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SHORT COMMUNICATION

Diagnosis of bordetellosis in pigs from North East India by PCR

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ABSTRACT

Aims: *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.
Methodology and Results: 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 hemagglutinin toxin genes (*fha*B and *fha*C) and fimbrial toxin genes (*fim*2 and *fim*3) primers to identify the specific toxin types of *B. bronchiseptica*. All the 47 isolates were positive for all the toxin genes. The specifity 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 indicate the amplicons are highly stable.
Conclusion, significance and impact of study: 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: Bronchoneumonia, *Bordetella bronchiseptica*, Toxin gene, PCR

INTRODUCTION

Bordetella bronchiseptica are small, aerobic, non-spore forming Gram-negative, pleomorphic coccobacilli 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

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Table 1: Details of PCR primers for detection of *fhaB, fha*C*, fim*2 and *fim*3 genes of *Bordetella bronchiseptica.*

Toxin genes	Primer sequences 5'-3'	Primer concentration (μM) each	Amplicon size (bp)	Reference/Gene bank accession No.
*fha*B	For 5′ - ggaaaattctgaattcccgcgc - 3′ Rev 5′ - cggtggaattctcgctcacgg - 3′	0.5	320	Dubuisson *et. al.,* (2000), (AF111796)
*fha*C	For 5′ - atgactgacgcaacgaaccgtttcc - 3′ Rev 5′ - gcgttctcgccgggctcagaaactg - 3′		1767	Dubuisson *et. al.,* (2000), (AF111794)
*fim*2	For 5′ - tacccatgcaagtccctttc - 3′ Rev 5′ - ggctcgaagtacgctttcac - 3′		301	Present study, (X74119)
*fim*3	For 5′ - tgcccaagatttccaagaac - 3′ Rev 5′ - cagggatagacgacggaaaa - 3′		454	Present study, (X74120)

rapid, sensitive and specific detection. The major virulence factors of *B. bronchiseptica* include adhesins namely filamentous haemagglutinin (*fha*), fimbriae (*fim*2*, fim*3*, fim*X *and fim*A) and pertactin (*prn*), and exotoxins as bifunctional adenylate cyclase haemolysin (*achly*), dermonecrotic toxin (*dnt*) and tracheal colonization factor (*tcf*A) (Cotter and Miller, 2001; Shina *et. al*., 2002). In the present study the published primer sequences namely *fha*B and *fha*C and primers designed namely fimbrial subunit genes (*fim*2 and *fim*3) were evaluated for detection of major virulent toxin genes in *B. bronchiseptica* isolates from North East India.

MATERIALS 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 piggery 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 (control) during slaughtering were scientifically processed for microbiological investigation.

Isolation and biochenical identification of *B. 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, Simmon'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 $MgCl_2$; 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 (*fha*B and *fha*C) toxin genes were used from the published sequences of Dubuisson *et. al*., (2000). The primers for fimbrial subunit genes (*fim*2 and *fim*3) 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 *fha*B, *fha*C, *fim*2 and *fim*3 genes were commercially synthesized (Clonitec, USA).

The specifity 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 amplication 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. *B. bronchiseptica* strain (ATCC®4617™, procured from Himedia biosciences, Mumbai) was used as positive control. Laboratory maintained *P. multocida* isolate

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(Division of Animal Health, ICAR complex for NEH region, Umiam, Meghalaya) was used as negative control.
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 *fha*B (Figure 1), *fha*C (Figure 2), *fim*2 (Figure 3) and *fim*3 (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

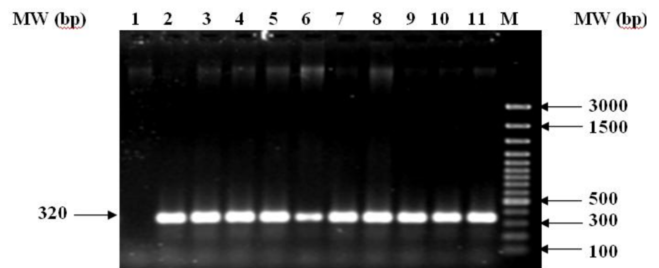

Figure 1: PCR detection of *fha*B toxin gene of *B. bronchiseptica*; lane 1: negative control (*P. multocida*); lanes 2: Positive control *B. bronchiseptica* (ATCC®4617™) strain; lanes 3-11: *B. bronchiseptica* field isolates showing fragment (320 bp) of *fha*B toxin gene; M: 100 bp DNA ladder mix (MBI Fermentas)

