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RESEARCH ARTICLE

# Comparative Serological and Random Amplified Polymorphic DNA Typing for *Bordetella avium* Isolates in China

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**ABSTRACT**

To study the similarity among *Bordetella avium* isolates in China, antigens and diagnostic antiserum of 22 *B. avium* isolates were prepared for serotyping, and a set of 20 commercially available primers was screened out to identify suitable primers for random amplified polymorphic DNA fingerprinting (RAPD) analysis in this study. Twenty-two *B. avium* isolates were divided into two serovars (A and B) based on their reaction in the plate-agglutination test. Four primers R1, R2, R4 and R10 resulted in informative fingerprints and were used to evaluate the *B. avium* isolates. Based on their RAPD patterns, a dendrogram allowed the separation of the *B. avium* isolates into six genetic similarity clusters. However, no direct correlation was observed between serotypes and RAPD typing among the isolates.

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## INTRODUCTION

*B. pertussis, B. parapertussis, B. bronchiseptica* and *B. avium* are all members of the genus *Bordetella*, which can cause upper respiratory diseases with similar tracheal lesions and outward symptoms (Spears *et al*., 2003). They are close phenotypically, possess common antigens and share a high degree of DNA similarity. Among all the medically important species of *Bordetella*, *B. avium* has the least virulence factors. A phylogenetic analysis based on 16S rRNA genes proved that *B. avium* is most distantly related to all other *Bordetella* species (Sebaihia *et al*., 2006).

*B. avium* is the etiologic agent of coryza or rhinotracheitis in poultry, including turkeys, chickens, finches, budgerigars, noble macaws, Japanese quails and ostriches, etc. (Raffel *et al*., 2002; Odugbo *et al*., 2006). Human cases of respiratory disease associated with *B. avium* have only recently been reported in patients with chronic obstructive pulmonary disease (Spilker *et al*., 2008; Harrington *et al*., 2009).

Many aspects of the biology of *B. avium* have been studied, including colony morphology, biochemical characterization, hemagglutination, dermonecrotic toxin, and tracheal cytotoxin, etc. (Jackwood *et al*., 1991; Gentry-Weeks *et al*., 1992; Kirby *et al*., 2004; Temple *et al*., 2010; Loker *et al*., 2011). Serotyping based on antigen differences is a very useful epidemiologic tool in etiological research; however, it has not been used in *B. avium* isolates. Although a number of molecular methods, such as restriction enzyme analysis (REA), random amplified polymorphic DNA (RAPD) fingerprinting, ribotyping and macro-restriction analysis by pulsed-field gel electrophoresis (PFGE) have been used to study differences in epidemiology among different isolates of bacteria (Wulff *et al*., 2006; Morandi *et al*., 2010), only REA and ribotyping have been used to distinguish isolates of *B.* avium from *B. hinzii* (Sacco *et al*., 2000). Typing and differentiating inter- and intraspecies differences are of great importance for veterinary diagnostic laboratories. Compared to other important members of *Bordetella* (Shin *et al*., 2007), reports regarding serotyping and genetic typing of *B. avium* are scarce.

Previous reports indicated that REA fingerprints depend on the enzyme used, and some fragments were too large to be resolved under the conditions used(Shin *et al*., 2007). Similar conditions were related to PFGE where some isolates could not be typed by PFGE in spite of the formaldehyde step (Leclair *et al*., 2006). As a genotyping method, RAPD has been used to study the genetic relatedness of many other bacteria isolates, but its usefulness in genotyping of *B. avium* remains unknown. Based on the whole genome analysis, the main advantages of RAPD lay in its rapidity and applicability to any organism without prior information on the nucleotide sequence.

The purpose of this study was to evaluate the serological differences through plate agglutination test and genetic diversity among *B. avium* isolates by RAPD analysis, at the same time, to compare the differences between the two methods in classifying *B. avium* isolates in China.

## MATERIALS AND METHODS

**Bacterial isolates:** Twenty-two *B. avium* isolates from different years were evaluated in this study. The origin, source, and date of isolation of them are presented in Table 1. All isolates were identified as *B. avium* using standard methods, including colony morphology, Gram staining, hemagglutination, plate-agglutination test and biochemical tests. They were stored at -70℃ until used.

