SHORT COMMUNICATION

Diagnosis of Bordetellosis in Pigs from North East India by PCR

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ABSTRACT

Bordetella bronchiseptica is an etiologic agent of bronchopneumonia and progressive atrophic rhinitis (PAR) in swine. Both toxigenic and nontoxigenic B. bronchiseptica strains have been associated with bronchopneumonia. Monitoring and investigation of outbreaks involving these bacteria require sensitive and accurate identification and reliable determination of the isolates. In the present study, we report the development, optimization and performance characteristics of polymerase chain reaction (PCR) for B. bronchiseptica strains. A total of 47 isolates of B. bronchiseptica were biochemically identified from 90 pigs suffering from bronchopneumonia maintained in a semi intensive rearing system of organized piggery in Meghalaya. PCR was employed with filamentous haemagglutinin toxin genes (thaB and thaC) and fimbrial toxin genes (fim2 and fim3) primers to identify the specific toxin types of B. bronchiseptica. All the 47 isolates were positive for all the toxin genes. The specificity of designed primer pairs was tested by screening some common bacterial species related to the respiratory tract namely, Pasteurella multocida, Staphylococcus aureus and Streptococcus spp. No DNA amplifications of the organisms tested could be seen in the specificity test. Amplicon mobility in agarose gels indicates the amplicons are highly stable. The data presented, establish this PCR as a reliable method for identification and study of adhesins of B. bronchiseptica that may greatly simplify investigations of swine bronchopneumonia and PAR for Indian isolates.

Keywords: Bronchopneumonia, Bordetella bronchiseptica, Toxin gene, PCR

INTRODUCTION

Bordetella bronchiseptica are small, aerobic, non-spore forming Gram-negative, pleomorphic cocccobacilli that cause respiratory tract infections in animals, humans and birds (Dubuisson et al., 2000). The organisms are worldwide in distribution. B. Bronchiseptica is widely distributed in swineherds with clinical respiratory disease and sometimes in association with P. multocida. The toxins produced by B. bronchiseptica are involved in this disease condition (Foged, 1992; Jutras & Martineau, 1996). The colonization is initiated by B. bronchiseptica, as the bacterium express a battery of adhesins, like fimbriae, filamentous haemagglutinin (Cotter and Miller, 2001) to colonize the upper respiratory tract of various host species and thus predisposing the colonization by P. multocida. The organism has received considerable importance as human pathogen in light of increasing reports on its isolation, especially from immunocompromised hosts with respiratory tract infections. The isolation of B. bronchiseptica from the respiratory tract or from the blood of human immunodeficiency virus (HIV)-infected patients with respiratory diseases is also being increasingly reported (Hovette et. al., 2001). This circumstance has prompted some investigators to propose the inclusion of B. bronchiseptica in the list of opportunistic pathogens causing diseases associated with exposure of HIV-infected patients to animals (Woodward et. al., 1995; Pajuelo et. al., 2002).

PCR has been used increasingly as an additional tool for the identification as well as detection of virulent toxin genes of microorganisms because of its rapid, sensitive and specific detection. The major virulence factors of B. bronchiseptica include adhesins namely filamentous
Material and Methods

Sample Collection and Examination

The crosses of New Hampshire and local pigs of different age groups having symptoms of anorexia, dyspnoea, oculo-nasal discharge, high temperature (40.5°C), twisting of the snout and death at later stages were taken as a part of study. The samples were collected from an organized piggy in Meghalaya. The atmospheric temperature and humidity was recorded between 25-35°C and 60-70% respectively with heavy rainfall during that period. Out of 90 pigs maintained at the farm, 57 (63.3%) were affected including 18 dead animals. In every case of death, postmortem was performed within one to two hours duration. All the internal organs were thoroughly examined and any macroscopic and gross lesions observed were recorded. The nasal swabs from infected pigs, heart blood, lymph node, lungs, and liver samples collected from dead animals after post-mortem examination or from the acute cases of nasal discharge and from healthy piglets during slaughtering were scientifically processed for microbiological investigation.

