OPTIMISATION OF THE PCR FOR DETECTION OF APXIVA GENE OF ACTINOBACILLUS PLEUROPNEUMONIAE

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Received: November 2, 2010 Accepted: February 16, 2011

Abstract

PCR technique used for the detection of apxIVA gene of Actinobacillus pleuropneumoniae (App) was developed. The optimisation of the technique was conducted on App ATCC 27088 strain. The concentration of primers, Mg²⁺, and Taq polymerase, as well as the annealing temperature and the number of cycles were optimised. The test could detect up to 10⁻⁷ dilution, which equals 3×10⁶ cfu/mL. The specificity of the PCR was verified with the use of genetic material of other pathogens existing in pigs' respiratory tract. Based on the obtained results it can be assumed that the developed PCR test can be used for the detection of the apxIVA gene, in both pure culture of App and lung tissue.

Key words: Actinobacillus pleuropneumoniae, apxIVA gene, PCR.

Actinobacillus pleuropneumoniae (App), Gram-negative bacteria belonging to Pasteurellaceae family, is the causative agent of pleuropneumonia, one of the most important world-wide spread bacterial diseases of the respiratory tract of pigs of all ages. App strains were classified into two biovars on the basis of nicotinamide adenine dinucleotide (NAD) requirement for their growth. Biovar 1 includes NAD-dependent strains, while biovar 2 contains less common NAD-independent strains (9).

App produces many virulence factors involved in disease development. One of the most important is exotoxin named Actinobacillus pleuropneumoniae toxin (Apx), which belongs to the pore-forming RTX-toxin family (1, 9). App synthesises four Apx toxins: ApxI, ApxII, ApxIII, and ApxIV. They are secreted in various combinations by the different serotypes, but only ApxIV is produced by all App serotypes (9). The mentioned toxin is specific for App species because of the absence of apxIV gene in the species related to App (6). The Apx toxins are encoded by four genes C, A, B, and D, located in operons in the presented order. The C and A genes are responsible for the active toxin protein synthesis, while the B and D genes code for proteins enabling toxin secretion (5).

The aim of this study was to develop and optimise PCR test for identification of apxIVA gene of App, directly in the lungs and bacterial cultures.

Material and Methods

Bacterial strains. App ATCC 27088 strain was used for test standardisation as a control material. App was grown on PPLO agar, supplemented with NAD (10 mg/mL) and horse serum, for 24 h at 37°C in 8% CO₂ atmosphere. Ten fold dilutions of the strain were used. The dilutions were equivalent to 3×10⁰, 3×10¹, 3×10², 3×10³, 3×10⁴, 3×10⁵, 3×10⁶, 3×10⁷, 3×10⁸, and 0 colony forming units per milliliter (cfu/mL).

DNA extraction. DNA from decimal dilutions of App ATCC 27088 strain was extracted according to the protocol of Genomic Mini DNA isolation kit (A&A Biotechnology, Poland). Genetic material was used in PCR test or stored at -80°C for further experiments.

Primer. Primers used in this study were taken from paper of Schaller et al. (10). Sequences of the primers are shown in Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>Sequence of primers used to proliferate apxIVA gene</td>
</tr>
<tr>
<td>Name</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>APXIVA-1L</td>
</tr>
<tr>
<td>APXIVA-1R</td>
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PCR conditions. The best concentration of primers, Mg$^{2+}$, and Taq polymerase, as well as the annealing temperature and the number of cycles were taken into consideration during optimisation of the PCR test. Primer concentrations tested were as follow: 0.4 μM, 0.6 μM, 0.8 μM, 1 μM, and 1.2 μM. Optimal concentration of Mg$^{2+}$ was selected from 2 mM, 2.25 mM, 2.5 mM, 2.75 mM, and 3 mM. Concentration of polymerase Taq was examined at 0.2 U, 0.35 U, and 0.5 U per reaction. The annealing temperature was determined using temperature gradient, with range from 51°C to 58°C. PCR test was examined at 35, 40, and 45 cycles.

A single PCR reaction was performed in a final volume of 25 μl containing: 2.5 μl of template DNA, 2.5 μl of 10×Taq buffer [100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl], 25 mM MgCl₂ solution, 0.5 μl of 10 mM dNTPs, 1 U/μl Taq DNA polymerase (Fermentas, Lithuania), primers (20 μM of each), completed with the appropriate amount of water free from DNase and RNase. All PCR tests were conducted with the use of T3 Thermocycler (Biometra, Germany).

PCR sensitivity. The sensitivity of the PCR was evaluated using decimal dilutions of the App reference strain. The 10-fold dilutions were simultaneously spread on PPLO agar and used for DNA extraction to define the amount of bacteria (cfu/mL) in each dilution.

