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# Isolation and the Analysis of 16S rDNA Sequence of Swine *Bordetella bronchiseptica*

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**Abstract:** A strain named SCBI-1, isolated from a sick pig was diagnosed by cultural characteristics and morphologic observation with its pathogenicity studied by mice virulent experiment and was accurately identified by biochemical assay and the analysis of 16S rDNA sequence. Drug resistance was analysed by sensitive assay. Homology of the 16S rDNA between SCBI-1 and *Bordetella bronchiseptica* is 99.3-99.9%, the study successfully isolated a strain of *Bordetella bronchiseptica* which could lead a mouse to death within 16 h and is resistant to penicillin, rifampicin, clindamycin, ampicillin and cefalotin.

**Key words:** Swine *Bordetella bronchiseptica*, biochemical assay, 16S rDNA, sensitive assay, penicillin, mouse

## INTRODUCTION

*Bordetella bronchiseptica*, the Gram-negative bacterial pathogen and the etiologic agent of upper respiratory infections in a wide range of mammalian hosts, is associated with a number of veterinary syndromes such as atrophic rhinitis and pneumonia in pigs (Burgos *et al*., 2010; Sukumar *et al*., 2010; Nicholson *et al*., 2009). Atrophic Rhinitis (AR) is divided into non-progressive AR which is caused by *B. bronchiseptica* and progressive AR which is caused by toxigenic strains of *Pastrurella multocida* (Giles, 1986). B. *bronchiseptica* could produce the dermonecrotic toxin which induces mucosal damage in swine nasal tissue and causes turbinate atrophy and pneumonic lesions characterized by necrosis, hemorrhage, neutrophil accumulation and eventually brosis (Brockmeier and Register, 2007). Meanwhile, it could infect humans, especially people who have weak immune system such as AIDS patient which highly attracts international attention (Valencia, 2004).

A disease characterized by cough, difficult breath, necrosis, hemorrhage and pneumonia occurred in a pig farm in Mingshan of Sichuan province. The lung and liver tissue samples of a swine herd which had the clinical signs and gross pathological lesions were collected sterilely for pathogenic detection.

The current research was to isolate a suspicious strain of *B. bronchiseptica* from a representative sick pig featured by the clinical signs and gross pathological lesions and identify the isolated strain based on morphology, chemical characteristics and genomic sequence analysis.

## MATERIALS AND METHODS

**Bacterial isolate and culture conditions:** The lung and liver tissue samples of a swine herd which had clinical signs and gross pathological lesions were collected sterilely for pathogenic detection. The samples were firstly grown on Tryptose Soya Agar (TSA) plates supplemented with 5% fetal calf serum (Gibco) and 0.01% Nicotinamide Adenine Dinucleotide (NAD). Plates were incubated at 37°C in 5% $CO_2$ for 24-48 h. Colony, characterized by smooth, moist, semi-transparent and dew size were extracted to purification cultivation. The isolated and purified suspicious strain, named after SCBI-1. The pure culture was kept for futher study.

**Cultural characteristics assay:** The isolates were respectively cultivated on Luria-Bertani plates, MacConkey agar plates and blood agar plates at 37°C in 5% $CO_2$ for 24-48 h. Growth situation on different plates were observed and recorded.

**Morphologic observation:** To observe the morphology of the isolates, a solo purified strain was extracted to Gram stain.

**Virulence test in mice:** The project was supervised and supported by China Animal Protection Association. Twelve mice (each weighted about 20 g) were randomly divided into three groups named after A-C. The three groups were respectively injected with 0.2 mL bacterium suspension using PBS to dilute to $10^9$ CFU $mL^{-1}$, 0.2 mL Luria-Bertani broth without any bacterium and 0.2 mL PBS. The three groups were raised separately with careful

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observation. The dead mice were instantly dissected, pathological variation was recorded. The liver and lung tissue of the dead mice were sampled sterilely to identify the pathogens.

**Biochemical assay:** Glucose, mannose, sucrose, lactose, arabinose, maltose, sorbitol, MR, indole, oxidase, catalase and citric acid were used to test the biochemical characteristics of SCBI-1.