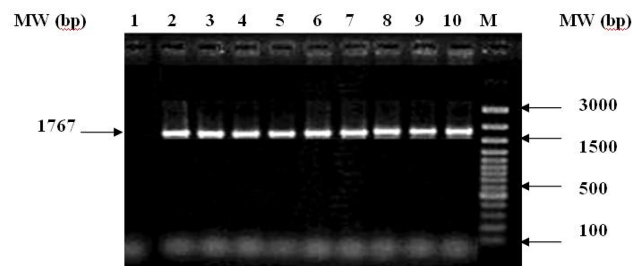

Figure 2: PCR detection of *fha*C toxin gene of *B. bronchiseptica*; lane 1: negative control (*P. multocida*); lanes 2: Positive control *B. bronchiseptica* (ATCC®4617™); lanes 3-10: *B. bronchiseptica* field isolates showing fragment (1767 bp) of *fha*C toxin gene; M: 100 bp DNA ladder mix (MBI Fermentas)

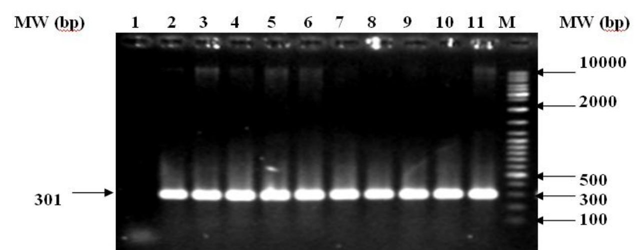

Figure 3: PCR detection of *fim*2 toxin gene of *B. bronchiseptica*; lane 1: negative control (*P. multocida*); lanes 2: Positive control, *B. bronchiseptica* (ATCC®4617™) strain; lanes 3-11: *B. bronchiseptica* field isolates showing fragment (301 bp) of *fim*2 toxin gene; M: 100 bp DNA ladder mix (MBI Fermentas)

undertaken to know the prevalence of bronchopneunomonic 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

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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.

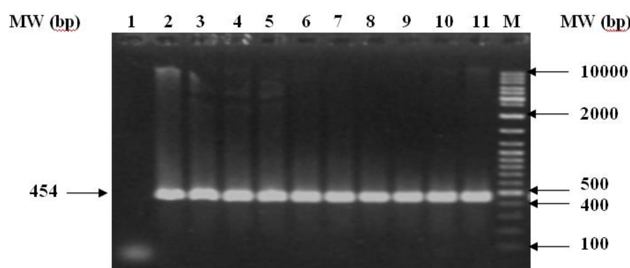

Figure 4: PCR detection of *fim*3 toxin gene of *B. bronchiseptica*; lane 1: negative control (*P. multocida*); lanes 2: Positive control, *B. bronchiseptica* (ATCC®4617™) strain; lanes 3-11: *B. bronchiseptica* isolates showing fragment (454 bp) of *fim3* toxin gene; M: 100 bp DNA ladder mix (MBI Fermentas)

PCR based detection of pathogens by amplification of specific DNA sequences theoretically allows the detection of single bacterium. The detection of *fhaB, fhaC, fim2 and fim3* genes suggests the presence of major attachment factors in all the *B. bronchiseptica* isolates. In the present study, the polymerase chain reaction using the primer pairs for *fha*B and *fha*C (published primer sequences) and *fim*2 and *fim*3 (designed primer sequences) showed high specificity as none of the products or any other amplicons appeared when same primers were used against *Pasteurella multocida*, *Staphylococcus aureus* and *Streptococcus spp.* as common respiratory pathogen. The PCR method described and the primer pairs used in this study might be used in the identification as well as the detection of major virulent genes in *B. bronchiseptica* especially for Indian isolates.

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.

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