APPLICATION OF PCR BASED ON \textit{apxIVA} GENE FOR RAPID DETECTION OF \textit{ACTINOBACILLUS PLEUROPNEUMONIAE}

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Received: October 17, 2011    Accepted: November 15, 2011

Abstract

PCR technique, based on \textit{apxIVA} gene of \textit{Actinobacillus pleuropneumoniae}, was used to test both bacterial colonies and fresh lung tissue specimens obtained for routine diagnosis. In total, 365 field isolates (previously recognised bacteriologically as \textit{A. pleuropneumoniae}) and 129 fresh lung tissue samples were tested. PCR test confirmed that 94.5% of the studied isolates belonged to \textit{A. pleuropneumoniae}. From all tested tissue specimens, 49.6% samples were positive in PCR while only 38% were positive in bacteriological examination. Furthermore, results of 88.4% tested tissue samples were identical in both PCR and bacteriological examinations. In case of 11.6% of tissue specimens only PCR gave positive results. Based on the obtained results, it can be assumed that the \textit{apxIVA} PCR assay, which is a very specific and sensitive technique, can be helpful in rapid and sensitive diagnosis of pleuropneumonia.

Key words: swine, \textit{Actinobacillus pleuropneumoniae}, \textit{apxIVA} gene, PCR.

The pleuropneumonia, caused by \textit{Actinobacillus pleuropneumoniae} (App) species, is one of the most important swine respiratory diseases. This disease can occur in pigs of all ages. Depending on the strain serotype, the immune status of the host and the number of bacteria reaching the lung, the course of pleurpneumonia may take peracute, acute, subacute, or chronic forms (1, 6, 15). Each serotype of App produces at least two or combination of three extracellular toxins (ApI, ApII, ApIII, and ApIV), which are members of the RTX (repeat in the structural toxin) family (8). The characteristic of Ap toxins is presented in Table 1. As it is demonstrated, only the gene encoding ApIV protein is present in all serotypes of App.

Classical diagnosis of bacterial disease depends on isolation of the etiological agent, followed by characterisation of its biochemical and morphological properties. Sometimes isolation of the bacterial strain can be impossible due to the nature of the pathogen or previous antibiotic treatment of infected animals. For example isolation of App is very time-consuming and can cause many difficulties, particularly in the case of chronic form of pleuropneumonia. In such situation bacteriological methods may be relatively insensitive.

Therefore, fast and reliable tools for the diagnosis of pleuropneumonia are crucial for veterinary diagnostic laboratories serving the swine industry. The molecular methods for the detection of App have been continuously developed and each has its strong and weak points (6). New approaches are based on the detection of \textit{apxIV} gene, due to the fact that it is species-specific (present in all App serotypes), as mentioned before (4, 5, 13, 14). Therefore, this gene is a significant diagnostic marker to identify the App species.

The aim of this study was to use PCR test, elaborated by ourselves (17), that can identify App, directly in lung samples as well as in bacterial colonies.

Material and Methods

Bacterial strains and culture conditions. App ATCC 27088 and App ATCC 27090 strains were used as control material. A total number of 365 field isolates collected between 1995 and 2010, firstly recognised as App, were tested. Bacterial isolates were cultured in horse blood agar with a perpendicular streak of \textit{S. aureus}. Colonies with characteristic morphology were transferred to medium for pleuropneumonia-like organisms (PPLO agar), supplemented with 10 mg/mL of NAD and 10% horse serum. The plates were incubated at 37°C for 24 h in atmosphere 8% CO2.