**PCR amplification of *16S rDNA* gene:** Total genomic DNA was extracted by boiling method and supernatant was used for PCR amplification. The *16S rDNA* gene expected to 1500 bp was amplified using the universal primers, forward: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse: 5'-GGTTACCTTGTTACGACTT-3' (Bal and Bal, 2012). PCR was performed in a 20 μL reaction mixture containing: 1.0 μL of DNA template, 10.0 μL of 2×Taq PCR Master Mix, 8.0 μL of $ddH_2O$, 0.5 μL of each primer. Amplification conditions were as follows: an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 35 sec annealing at 55°C for 40 sec, polymerization at 72°C for 1.5 min and a final extension at 72°C for 7 min. Amplified bands were separated by gel electrophoresis (1% agarose gel) and visualized on a gel documentation system. According to the manufacturer's instructions, the QIAquick Gel Extraction kit was used to collect PCR products which were subsequently connected with pMD19-T simple Vector. The ligation reaction mixture were transferred into DH5α competent cells.

**DNA sequencing and homology analysis:** Plasmid Extraction kit was used to extract recombinant plasmid. The positive recombinant plasmid were sent to Takara for sequencing. Sequences were compared to the sequences available in the GenBank database using BLAST. Then, homology of sequences was analysed and a phylogenetic tree was constructed by DNAStar Software.

**Drug sensitive assay:** Ceftriaxone, cefotaxime, cefuroxime, norfloxacin, SMZco, cefazolin, cefoperazone, cephaloridine IV, vancomycin, gentamycin were employed to test the sensitivity of the purified strains about medicines judging by diameter inhibition zone.

## RESULTS AND DISCUSSION

**Cultural characteristics, morphology and virulence:** SCBI-1 could grow on Luria-Bertani plates, MacConkey agar plates and blood agar plates. Meanwhile, SCBI-1 is a Gram-negative, characterized by single, consistent morphology without capsule, flagella and spore nearly roundness and dyeing both of ends. Furthermore, mice challenged with SCBI-1 were dead within 16 h however, the other two groups were normal. The bacterium corresponding to isolates were isolated from the dead mice which indicated that SCBI-1 is virulent.

Table 1: The results of biochemical assay

| Reagents | Results | Reagents | Results | Reagents | Results |
|---|---|---|---|---|---|
| Glucose | - | Mannose | - | Sucrose | - |
| Lactose | - | Arabinose | - | Maltose | - |
| Sorbitol | - | MR | - | Indole | - |
| Oxidase | + | Catalase | + | Citric acid | + |

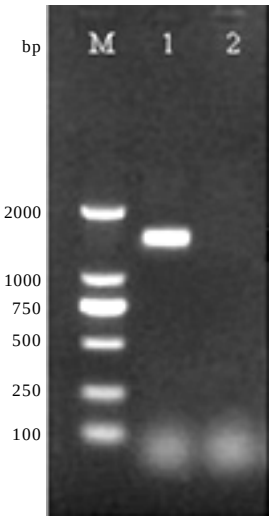


Fig. 1: Electrophoresis figure of PCR product. M: Molar mass marker; 1: PCR product; 2: Negative contrast

**Biochemical assay:** SCBI-1 did not ferment and decompose any carbohydrates. MR and indole test were negative. Oxidase, catalase and citric acid test were positive (Table 1). It accorded with *B. bronchiseptica* in Bergey's manual of systematic bacteriology (Dong and Cai, 2001).

**PCR amplification of *16S rDNA* gene:** The PCR was successfully amplified an amplicon as predicted size 1500 bp (Fig. 1).

***16S rDNA* gene sequencing and homology analysis:** According to the BLAST, the homology of the 16S rDNA sequence between SCBI-1 and Bordetella is high. Further comparision between SCBI-1 and two Bordetella and several gram negative bacterium via DNAStar Software indicates that the identity of the 16S rDNA sequence between SCBI-1 and *Bordetella bronchiseptica* is highly 99.9% while others is lower (Table 2).

