, which were frozen at -20ºC in 20 µl aliquots. This storage method prevented repeating thawing and freezing of the reagents, which could affect the PCR results. The optimized and standardized amplification conditions were used in all subsequent PCR tests.

M-PCR amplification with purified DNA templates from the reference strains of *L. intracellularis* and *B. hyodysenteriae* consistently generated two distinct products of the expected molecular weights as detected by agarose gel electrophoresis (Fig. 1).

![Fig. 1. Simultaneous identification of *B. hyodysenteriae* tlyA and *L. intracellularis* 16S rRNA genes using a M-PCR system with H₁/H₂ and A/B primers, respectively. Lane M: 100 bp DNA ladder; lane 1: M-PCR test for identification of *B. hyodysenteriae* and *L. intracellularis* (amplicons 658 bp and 319 bp, respectively); lanes 2-7: field strains of *B. hyodysenteriae* positive for the tlyA sequences (amplicon 658 bp); lane 8: DNA-negative PCR mix control.](image)
In pure culture dilutions (from $10^{-1}$ to $10^{-10}$) of *B. hyodysenteriae* B204 strain we were able to detect by the plate counting method in average 203 CFU/ml in dilution $10^{-6}$ and 14 CFU/ml in dilution $10^{-7}$. The PCR detection limit of 10-fold dilutions of *B. hyodysenteriae* cell cultures inoculated to an equal amount of faeces were detectable at $10^{-4}$, which was uncountable on agar plate due to the high number of spirochetal cells.

The sensitivity of single and M-PCR for the detection *L. intracellularis* in faeces was 1.1x10$^6$ of bacterial cells at $10^{-1}$ dilution of filtrate. Modified one tube nested PCR allowed to detect 1.1x10$^4$ *L. intracellularis* per 0.1 g of faeces at the $10^{-3}$ dilution.

**Specificity of the multiplex PCR.** The specificity of the M-PCR for the tlyA-encoded haemolysin gene of *B. hyodysenteriae* and 16S rRNA sequences of *L. intracellularis* were first tested with mucosal scrapings originating from acute haemorrhagic cases of SD and PE. For control purposes, pure cultures of the reference strains B204 and B-78 of *B. hyodysenteriae* and 3 filtrates obtained from infected with *L. intracellularis* intestines were used as positive controls. The specific M-PCR products were obtained only with the reference strains. The specificity of M-PCR was also tested with other bacterial species which were isolated from pigs (the number in brackets refers to the number of isolates tested): *Salmonella choleraesuis* [1], *Salmonella typhimurium* [1], *Escherichia coli* [4], *Haemophilus parasuis* [3], *Actinobacillus pleuropneumoniae* [3], *Streptococcus suis* type 2 [5], *Mycoplasma hyopneumoniae* [1]. All these bacteria were negative in M-PCR.

**Bacterial cultures.** Out of 91 fragments of large intestine, 14 were randomly selected and used for further studies to isolate *B. hyodysenteriae* on TSA agar. The plates were inoculated with 14 mucosal scrapings collected from the colon and/or caecum. Eleven from these samples gave characteristic bacterial growth on the surface of TSA agar. Additionally, the obtained bacterial cultures were tested by dark-field microscopy. In the same number of large intestines live spirochaetes were confirmed to be present. We also proved that the 11 of 14 bacterial cultures were positive for *B. hyodysenteriae* by single and M-PCR.

**Application of PCR for clinical specimens.** The results of the field study indicated that from total of 606 faecal samples 256 were positive and 350 negative for genetic material of *L. intracellularis* in case of chronic form of PE. Of the 92 intestinal specimens, 22 were positive and yielded the 270 bp product with the *L. intracellularis* 16S rRNA specific PCR assay. No PCR amplicon was obtained from the remaining 70 samples of the examined intestines. Of 406 faecal samples suspected of SD, 126 samples yielded the 658 bp product of the tlyA-encoded haemolysin gene of *B. hyodysenteriae* whereas the 280 samples were negative in the PCR test (Table 2). Similarly, amplification of *B. hyodysenteriae* 16S rRNA specific sequences by PCR yielded the 658 bp product in case of 35 intestinal specimens; the remaining 56 intestines were negative (Table 2).