PCR specificity. In order to estimate the specificity of the test, genetic material extracted from common pathogens existing in swine respiratory tract, including: Actinobacillus porcinus, Haemophilus parasuis, Mycoplasma hyopneumoniae, (DNT+) Pasteurella multocida, (DNT-) Pasteurella multocida, Streptococcus suis, Bordetella bronchiseptica, swine influenza virus (SIV), and Staphylococcus aureus, was used.

Electrophoresis. The amplified products were separated by electrophoresis in 2% agarose gel with ethidium bromide (1μg/mL) in 1×TAE (Tris-acetate-EDTA) buffer and visualised by UV transillumination using EC3 Chemi HR 410 Imaging System (Ultra-Violet Products Ltd., UK). A GeneRulerTM 100bp DNA Ladder Plus (Fermentas, Lithuania) was used as a molecular size standard.

Results

Sequences of the designed primers amplified a DNA fragment of 442 bp.

As it is presented in Fig. 1, all tested primer concentrations gave positive result. However, due to obtaining the most visible product at the concentration of 0.8 μM, it was chosen as optimal.

The use of each tested concentrations of Mg$^{2+}$, similarly as in case of the optimisation of primers concentration, allowed obtaining visible products in agarose gel, as presented in Fig. 2. Nevertheless, 2.5 mM concentration of Mg$^{2+}$ was considered as the most effective.

During PCR optimisation the most efficient concentration of Taq polymerase was 0.35 U per reaction, as it is shown in Fig. 3.

Summarising the optimisation of the best concentration of PCR assay substrates (primers, Mg$^{2+}$, and Taq polymerase, Figs 1-3), the final volume of individual component per one reaction mixture was selected, as it shown in Table 2.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Volume (μl)</th>
</tr>
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<tbody>
<tr>
<td>water</td>
<td>14.65</td>
</tr>
<tr>
<td>10×Taq buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.5</td>
</tr>
<tr>
<td>primer IVAF</td>
<td>1.0</td>
</tr>
<tr>
<td>primer IVAR</td>
<td>1.0</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.35</td>
</tr>
<tr>
<td>template DNA</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 2 Composition of the reaction mixture

<table>
<thead>
<tr>
<th>Table 3</th>
<th>PCR assay reaction conditions</th>
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<tr>
<td>Step</td>
<td>Temperature</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>92°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>92°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>57°C</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
</tr>
<tr>
<td>Final synthesis</td>
<td>72°C</td>
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Basing on electrophoresis readings of the lowest detectable dilution of DNA of App reference strain amplified by PCR conducted at different annealing temperatures, the temperature of 57°C was selected as optimal for further work (Fig. 4).

Among numbers of cycles tested (35, 40, 45) during the optimisation of PCR assay, the most efficient was 45 cycles, as it is shown in Fig. 5.

Based on the results of the optimal annealing temperature and the number of cycles tested, presented on Figs 4 and 5, respectively, the most appropriate amplification conditions useful for routine detection of ApxIV toxin gene in clinical samples were estimated. They are summarised in Table 3.

The validation of the elaborated PCR test included also the estimation of its sensitivity. The obtained results are presented in Fig. 6. As it is shown, this test was extremely sensitive. The positive signal was observed in dilution as high as 10⁻⁷, which equal 30 cfu/mL (Fig. 6).
Fig. 1. Optimisation of primer concentration. M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus; number corresponds to concentration of primers in μM.

Fig. 2. Optimisation of Mg²⁺ concentration. M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus; number corresponds to concentration of Mg²⁺ in mM.

Fig. 3. Optimisation of Taq polymerase. M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus, 1 – 0.2 U/reaction, 2 – 0.35 U/reaction, 3 – 0.5 U/reaction.

Fig. 4. Optimisation of annealing temperature. M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus; number corresponds to temperature (°C).

Fig. 5. Number of cycles optimisation. M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus, 1 – 2×10⁷ cfu/mL, 2 – 1.8×10⁸ cfu/mL.

Fig. 6. Estimation of PCR sensitivity. M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus. Lanes 1 – 11 – consecutive 10-fold dilutions of App reference strain.
Figure 7 demonstrates the results of PCR specificity testing. As it is shown below, except for App reference strain, genetic material obtained from common swine pathogens used to determine the specificity of the test was not amplified with primers chosen. This indicates that elaborated test was highly specific and very specific. Therefore, it is appropriate for routine detection of App in clinical material.

Discussion

Bacteria belonging to Pasteurellaceae family are classified in Haemophilus, Actinobacillus, and Pasteurella genera. Many taxonomic studies have highlighted difficulties in distinguishing bacteria from Pasteurellaceae family (6).

Despite of characteristic clinical signs and results of postmortem examination, the “gold standard” for App diagnosis is still isolation of the bacteria and their biochemical identification using the CAMP reaction and determination of urease activity. Pleuropneumonia can be suspected on the basis of characteristic pulmonary lesions. However, appearance of the lesions caused by other porcine actinobacilli may be impossible to differentiate from those of pleuropneumonia. The changes observed in pigs that died due to acute pasteurellosis may be sometimes similar to those characteristic for pleuropneumonia. Moreover, in the last years atypical App isolates (for example, urease negative strains), for which classical diagnosis may be insufficient, have been described. Identification of the NAD-independent App isolates also causes some problems. These isolates might be mistaken as A. suis. Therefore, to avoid false results, the accurate identity of App strains should be verified by PCR (8).