Isolation and biochemical identification of Bordetella bronchiseptica

All the samples were inoculated in sterile 10% sheep blood agar and incubated aerobically for 24 h at 37°C. Bacterial colonies were purified based on the size, shape, color and patterns of haemolysis on blood agar and were subjected to motility test and Gram’s staining. In addition an array of biochemical tests namely catalase, cytochrome oxidase, indole production, hydrogen sulphide production, nitrate reduction, Simon’s citrate utilization, growth in triple sugar iron agar slants and urease production were performed to identify isolates as per standard protocol (Holt et al., 1994).

Detection of virulent toxin genes in B. bronchiseptica by PCR

A single colony of B. bronchiseptica was pulled from blood agar plate, suspended in 100 μL of Milli-Q water, gently vortexed and boiled approximately at 100°C for 10 min in water bath. The cell debris was removed by centrifugation at 10,000 rpm for 5 min at 4°C and the top clear supernatant was used as source of template DNA. The PCR amplification was carried out in a iCycler (BioRad, USA) in 25 μL reaction volume containing 12.5 μL of 2X PCR master mix [4 mM MgCl2; 0.4 mM of each dNTPs (dATP, dCTP, dGTP, dTTP); 0.5 units/μL of Taq DNA polymerase; 150 mM Tris-HCl PCR buffer (pH 8.5)]; 0.5 μM of each (Forward and Reverse) primers and 2.5 μL of template DNA.

Primers for filamentous hemagglutinin (fhaB and fhaC) toxin genes were used from the published sequences of Dubuissson et al., (2000). The primers for fimbrial subunit genes (fim2 and fim3) were designed from the gene sequences available from EMBL gene bank by using primer3 software. The designed primers were checked for their complimentarity by using the Neucleotide-neucleotide BLAST (blastn) program. After evaluation, the primer pairs for fhaB, fhaC, fim2 and fim3 genes were commercially synthesized (Clontec, USA).

The specificity of designed primer pairs was tested by screening some common bacterial species related to the respiratory tract namely, Pasteurella multocida, Staphylococcus aureus and Streptococcus spp. The detailed PCR primers used in this study are listed in Table 1. After initial denaturation at 94°C for 5min, the amplification cycle had denaturation at 94°C, annealing [46°C (fhaB), 55°C (fhaC), 59°C (fim2) and 59°C (fim3)] and extension at 72°C for 1 min each respectively with a repeat of 35 cycles. Final extension was done for 10 min. Bordetella bronchiseptica strain (ATCC®4617™), procured from Himedia biosciences, Mumbai) was used as positive control. Laboratory maintained P. multocida isolate (Division of Animal Health, ICAR complex for NEH region, Umiam, Meghalaya) was used as negative control.

Table 1: Details of PCR primers for detection of fhaB, fhaC, fim2 and fim3 genes of Bordetella bronchiseptica.

<table>
<thead>
<tr>
<th>Toxin genes</th>
<th>Primer sequences 5'–3'</th>
<th>Primer concentration (μM each)</th>
<th>Amplicon size (bp)</th>
<th>Reference/Gene bank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>fhaB</td>
<td>For 5'-ggaaaattctggaatcctggcgc-3'</td>
<td>320</td>
<td>1767</td>
<td>Dubuissson et al. (2000), (AF111796)</td>
</tr>
<tr>
<td></td>
<td>Rev 5'-gggttgaactctcgcacggg-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fhaC</td>
<td>For 5'-atgactgcagcaaacagttccc-3'</td>
<td>0.5</td>
<td>301</td>
<td>Dubuissson et al. (2000), (AF111794)</td>
</tr>
<tr>
<td></td>
<td>Rev 5'-ggtcttcgcccggctcgccagaaactg-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fim2</td>
<td>For 5'-tcaccatctcagttccttc-3'</td>
<td></td>
<td>454</td>
<td>Present study, (X74119)</td>
</tr>
<tr>
<td></td>
<td>Rev 5'-ggctgagtaagcagttcacc-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fim3</td>
<td>For 5'-tgcccaagtctcaagagaac-3'</td>
<td></td>
<td></td>
<td>Present study, (X74120)</td>
</tr>
<tr>
<td></td>
<td>Rev 5'-cagggatatagcgcagggaaaa-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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The PCR amplicons (5μL) were separated by electrophoresis in 1.5% Agarose (Promega, USA) gel with TAE 1X (Tris-Acetate-EDTA; pH 8.0) running buffer at 60V. The gel was stained with 0.4 μg/mL ethidium bromide (Pharmacia Biotech), visualized and photographed in gel documentation system (Image Master® VDS, Pharmacia Biotech, Sweeden).