**Table 1:** *B. avium* isolates included in this study

| Isolates | Time of isolation | Host | Geographic origin |
|---|---|---|---|
| Ba1 | 1991.07 | Chicken | Taian, China |
| Ba2 | 1992.10 | Chicken | Taian, China |
| Ba3 | 1993.09 | Chicken | Taian, China |
| Ba4 | 1994.11 | Chicken | Taian, China |
| Ba5 | 1994.11 | Chicken | Qufu, China |
| Ba6 | 1994.11 | Chicken | Taian, China |
| Ba7 | 2000.12 | Chicken | Feicheng, China |
| Ba8 | 2002.07 | Chicken | Guanxian, China |
| Ba9 | 1992.06 | Pheasant | Taian, China |
| Ba10 | 1992.03 | Duck | Feicheng, China |
| Ba11 | 2001.09 | Chicken | Feicheng, China |
| Ba12 | 2001.05 | Chicken | Jining, China |
| Ba13 | 2002.10 | Chicken | Zibo, China |
| Ba14 | 2004.09 | Chick Embryo | Taian, China |
| Ba15 | 2007.10 | Chick Embryo | Weifang, China |
| Ba16 | 2008.09 | Chicken | Dongping, China |
| Ba17 | 2009.06 | Chick Embryo | Laoling, China |
| Ba18 | 2009.10 | Chick Embryo | Muping, China |
| Ba19 | 2009.10 | Chick Embryo | Weifang, China |
| Ba20 | 2009.11 | Chicken | Heze, China |
| Ba21 | 2010.07 | Chick Embryo | Anqiu, China |
| Ba22 | 2010.09 | Chick Embryo | Pingyuan, China |

**Preparation of antigens:** *B. avium* isolates (22) were grown on the Bordet-Gengou (BG) agar (Difco, USA) plate, containing 5% defibrinated sheep blood, incubated at 37°C for 24 h, then harvested with sterilized phosphate-buffered saline (PBS) solution (pH 7.4) and adjusted to a concentration of $3.0\times10^9$ CFU/ml. Formalin was added to the suspensions to a final concentration of 0.3% and incubated at 37°C for 18 h again. Bacterial cells were pelleted by centrifugation at 6000 r/min for 15 min, and washed 3 times with PBS. After stained with crystal violet, the suspensions were diluted to the original concentration and stored at 4°C.

**Preparation of antiserum:** The 22 *B. avium* isolates were cultured, harvested and inactivated as described above. Antisera against *B. avium* isolates were prepared as described previously (Guo *et al*., 2010). Briefly, 1 ml of formalinized-whole-cell (FWC) suspensions and an equal volume of Freund's complete adjuvant were mixed and injected into rabbits subcutaneously at three sites of the neck and back. Two weeks later, rabbits were subcutaneously given 1 ml of FWC suspensions and an equal volume of Freund's incomplete adjuvant, followed by two times the dose in two weeks. At last 1 ml of FWC suspensions were administered intravenously. Antisera were collected four days after the last injection and named S1, S2, S3…S21, and S22, respectively.

**Serotyping:** Antiserum of each *B. avium* isolate was used to cross react with the 22 culture suspensions in a plate-agglutination test. Based on the titers recorded, all of the antisera were absorbed with an equal volume of whole cells of isolate which had low titers with the antiserum. If the antiserum could still react with their immune isolates but not react with the absorbed culture any more after absorption, they would be named S'1, S'2, S'3…S'21, and S'22 as diagnostic antiserum. They were used to react with 22 isolates in a plate-agglutination test for serotyping.

**RAPD typing:** Bacterial isolates were inoculated into 5 ml of fresh brain heart infusion broth (Difco, USA) and incubated at 37°C for 24 h. Genomic DNA from each isolate was extracted using a commercially available kit (Tiangen, China) according to recommendations of the manufacturer as reported previously (Poorbaghi *et al*., 2012). DNA concentrations were detected and the integrity was checked by electrophoresis in 0.8% agarose gel.

A set of 20 commercially available primers (Oligo 10-mer; Genscript, China) was screened out to identify suitable primers for RAPD analysis of *B. avium* isolates. Primers R1 (TGCCCGTCGT), R2 (CAGGCCCTTC), R4 (ACGACCGACA), and R10 (CCTTGACGCA) resulted in informative fingerprints and were used to evaluate the isolates.

PCR mixtures (25 µl) contained 2.5 µl of 10× enzyme assay buffer, 100 µM each of dATP, dCTP, dGTP, and dTTP, 2 nM of random primer (10bp), 1.5 mM $MgCl_2$ and 1.5 U of Taq DNA polymerases. Template concentrations were standardized. Reactions were carried out in a thermal cycler PCR system FTC-200 (FedBio, England) using the following conditions: an initial denaturation for 10 min at 94°C; 35 cycles of 3 min at 34°C, 1 min at 72°C and 30s at 94°C; followed by a final extension of 10 min at 72°C. Following PCR, 10 µl of the reaction mixture was analyzed by electrophoresis in a 2% agarose gel containing 500 ng/ml ethidium bromide. Marker D2000 was used to determine molecular size. At the same time, a negative control (RAPD assay without DNA) was performed to check for no spurious amplification.