**Table 2**

Detection of *B. hyodysenteriae* and *L. intracellularis* using different PCR methods

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Single PCR for <em>B. hyodysenteriae</em></th>
<th>Single PCR for <em>L. intracellularis</em></th>
<th>Nested PCR for <em>L. intracellularis</em></th>
<th>M-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total number of samples</td>
<td>Number of positive samples</td>
<td>Total number of samples</td>
<td>Number of positive samples</td>
</tr>
<tr>
<td>Faeces</td>
<td>406</td>
<td>126</td>
<td>606</td>
<td>-</td>
</tr>
<tr>
<td>Mucosal scrapings</td>
<td>91</td>
<td>35</td>
<td>92</td>
<td>-</td>
</tr>
<tr>
<td>Mucosal scrapings from pigs with acute form of SD* and PE**</td>
<td>14</td>
<td>11</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total number of specimens tested</td>
<td>511</td>
<td>172</td>
<td>703</td>
<td>5</td>
</tr>
</tbody>
</table>

- negative results of PCR, * acute form of SD, ** acute form of PE.
The strongly β-haemolytic spirochaetes were present in 14 of 91 mucosal scrapings collected from pigs with signs of SD. All field isolates were re-cultivated until pure bacterial cultures were obtained. The isolates were then investigated by standard and M-PCR and the 658 bp product with the B. hyodysenteriae tlyA-encoded haemolysin gene was seen and no amplicon corresponding to the L. intracellularis 16 rRNA marker was found (Table 2).

**Discussion**

The M-PCR assay developed in this work proved to be efficient as an alternative method to the conventional PCR technique. This test allows simultaneous detection of pathogenic L. intracellularis and B. hyodysenteriae amplifying regions of the p78 gene and the tlyA haemolysin gene, respectively. The use of two sets of primers in a single PCR mixture yielded specific amplification products after M-PCR assay with bacterial DNA. The primers derived from the cloned DNA segment of L. intracellularis have been previously shown to be sensitive and specific for the detection of these bacteria (11). The primers for the 16S rRNA gene were selected because this sequence was highly conserved among all pathogenic L. intracellularis strains. The other set of specific primers was used to amplify directly from the intestinal material the unique DNA sequence of the tlyA gene of B. hyodysenteriae. The proteins encoded by the B. hyodysenteriae tly genes are well conserved. The M-PCR products obtained with the purified DNA from L. intracellularis and B. hyodysenteriae and with the total DNA recovered from intestinal specimens corresponded to the predicted molecular weight of the genomic fragments from each of the bacterial species tested. The PCR analysis of the positive controls of L. intracellularis and B. hyodysenteriae and positive intestinal samples from pig affected either with PE or SD yielded consistent results with the PCR analyses performed separately with the single primer pair specific for the respective genes.

One of the most important advantages of the M-PCR developed here is the identification of L. intracellularis and B. hyodysenteriae without limitations associated with standard bacteriological methods. Moreover, detection of these microorganisms with the use of nucleic acid-based assays is not affected by viability of bacterial cells. The bacterial viability is always a concern of standard cultivation procedures due to e.g. antimicrobial residues present in intestinal specimens. The M-PCR assay is preferable to alternative diagnostic methods in a number of ways. PE can be diagnosed post-mortem by examining for the presence of typical gross and histologic lesions and detection of the intracellular organism with silver staining (21). Unfortunately, this method is not specific for L. intracellularis, therefore only a reliable post-mortem diagnostic method may greatly enhance the ability to the monitoring of the disease in clinical or epidemiologic studies. Immunoochemical staining is specific for PE and can be used for the ante-mortem faecal immunofluorescence assay (14, 20, 22). However, the monoclonal antibodies required for this technique are not commercially available. In addition, the faecal immunofluorescence test is not sensitive for PE, therefore immunohistochemical staining limits the diagnosis to post-mortem specimens only. Cultivation of the intracellular organism is also difficult to be used as a routine diagnostic test (14, 29).

