EVALUATION OF PCR TEST FOR DETECTION OF DERMONECROTOXIN OF *BORDETELLA BRONCHISEPTICA*

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Abstract

The aim of the study was the evaluation and optimisation of PCR test for the detection of dermonecrotxin gene (*DNT*) of *Bordetella bronchiseptica*. For the optimisation of the test, vaccine strain B16 was used. The optimisation procedure included: estimation of optimal Mg\(^{2+}\) concentration, annealing temperature, numbers of cycles, as well as sensitivity. The specificity of PCR test was checked with DNA of other pathogens existing in pigs’ respiratory tract. The elaborated test was specific and sensitive to detect *DNT* gene of *B. bronchiseptica*, in both clinical samples, as well as in pure culture of the bacteria.

Key words: *Bordetella bronchiseptica*, dermonecrotxin gene, PCR.

*Pasteurella multocida* (*Pm*) and *Bordetella bronchiseptica* (*Bbr*) are aetiologic agents of atrophic rhinitis (AR) in pigs, which cause noticeable economical losses in pigs’ farms (5, 12). Acting together they could cause progressive AR (PAR), while *Bbr* alone is responsible for non-progressive form of the disease (NPAR). Both mentioned pathogens have variety of agents, such as dermonecrotxin, fimbriae, cytotoxin, adenylate cyclase toxin, which play significant role in the pathogenesis of the disease (7, 11). From a diagnostic point of view, it is very important to detect both, *Pm* and *Bbr* using specific and sensitive method. Up to now, in the detection of the mentioned pathogens classical bacteriology methods, complemented with biochemistry are used (5). They are still a “gold” standard, but they are laborious and time consuming. In the case of *Pm*, the ELISA for serological control of pigs farm status is also widely use (3, 6). Among *Bordetella* sp., there is a high similarity of antigens structure so the detection of *Bbr* antibodies by the ELISA is insufficient. Additionally, in the detection of pathogenic *Pm*, PCR test, based on *Pm* dermonecrotxin (*DNT*) gene sequence, is widely used (3, 6, 9). *DNT* is a thermolabile protein responsible for turbinate atrophy by causing degenerative changes in osteoblasts and impairing bone formation.

Since in the case of *Bbr* infection, molecular diagnosis was not implemented in Poland so far, the aim of the study was the evaluation and optimisation of sensitive and specific PCR test for the detection of *Bbr* directly in clinical samples, such as nasal swabs, and in bacterial culture in order to diagnose NPAR and improve PAR diagnosis, as well as to analyse pigs’ farm status. The PCR elaborated test is based on the sequence of *Bbr DNT* gene.

Material and Methods

**Bacterial strains.** In this study *Bbr* vaccine strain (B16) was used as reference strain. G20G medium supplemented with gentamycin (10 mg/mL), penicillin (10 mg/mL), nystatin (10 mg/mL), and nitrofurantoin (10 mg/mL) was used for the multiplication of the strain. *Bbr* was grown in the medium during 24-72 h, at 37°C, with 7.5% CO\(_2\) atmosphere.

**DNA extraction.** DNA extraction was performed directly from decimal dilution of B16, according to the protocol of Genomic Mini (A&A Biotechnology, Poland). Genetic material was used directly in PCR reaction, or stored at -80°C for further analysis.

**Primers.** Primers dntF and dntR used in PCR test were designed by analysing sequence of *DNT* gene of *Bbr* from GeneBank (BB3978). To select the best sequence of primers, computer programme LaserGene was used. The length of both primers - the forward and the reverse, was of 20 nucleotides. Sequences of the used primers are shown in Table 1.

**Reaction conditions.** For optimisation the conditions of the PCR test, different concentration of primers, as well as Mg\(^{2+}\), annealing temperature, and number of cycles were tested.

The concentrations of 0.4 pM; 0.6 pM; 0.8 pM; 1.0 pM; 1.2 pM; 1.4 pM; 1.6 pM; 1.8 pM; 2.0 pM of each primers was thoroughly examined. The concentration of Mg\(^{2+}\) was examined at 0.25 mM; 0.75
mM; 1.25 mM; 1.75 mM; 2.25 mM; 2.75 mM; 3.25 mM, and 3.75 mM thoroughly.