Tissue samples. A hundred and twenty-nine lung tissue samples were obtained from biological material sent to Department of Swine Diseases for routine bacteriological diagnosis (lungs with and without lesions characteristic for pleuropneumonia). These samples were used for direct isolation of bacterial DNA, which was tested by PCR or stored at -80°C for further investigations.
Table 1
Phenotypical characterisation of Apx toxins (8, 14)

<table>
<thead>
<tr>
<th>Activity</th>
<th>Molecular weight (kDa)</th>
<th>Serotype</th>
</tr>
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<tbody>
<tr>
<td>ApxI</td>
<td>strong</td>
<td>strong</td>
</tr>
<tr>
<td>ApxII</td>
<td>weak</td>
<td>moderate</td>
</tr>
<tr>
<td>ApxIII</td>
<td>none</td>
<td>strong</td>
</tr>
<tr>
<td>ApxIV</td>
<td>weak</td>
<td>not determined</td>
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Fig. 1. Agarose gel electrophoresis of PCR amplification products from App strains (1 – App ATCC 27088, 2 – App ATCC 27090, 4-18 – field isolates); 3 – Negative control; M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

Fig. 2. Agarose gel electrophoresis of PCR amplification products from tissue samples (1 – App ATCC 27088, 2-15 – lung specimens); 16 – Negative control; M – DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

The specific amplicon of 442 bp was present also in the direct extracts from lung tissue samples, as it is shown on Fig. 2.

**DNA extraction and PCR.** Total genomic DNA was extracted according to the protocol of Genomic Mini kit (A&A Biotechnology, Poland). Genetic material was used in PCR test. Strains of App were characterised by amplification of the *apxIVA* gene using the primers, which were elaborated by Schaller *et al.* (13) (APXIVA-1L – 5’-TGGCACCTGACGGTGATGA-3’, APXIVA-1R – 5’-GGCCATCGACCTCAACCAT-3’). A single PCR was performed as follows: a final volume of 25 µl containing: 2.5 µl of template DNA, Taq Buffer (10 mM Tris-HCl, pH 8.8 at 25°C, 50 mM KCl), 2.5 mM of MgCl₂ solution, 200 µM of dNTPs, 0.35 U of Taq DNA Polymerase (Fermentas, Lithuania), primers (0.8 µM of each), completed with the appropriate amount of water free from DNase and RNase. All PCRs were conducted with a use of T3 Thermocycler (Biometra, Germany). Amplification was performed in 45 cycles comprising of: 30 s at 92°C, 30 s at 57°C, and 30 s at 72°C. The first cycle was preceded by an initial denaturation lasting 5 min at 92°C, and the last cycle was followed by 10-min extension at 72°C (17). After amplification, 10 µl of the product was subjected to electrophoresis for 35 min at 300 V in 2% agarose gel stained with ethidium bromide. Amplification products were visualised and
The apxIVA-PCR amplified a product of 442 bp with App reference strains and with all tested App isolates. They were mostly classified as serotypes 2, 6, 9, and 4, but a few strains belonging to serotypes 1, 5, 7, 8, 10, and 11 were also included (Fig. 1).

From all 365 tested strains, which were assigned to App species on the basis of their morphology and biochemical tests, 345 (94.5%) were confirmed by amplification of the apxIVA gene. However, 20 (5.5%) examined strains were PCR negative.

The apxIVA product was amplified from 64 (49.6%) lung tissue specimens, while 65 (50.4%) remaining samples were negative.

In case of bacteriological examination, 49 (38%) tested samples were positive and the remaining 80 (62%) gave a negative result. It should be stressed that identical results in the PCR and bacteriological examination were obtained from 114 (88.4%) tissue samples.

Discussion

In the respiratory tract of pigs, bacteria belonging to the Pasteurellaceae family may occur among other isolated pathogens. Distinction of these species from the obligate App pathogen can be difficult due to similar results obtained in biochemical and serological tests (2, 9, 15). Problems with isolation and identification of App imply the necessity of elaboration of new, rapid, sensitive, and specific methods.

We developed a method allowing easy identification of App based on species specific apxIVA gene amplification. Using the PCR, we confirmed that 94.5% of the tested strains were App. However, 5.5% of them were excluded from App species. These results are in favour of the molecular method, and shows that bacteriological tests may be insufficient because of their lower sensitivity and specificity.