On the other hand, M-PCR has also several limitations and the method cannot be applied in all cases, especially when faeces are used as a source of DNA for the diagnostic procedure. It has to be mentioned that all experiments performed under the optimal conditions gave positive results mainly when the biological material from mucosal scrapings of the colon, caecum and ileum originated from acute forms of PE (watery diarrhoea) were applied, the M-PCR assay resulted in no product corresponding to the 319 bp of L. intracellularis. In this situation all the examined faecal samples were monitored by one tube nested PCR which allows to detect the 16S rRNA sequences of L. intracellularis as well as by using a single PCR reaction for the detection of the B. hyodysenteriae haemolysin tlyA gene. It is worth to mention that the molecular diagnostic method for PE such as nested PCR assay offers several advantages over the previously described techniques. The standard nested PCR is sensitive and specific for PE (10, 11). However, this assay requires a reamplification step, making the test more time consuming than multiplex PCR. In addition, two amplifications may result in contamination of samples with amplicons from previous reactions. To omit the risk of the contamination we decided to change the standard method consisting of 2 steps which was performed in two separate reaction tubes into the single tube method where both amplifications were done in one closed tube. In our opinion, one tube nested PCR can be used as a good alternative test for L. intracellularis screening in pig faeces because it was able to detect as few as 1.1x10^8 bacterial cells per 0.1 g of faeces. By using only single PCR 1.1x10^9 bacterial cells per 0.1 g of faeces were identified. Jones et al. (11) and Mc Cormick et al. (18) were able to detect 10^2-10^4 bacterial cells per gram of faeces by single PCR with primer pairs A/B. The detection limit for the nested PCR procedure was not reported. Möller et al. (23) proved that combination of a boiling procedure for DNA extraction with nested PCR was able to detect 2x10^9 bacterial cells per gram of faeces. Such kind of differences in the detection of L. intracellularis in faeces using single PCR may be explained by inhibitory factors within faecal specimens that could hamper the PCR reaction (33). The differences could also be due to various reference positive material used for analysis of the sensitivity of the tests. In the present study, the filtrates originated from infected intestines with known number of bacteria (1.1x10^7 bacteria cells/ml) were used. Comparing to our
results, the authors mentioned above used the pure cell cultures of *L. intracellularis*.

The limited sensitivity and a low number of organisms in faeces showed that M-PCR system is not able to detect specifically regions of *L. intracellularis* 16S rRNA sequences. In order to reduce the inhibitory influence of faecal components without reducing the sensitivity of the test, the previously described modified one tube nested PCR was performed (19, 26). The reported results indicated that re-amplification with nested primers increased the sensitivity of the bacterial DNA detection in faeces as much as 100-fold (3).

The sensitivity of the single and M-PCR for the detection of *B. hyodysenteriae* in 10-fold dilutions was estimated on the level of $10^{-4}$ which was not possible to count by the plate counting method due to a high number of spirochaetal cells on the TSA selective medium. We revealed that only two dilutions were countable: $10^{-6}$ with 203 CFU/ml and $10^{-7}$ with 14 CFU/ml. Elder *et al.* (6) showed that the sensitivity of the PCR assay was between 1 and 10 organisms per 0.1 g of faeces, or 1000 times that of bacteriological culture on TSA agar, allowing definitive diagnosis of SD even when the organism was at undetectable levels by culture methods. Kunkle and Kinyon (13) demonstrated that the number of *B. hyodysenteriae* in porcine faeces at the onset of SD ranged between $2 \times 10^6$ and $2 \times 10^{10}$ CFU/g when the organism were cultured with the selective TSA. In contrast, subclinically affected animals may shed recoverable numbers of spirochetes only sporadically and in much lower number than animals with clinical SD, often resulting in false negative culture results. Animals from field cases of SD may also contain drug residues that adversely affect the recovery of viable *B. hyodysenteriae* by the culture method. On the other hand, factors which affect the results of routine bacteriological examinations are not critical to the detection of *B. hyodysenteriae* by single and M-PCR.

In summary, the results of this study demonstrated that the M-PCR method is a useful tool for rapid diagnosis of a etiological agents only of acute onset of SD, and that the detection of *B. hyodysenteriae* in faeces from acute and chronic cases of the disease.

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