RESULTS

Isolation and identification of B. bronchiseptica

On sheep blood agar, bacterial colonies were found to be very small, light white, round, domed shape and hemolytic. The colonies increased in size after 48 h of incubation. Bacteria were observed to be gram negative, motile small rods, able to grow on MacConkey agar and positive for citrate, oxidase, catalase, nitrate and urease, with no reaction at all in the butt of a triple sugar iron agar slant were identified as B. bronchiseptica. Upon detailed bacteriological investigation from 18 dead animals and 50 nasal swabs from live ailing pigs, 47 B. bronchiseptica were isolated and identified. No B. bronchiseptica could be isolated from healthy (control) animals.

Detection of virulent toxin genes in B. bronchiseptica by PCR

All the major virulent genes of B. bronchiseptica could be detected by PCR analysis. The primer pairs used in the PCR analysis amplified the desired amplicon size from all the 47 B. bronchiseptica isolates. All the B. bronchiseptica isolates produced an amplicon sizes of 320 bp, 1767 bp, 301 bp and 454 bp respectively, representing fhaB (Figure 1), fhaC (Figure 2), fim2 (Figure 3) and fim3 (Figure 4) genes. Specificity of the primer was confirmed, as there was no amplification of any product when DNA templates from Pasteurella multocida, Staphylococcus aureus, Streptococcus spp, were used.

DISCUSSION

Meghalaya is a poorly developed state in the remote North Eastern region of India. Recent Animal Husbandry initiatives have led to the establishment of swine industry as a profitable enterprise and the farmers are greatly dependent on this. But the success has badly been thwarted by the disease occurrence, the result of which is severe economic losses to the farmers. Farmers often report outbreaks due to infectious diseases affecting the upper respiratory tract of pigs and practice sacrificing of the affected pigs for immediate disposal in the open market to reduce further loss.

Scientific management, breeding and prevention of various diseases affecting pigs are the three pillars on which success of pig husbandry depends. Of late, due to importation of some improved breeds of pigs from Europe to this region, the problem of pig diseases likely to precipitate further. So far, no systematic study was undertaken to know the prevalence of bronchopneumonic conditions, the causative organisms, diagnosis and control in the state. Onpreliminary studies, Shome et. al., (2006) found the involvement of B. bronchiseptica exclusively as the causative agent responsible for atrophic rhinitis in pigs in Meghalaya based on isolation and phenotypic characteristics of the isolates. A perusal of literature showed rare reportings of PCR based detection of virulent
genes especially for *B. bronchiseptica*. Hozbor et al., (1999) developed a PCR that enabled not only discriminative detection of three *Bordetella* species, *B. pertussis, B. parapertussis,* and *B. bronchiseptica* (Bspp PCR), but also specific detection of *B. bronchiseptica* (Bb PCR). Pajuelo et. al., (2002) identified a *B. bronchiseptica* strain isolated from AIDS patient by analyzing the isolate for the presence of *B. bronchiseptica* specific DNA sequences of 600 bp DNA fragment encompassing the linker-encoding sequences and some of the transmitter-encoding sequences of *bvgS* gene by polymerase chain reaction.

**CONCLUSION**

The PCR described in the present study may prove to an improvement of the present methods for surveillance of bordetellosis and may provide a more accurate means for the diagnosis of *B. bronchiseptica* especially from India.

**ACKNOWLEDGEMENT**

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