Numerous publications describe the use of PCR assay as a tool to identify App strains (2, 3, 6, 7, 9, 11-13). These PCR’s are based on several different genes occurring in these microorganisms. However, in most of them only DNA from bacterial culture was used, which is disadvantageous because of the difficulty in the culture of App. This bacterium forms small colonies, up to 1 mm, after 24-48 h incubation in blood agar supplemented with NAD or in the presence of its donor. Furthermore, isolation of this microbe can be problematic due to accompanying microbial flora (15, 16). Therefore, the possibility of final diagnosis of pleuropneumonia based on direct testing of tissue specimens is a good point of the presented technique.

The apxIVA gene was also selected as the basis for PCR assays by other authors (6, 7, 13). ApxIV, belonging to the pore-forming RTX toxin family, has been reported to be expressed only in vivo. The haemolytic activity of the ApxIVA recombinant is weak, and it has a cohaemolytic synergy with the sphingomyelinase (β-toxin) of Staphylococcus aureus (14). Deletion of apxIVA gene led to the attenuation of the virulence of App, therefore, the presence of the ApxIVA is essential for the expression of the full virulence of the bacterium (10). They obtained similar results to those presented in our study. They detected the specific amplicon in all examined App serotypes and confirmed its absence in related species studied.

Chiers et al. (3) used the PCR based on the nucleotide sequence of a dsbE-like gene for the detection and identification of App. The dsbE-PCR amplified a product of 343 bp with all App reference and field strains. This PCR did not react with any other tested species, except for A. lignieresii. It was evaluated on mixed bacterial cultures of the tonsils or conchae of pigs. In general, the results obtained from tissue suspension were better (more positive results) than those from swabs.

A year later, Chiers et al. (2) confirmed usefulness of the dsbE-PCR in the diagnosis of the subclinically infected animal. App was verified by PCR in cultures from all 12 experimentally infected pigs, while routine bacteriological methods were effective only in 75%.

Hernanz Moral et al. (9) used the aroA gene as the target DNA, which was amplified by PCR assay. They analysed either cultured bacteria or DNA samples. This PCR identified all serotypes of App by amplification of 1,025 bp fragment. Except for A. equuli no other species tested by them yielded positive reaction.

Based on cpx and cps genes, Lo et al. (11) developed a multiplex PCR assay to reveal the presence of App. The length of product gain from amplification of cpx and cps genes was 0.7 and 1.1 kb, respectively. This PCR was used to amplify DNA from the reference strains of App representing 12 different serotypes. With the exception of serotype 4, all serotypes gave a band of 0.7 kb and the band was amplified only from serotype 5 DNA the 1.1 kb. The usefulness of this PCR was confirmed by testing field isolates of App and tissue samples collected from pigs recently infected or frozen 6 years earlier. All isolates as well as lung samples gave positive results. In addition, nine isolates were classified as serotype 5 (present of 1.1 kb band).

Savoye et al. (12) used PCR based on the amplification of the omIA gene of App. All of the 13 App reference strains and 27 field isolates were PCR positive, while the other tested species, belonging to the Pasteurellaceae family, or those encountered in the respiratory tract of the pig, were all omIA negative. In these studies, the number of PCR positive samples was three times higher than from App isolation by bacteriological techniques.

In conclusion, PCR is a tool, which can significantly help in the diagnosis of pleuropneumonia.
It is more sensitive than culture of App and the result is not affected by the presence of contamination. In our study we confirmed it by obtaining positive PCR results from 15 (11.6%) tissue specimens of which App isolation was impossible. PCR may therefore be very helpful in the identification of the strains, and sufficient to diagnose the disease also in the absence of the possibility of using bacteriological methods. Failing to culture the App for susceptibility testing should not be of concern, because this bacterium is susceptible to most broad-spectrum antibiotics (11).

It should be remembered that App, except of causing pleuropneumonia, may also be an aetiological agent of porcine respiratory disease complex (PRDC). Thus, with other methods allowing identification of the pathogens involved in PRDC (mainly porcine reproductive and respiratory syndrome virus, Mycoplasma hyopneumoniae, swine influenza virus, Pasteurella multocida, Streptococcus suis, Aujeszky’s disease virus) the apxIVA-PCR may have a great significance in its diagnosis.

References