As described previously (Kann *et al*., 2006; Ongor *et al*., 2011; Hryncewicz-Gwóźdź *et al*., 2011), the agarose gels were photographed under UV light using a Bio-Imaging System MiniBis Pro (DNR, Israel). Then the band patterns produced by different primers were analyzed using software Cross Checker and NTSYSpc 2.10e, which were also used to construct a dendrogram of the isolates (Najiah *et al*., 2011).

## RESULTS

**Serotyping:** Extensive cross-reactions were observed among 22 *B. avium* isolates, and the titers varied from 1:100 to 1:4800. Diagnostic antiserum of S′1, S′5, S′10, S′11, S′12, S′13 and S′15 were obtained after absorption. S′1, S′5, S′10 and S′15 reacted with isolates Ba1-Ba10

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and Ba14-Ba22, but not reacted with Ba11, Ba12 and Ba13. The S′11, S′12 and S′13 only reacted with Ba11, Ba12 and Ba13, not with other isolates. Based on the results above, 22 *B. avium* isolates were divided into two serovars: A (Ba1-Ba10 and Ba14-Ba22) and B (Ba11, Ba12 and Ba13).

**RAPD typing:** Both visual and computer analysis showed that all of the 22 *B. avium* isolates tested by RAPD generated clear DNA fragment patterns with primers R1, R2, R4 and R10 (Fig. 1). DNA fragments from different isolates varied from 150 bp to 4000 bp. Based on their RAPD patterns, the percentage of similarity among isolates was determined using the dice coefficient, and the clustering was performed by UPGMA (Fig. 2). The maximum similarity among banding patterns obtained from 22 *B. avium* isolates was 0.95, and the minimum similarity was 0.54. A dendrogram based on the RAPD patterns with primers R1, R2, R4 and R10 allowed the separation of the *B. avium* isolates into genetic similarity clusters. 22 *B. avium* isolates may be allocated into six main clusters (-Ⅵ) with the similarity index between the isolates within the cluster being 0.69 or higher (Fig. 2).

## DISCUSSION

Antiserum used in serotyping was obtained after immunization with the whole cells of 22 *B. avium* isolates. Extensive cross-reactions were observed among 22 *B. avium* isolates. Except its own antiserum, the isolate also reacted with the antisera of the other 21 *B. avium* isolates. It indicated that there were common antigens among 22 *B. avium* isolates as described previously (Sebaihia *et al.*, 2006), however, the numbers of them were not the same. It was further resolved from the titers of the plate-agglutination test that the antigen components among isolates Ba1 to Ba10 and Ba14 to Ba22 were similar, and the antigen components between Ba11 to Ba13 were homologous.

After the antiserum was absorbed by the whole cells of the same *B. avium* isolates which have cross-reaction, the antibodies to common antigens were eliminated. Diagnostic antiserum of S′1, S′5, S′10, S′11, S′12, S′13 and S′15 were obtained at last. These antisera could separate the 22 *B. avium* isolates from different areas into two serotypes, A and B. The plate-agglutination test is an accessible serological test. It is rapid, simple and useful for serotyping *B. avium* isolates. And it can also be conducted in any diagnostic laboratory as described previously (Guo *et al*., 2010).

In order to select suitable candidate primers for genotyping *B. avium* isolates, 20 commercially available arbitrary primers were screened out. Only four primers (R1, R2, R4 and R10) showed informative fingerprints, and were further used to evaluate the isolates. These four different primers yielded 18, 20, 20, and 17 DNA patterns, respectively. The 18 distinct DNA patterns produced by R1 had 24 different DNA bands with an average of 6.5