Optimal annealing temperature was defined by temperature gradient with the use of iCycler (BioRad). Range of temperature from 50°C to 65°C was automatically estimated (50, 51.1, 53, 55.7, 59.5, 62.3, 64, 65°C).

To estimate the optimal efficiency of PCR conditions different number of cycles (from 35 to 45) were determined. The total volume of reaction mixture was 25 µL, which include 2.5 µL of DNA and 22.5 µL of mix reagents. The mix contained 10xPCR Gold Buffer, 25 mM MgCl₂ solution, polymerase AmpliTaq Gold 5 U/mL, 10 mM dNTPs (Fermentas), and 20 pM of each primer and free from DNase and RNase water.

**PCR sensitivity.** For estimation of the sensitivity of PCR test decimal dilutions of B16 (10⁻¹⁻⁻⁻⁻) in PBS were prepared. Simultaneously they were used for DNA extraction and inoculated on blood agar to check CFU. Dilution 10⁰ has got 0.5 McFarland scale concentration.

**PCR specificity.** For control of the specificity of PCR test, a genetic material of other pathogens, which could exist in swine respiratory tract, such as DNT⁺ Pasteurella multocida, DNT⁻ Pasteurella multocida, Mycoplasma hyopneumoniae, Streptococcus suis, Haemophilus parasuis, Actinobacillus indolicus, Actinobacillus porcilis were examined.

**Electrophoresis.** Products obtained in the PCR were separated by electrophoresis in 2% agar gel with ethidium bromide in concentration of 1 µl/mL. The electrophoresis was done in 1xTAE buffer, at 350 mA. Ten microliters of reaction mixture and 2 µl of loading buffer 6xDNA Loading Dye (Fermentas) were inserted into each well. The molecular weight of the obtained products was determined on the basis of molecular weight of marker GeneRuler™ 100 bp DNA Ladder Plus (Fermentas). The agarose gels were photographed under UV light using EC3 Chemi HR 410 Imaging System (UK).

**Results**

Sequences of the designed primers (dntF, dntR) amplified a DNA fragment of 224 bp length. The optimal concentration of each primer was 1.0 pM, as it is shown at Fig. 1. The best efficiency of this test was at 2.75 mM Mg²⁺ concentration.

Temperature gradient showed that at the range of 50°C to 65°C all stripes were present (Fig. 2). The choice of 56°C as a amplification temperature was made on similarity to the melting temperature provided by producer (IBB PAN).

The best number of cycles was 45 cycles, as it is shown in Fig. 3.

Parameters of amplification conditions are shown in Table 2.

The sensitivity of PCR was 2.2x10² cfu (Fig. 4).

As it is shown at Fig. 5, the elaborated test is specific to Bbr genetic material only.

**Discussion**

Bacteria belonging to **Bordetellaceae** family are closely related Gram–negative β-proteobacteria (7). **B. pertussis (Bp)**, **B. parapertussis (Bpp)**, and **Bbr** are subtypes of a single genome species (when DNAs are 70% or more related, and with 5°C or less divergence in Tm values) (9). At the genetic level Bpp and Bp are derived from Bbr-like ancestor. Between Bbr and Bpp, a high degree of similarity between their genomes is shown, mainly around the replication origin. Comparison of Bbr and Bp genomes showed that rearrangements and deletions in Bp were similar to those in Bpp but much more serious, where the rearrangements were bounded in 88% by the insertion of sequence elements (7).

All bordetellae have a wide range of determinants playing role in host interaction and virulence. These factors include the filamentous haemagglutinin, fimbriae, a lot of autotransporters such as pertacin, tracheal colonisation factor and serum resistance protein, numerous toxins, type-III secretion system, LPS and flagella biosynthesis system.