Table 2: The homologous comparision of the *16S rDNA* gene among SCBI-1 and two *Bordetella* and several gram negative bacterium

| Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | 99.4 | 74.4 | 73.9 | 74.4 | 77.5 | 75.9 | 72.9 | 76.4 | 77.5 | 72.6 | 72.9 | 74.7 | 73.9 | 99.9 |
| 2 | 0.0 | - | 72.8 | 72.3 | 73.3 | 76.4 | 75.2 | 72.3 | 76.6 | 76.4 | 71.6 | 71.6 | 73.8 | 73.6 | 99.3 |
| 3 | 21.2 | 21.5 | - | 99.5 | 85.6 | 85.8 | 92.0 | 92.7 | 84.9 | 85.1 | 93.0 | 93.0 | 84.9 | 83.6 | 72.2 |
| 4 | 21.3 | 21.6 | 0.1 | - | 85.2 | 85.4 | 91.5 | 92.2 | 84.5 | 84.7 | 92.5 | 92.5 | 84.5 | 83.1 | 68.4 |
| 5 | 20.7 | 21.3 | 12.9 | 12.9 | - | 98.6 | 85.6 | 84.6 | 94.2 | 95.3 | 84.9 | 85.2 | 97.1 | 97.0 | 75.7 |
| 6 | 20.3 | 20.8 | 12.6 | 12.6 | 0.7 | - | 86.0 | 85.4 | 93.6 | 95.7 | 85.6 | 85.9 | 96.3 | 96.4 | 71.9 |
| 7 | 20.6 | 20.9 | 6.5 | 6.6 | 13.0 | 12.5 | - | 98.8 | 85.0 | 85.1 | 93.6 | 93.6 | 85.6 | 86.0 | 74.0 |
| 8 | 20.8 | 21.0 | 6.5 | 6.7 | 12.9 | 12.4 | 0.5 | - | 84.9 | 85.0 | 94.3 | 94.4 | 84.6 | 85.0 | 73.8 |
| 9 | 21.7 | 21.0 | 13.1 | 13.3 | 4.6 | 5.3 | 13.5 | 13.4 | - | 97.9 | 84.7 | 83.3 | 95.5 | 95.3 | 77.3 |
| 10 | 20.6 | 21.1 | 13.1 | 13.3 | 3.5 | 4.0 | 13.5 | 13.3 | 1.4 | - | 85.2 | 85.6 | 95.5 | 95.3 | 77.1 |
| 11 | 21.5 | 22.1 | 6.7 | 6.9 | 13.0 | 12.8 | 5.2 | 5.1 | 13.3 | 12.9 | - | 99.9 | 85.1 | 84.4 | 72.7 |
| 12 | 21.2 | 21.9 | 6.7 | 6.9 | 12.7 | 12.4 | 5.1 | 5.0 | 14.5 | 12.7 | 0.1 | - | 85.1 | 84.4 | 72.9 |
| 13 | 20.3 | 20.9 | 13.4 | 13.6 | 2.5 | 3.1 | 13.1 | 13.1 | 3.7 | 3.7 | 13.1 | 13.1 | - | 99.4 | 77.2 |
| 14 | 20.6 | 20.7 | 13.7 | 13.9 | 2.4 | 3.1 | 13.2 | 13.2 | 3.9 | 3.9 | 13.8 | 13.8 | 0.4 | - | 77.4 |
| 15 | 0.1 | 0.1 | 24.9 | 25.1 | 21.5 | 21.3 | 23.1 | 23.6 | 21.2 | 21.5 | 24.7 | 24.5 | 20.6 | 20.6 | - |

1: *B. bronchiseptica*, X57026; 2: *Bordetella bronchiseptica*, NR025949; 3: *Acbnobacillus pleuropneumonia*, AF 03305; 4: *Acbnobacillus pleuropneumonia*, NR044752; 5: *E. coli* EHEC Strains ATCC 43895 Z83205; 6: *E. coli* Strains KCTC 2441. EU014689; 7: *H. parasuis*, FJ667962; 8: *H. parasuis*, FJ667982; 9: *Klebsiella*, DQ 831003; 10: *Klebsiella*, EU545402; 11: *Pasteurella*, FJ405340; 12: *Pasteurella muttocida*, AF224297; 13: *Salmonella enterica*, AF227869; 14: *Salmonella enterica*, AF332600; 15: SCBI-1