The presented study focused on the development of PCR assay, which identifies App. Based on many different publications, amplification of apxIVA gene was selected as the most accurate. The gene encoding toxin, which biological function still remains unclear, is specific for App species and, as mentioned above, for all App serotypes (9).

There are many publications describing the identification of App strains based on PCR assay of apxIVA and other tests (2-4, 6, 7, 10, 11). Schaller et al. (11) characterised apxIVA gene. Using pairs of primers matching three different parts of the apxIVA gene, they obtained products from DNA of all App reference strains, while using DNA of other examined species no PCR product was detected. They confirmed the presence of apxIVA by Southern blot hybridisation of Clal-digested genomic DNA, using wide-range of probes designed for RTX gene screening. The probes were specific to the 5’-terminal, central, and 3’-terminal part of apxIVA. Probes for the central and 5’ part of apxIVA hybridised to DNA of all 12 studied App serotypes and gave weak hybridisation signal to DNA of A. lignieresii. Specific reaction with DNA of App strains was observed only when probe specific to the 3’ part of apxIVA was used.

Schaller et al. (10) examined the reference strains of the 14 serotypes and 194 field strains representing the major 12 serotypes. Using a primer pair spanning 3’-terminus region of apxIVA gene, a 422 bp fragment was amplified. DNA from all studied App strains were positive in PCR, while no amplification product was observed when DNA from 17 different bacterial species closely related to App were used as matrix. Furthermore, in several cases DNA from Actinobacillus sp. firstly characterised as App, were negative in PCR assay.

PCR conditions presented by Schaller et al. (10) differ from that described in our paper. Except primers sequence and concentration of tris-HCl and KCl, all other parameters were different. These authors used 1.5 mM MgCl2, 150 μM each dNTP, 0.25 μM each primer, and 0.5 U Taq polymerase per reaction, while our optimisation revealed better effect by using higher concentrations of the reagents listed above. On the basis of the results obtained in our studies, we also chose higher amplification temperature than recommended by Schaller et al. (10). These distinctions may result from different equipment and reagents supplied by different producers (Schaller et al. did not mention producers of reagents). Furthermore, the quality of the reagents has a crucial impact on received results.

Similar results to those presented by Schaller et al. (10, 11), were obtained by da Costa et al. (6). They detected the specific 388-bp product in all examined App serotypes, which was absent in related species studied. Furthermore, da Costa et al. (6) confirmed that nine samples taken from infected swine and four bacteria isolated from apparently healthy animals were App positive and had the apxIVA gene.

Cho et al. (2) also reported the presence of apxIV gene in 90 App field isolates and its absence in related bacteria belonging to the Pasteurellaceae family. The authors suggest that this gene is species specific and its presence has a potential value for use in diagnostic purpose.

The presence of the apxIV gene was also detected with the use of in situ hybridisation. Cho et al.
(3) demonstrated that the apxIV gene could be identified with a non-radioactive digoxigenin-labelled DNA probe in formalin-fixed paraffin wax-embedded lung tissues from 10 pigs naturally infected with App serotypes 2, 5, and 6 (three of each) and an untypable strain. Detection of hybridisation signal for apxIV gene within neutrophils, alveolar macrophages, and on the periphery of acute coagulative necrosis suggests the production of the ApxIV toxin in pleuropneumonic lungs.

Cho et al. (4) confirmed usefulness of the in situ hybridisation for the detection of the apxIV gene in the diagnosis of porcine pleuropneumonia. With the use of in situ hybridisation they obtained distinct positive signal in lung specimens from all pigs inoculated with the 12 App serotype reference strains.

Dreyfus et al. (7) used immunoblot analysis of sera from pigs experimentally infected with App. Seroconversion to ApxIV was measurable by immunoblot already at 20 d post infection, whereas sera from pigs inoculated with non-pathogenic *Actinobacillus* sp. that gave cross-reaction with LPS of App serotype 9 showed no signals in the ApxIV-immunoblots. They also developed the non-competitive indirect ELISA for the detection of anti-ApxIV-antibodies in sera of pigs. The results obtained with the use of ELISA were highly specific, as those of immunoblot.

Summarising, the results of our study were similar to those of other authors (2-4, 6, 7, 10, 11) and confirmed that specific and sensitive PCR test for the detection of the apxIV gene is a good tool in porcine pleuropneumonia diagnosis. The elaborated test was used in routine diagnosis of porcine pleuropneumonia - a total of 308 specimens (221 bacterial strains and 87 lung tissue samples), were tested. The obtained results of this examination will be presented in a separate article.

References