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer</th>
<th>Sequence 5’ → 3’</th>
<th>PCR product length</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bordetella</strong></td>
<td>dntF</td>
<td>GCCGTACTTGGGATAATAGA</td>
<td>224 bp</td>
</tr>
<tr>
<td><strong>bronchiseptica</strong></td>
<td>dntR</td>
<td>ATAAAGATGAATCGGCATTG</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Initial denaturation of dsDNA</td>
<td>95°C</td>
<td>10 min</td>
<td>1x</td>
</tr>
<tr>
<td>2 Denaturation</td>
<td>94°C</td>
<td>30s</td>
<td></td>
</tr>
<tr>
<td>3 Annealing</td>
<td>56°C</td>
<td>30s</td>
<td>45x</td>
</tr>
<tr>
<td>4 Elongation</td>
<td>72°C</td>
<td>20s</td>
<td></td>
</tr>
<tr>
<td>5 Final chain synthesis</td>
<td>72°C</td>
<td>7 min</td>
<td>1x</td>
</tr>
</tbody>
</table>
Fig. 1. Optimisation of Mg$^{2+}$ concentration. M - DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus, 1 – positive control, 2 - 10$^6$ (0.5 McFarland scale), 3 - 10$^{-4}$, X mix (negative control).

Fig. 2. Temperature gradient. M - DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus, 1 - 65°C, 2 - 64°C, 3 – 62.3°C, 4 – 59.5°C, 5 – 55.7°C, 6 - 53°C, 7 – 51.1°C, 8 - 50°C.

Fig. 3. Cycles optimisation: 35, 40, 45, respectively. M - DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus, 1 - positive control, 2 - 10$^6$ (0.5 McFarland scale), and next values: 3 - 10$^{-1}$, 4 - 10$^{-2}$, X- mix (negative control).
Differences among these systems in the three species mentioned above might influence the different host ranges and virulence profiles of these pathogens (7). Bp and Bpp are strictly connected with human host; however Bpp could also cause respiratory infection in sheep. Bbr has a wide range of hosts, including domestic pets, rodents, horses, monkey, humans, and numerous wild animals (12, 14).

Molecular techniques used for the identification of Bordetella species are mainly based on some gene sequences mentioned before. Hozbor et al. (2) developed PCR test based on flagellin structural gene (fla gene). They used four primers in different variations for the detection of each pathogen isolated from human cases. Register et al. (9) used primer sets in multiplex PCR to detect both, DNT gene of Pm and fla gene of Bbr. Shin et al. (13) studied polymorphism repeated regions in Bbr pertactin gene (prn) isolated from pigs in Korea. Pertactin is an outer membrane protein, which has adhesion properties. It plays also a role of antigen, which stimulates swine immunity system. The mentioned researchers suggest that the wide diversity in pertactin could be an evolutionary response to numerous and diverse selection pressures. They were also interested in further investigation on cross-protection between different pertactin variants. To distinguish Bbr from Bp, Register et al. (10) used RT-PCR based also on prn gene, but this test is not recommended for the identification of Bp without additional confirmatory testing. Nevertheless from the practical and economical point of view, in the case of AR detection of Bbr should be concentrated on the presence of the factor of greater importance. Koidl et al. (4) developed a method including a multiplex PCR system consisting of four independent PCRs for the qualitative detection of insertion sequence IS481, IS1001, the filamentous haemagglutinin gene, and the heterologous IC in a single LC glass capillary. These sequences are present in genomes in defining copy number. Differentiation was provided by melting-curve analysis of the products. This method was suitable for the routine laboratory work, allowing diagnosis of pertussis.

In our study, PCR for the detection of DNT gene in Bbr was evaluated. DNT is mainly responsible for cytotoxic properties, as well as for local inflammatory effects. It could also influence the colonisation of nasal epithelium cells by Bbr. DNT gene is present in all Bordetella sp. (1, 8, 14). In this case it
has not important significance, because from all of them only Bbr is specific swine respiratory tract pathogen.

Results of our study show that designed DNT Bbr set of primers are specific only for Bbr. For other pathogens, causing swine respiratory diseases, the results of PCR test were negative. The sensitivity of the elaborated test was high. Sensitivity of PCR performed directly from nasal swabs depends on complexity of a sample, as well as on the presence of unknown substances that inhibit the reaction. An important fact worth underlining is that Bbr grow on G20G medium very slowly. Sometimes the diagnosis is based on PCR test performed directly from swabs with the lack of bacteria isolation.

PCR test is a useful tool for a fast and accurate detection of the pathogen. Currently the PCR test for DNT Bbr detection is applied for the routine diagnosis of NPAR using nasal swabs or pure culture of the bacteria. This assay could back up the study on the frequency of PAR and NPAR in pigs’ herds.

References