Table 3: The result of the sensitive test

| Drug name | Diameter of inhibition (mm) | Results |
|---|---|---|
| Amikacin | 22 | Susceptible |
| Ciprofloxacin | 23 | Susceptible |
| Penicillin | - | Resistance |
| Kanamycin | 10 | Moderately sensitive |
| Rifampicin | - | Resistance |
| Clindamycin | - | Resistance |
| Gentamycin | 17 | Moderately sensitive |
| Levofloxacin | 17 | Moderately sensitive |
| Ampicillin | - | Resistance |
| Cefalotin | - | Resistance |

-: Means no inhibition zone

A phylogenetic tree was constructed based on Clustalx program, revealing that SCBI-1 and *Bordetella bronchiseptica* were on the same branch (Fig. 2). It deduces that SCBI-1 belongs to *Bordetella bronchiseptica*.

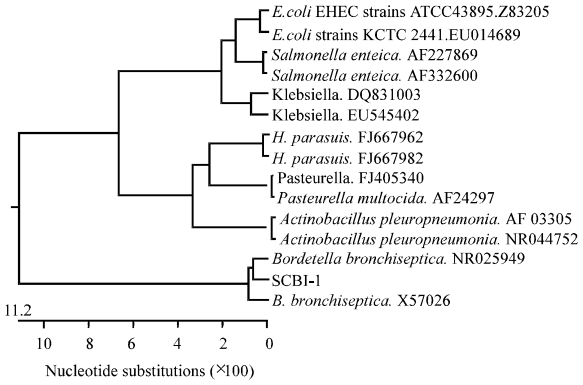


Fig. 2: Dendrogram of genetic relationships among 16S rDNA genotypes of bacteria classified. The tree showed the relationship based on partial sequences of the *16S rRNA* gene of selected clones. The sequence alignment was performed by means of the CLUSTAL X program and the tree was generated by the Neighbor-Joining Method

**Sensitive assay:** Sensitive assay showed that SCBI-1, *Bordetella bronchiseptica* is susceptible to amikacin, ciprofloxacin; moderately sensitive to kanamycin, gentamycin, levofloxacin and resistance to penicillin, rifampicin, clindamycin, ampicillin and cefalotin (Table 3).

The study faced great obstacles because of the small amount of wanted bacterium that grow slowly in pathological tissues and the interference of other microbe which reduced separation rate. However, the research isolated a suspicious strain, named after SCBI-1 from the typical parts with gross pathological lesions. The Gram-negative SCBI-1 could grow on Luria-Bertani plates, MacConkey agar plates and blood agar plates. Gram stain shows that the isolated strain is single, consistent morphology without capsule, flagella and spore nearly roundness and dyeing both of ends. Yet there are some defects that the result of gram stain may be confused with Enterobacteriaceae and it is hard to predict the phylogeny only based on biochemical assay. The *16S rDNA* gene as an operational taxonomic unit (Jinsheng *et al*., 2011) is selected to identify the suspicious strain in this study. The *16S rDNA* gene analysis which provides taxonomic relatedness and an estimate of the evolutionary distance between sequences is widely used to investigate the relationships of prokaryotes (Raje *et al*., 2010).

Based on cultural characteristics, morphology, mice virulent experiment, biochemistry assay and the analysis of 16S rDNA sequence that the homology of the 16S rDNA between SCBI-1 and *Bordetella bronchiseptica* is 99.3-99.9%, the study successfully isolated a strain of *B. bronchiseptica* which is susceptible to amikacin, ciprofloxacin; moderately sensitive to

kanamycin, gentamycin, levofloxacin and resistance to penicillin, rifampicin, clindamycin, ampicillin, cefalotin. It indicates that *B. bronchiseptica* is highly drug-resistant and the more antibiotics are used the stronger drug-resistance is.

## CONCLUSION

Therefore medicines, especially antibiotics should be used scientifically and alternately. Thus, this study offers materials to the exploration of *B. bronchiseptica* diagnostic techniques and vaccines.

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