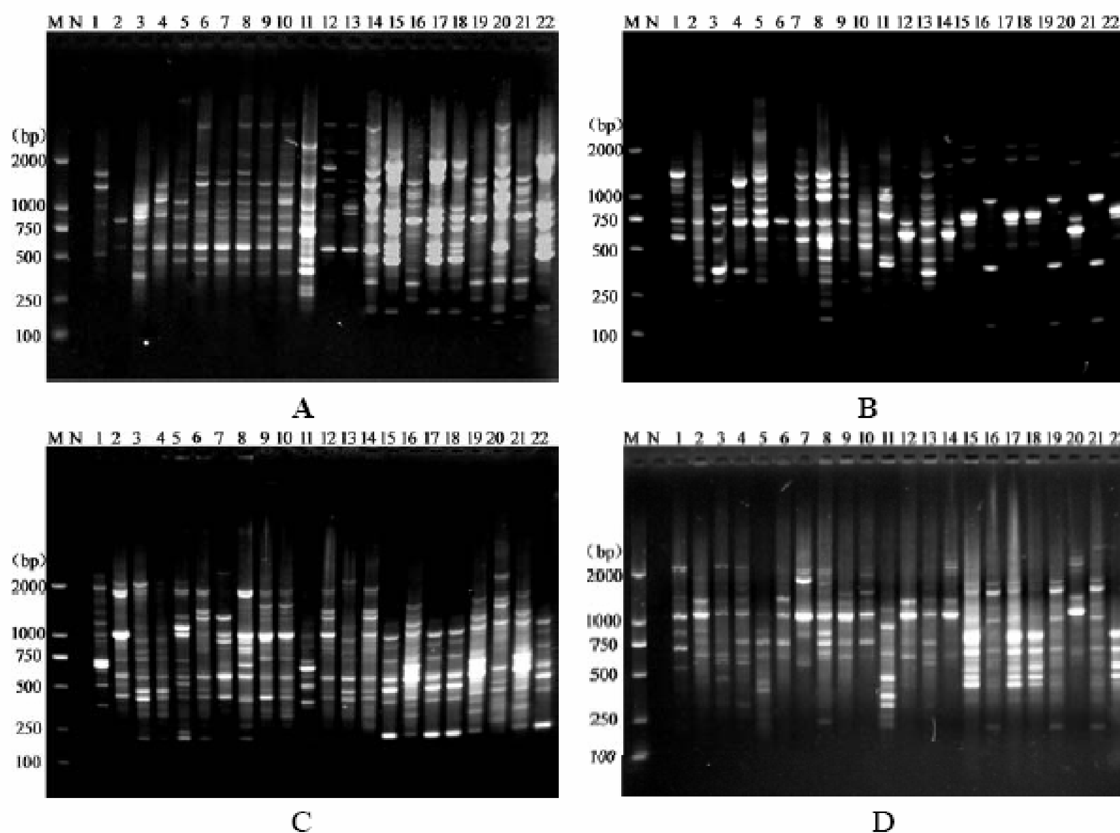


**Fig. 1:** RAPD patterns of 22 *B. avium* isolates generated with four primers: A, RAPD patterns generated with primer R1; B, RAPD patterns generated with primer R2; C, RAPD patterns generated with primer R4; D, RAPD patterns generated with primer R10. Lane M, DNA marker D2000; Lane N, negative control; Lanes 1 to 22, *B. avium* isolates Ba1 to Ba22.

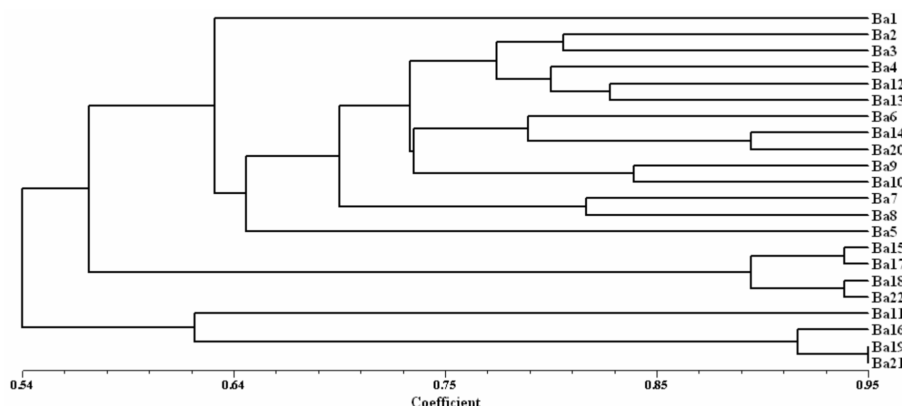


**Fig. 2:** Dendrogram of 22 *B. avium* isolates based on their RAPD patterns. The percentage of similarity among isolates was determined using the dice coefficient, and the clustering was performed by UPGMA.

bands each isolate. The 20 distinct DNA patterns produced by R2 had 23 different DNA bands with an average of 5.96 bands in each isolate. The 20 distinct DNA patterns produced by R4 had 20 different DNA bands with an average of 8.77 bands each isolate, while the 17 distinct DNA patterns produced by R10 had 20 different DNA bands with an average of 5.36 bands in each isolate.

It has been argued that RAPD procedure had poor reproducibility with some primers (Leclair *et al*., 2006). The reason may be due to primers, the criteria for faint bands or different experimental conditions. To confirm the reproducibility of the method, experimental conditions were optimized and remained unchanged when different primers were used. Faint bands were considered present only when the same band was obtained at least three times. Few isolates of those related distantly may vary in different clusters when the analysis was performed with primers lack of polymorphism. Thus, only primers who yield good polymorphism were used for RAPD analysis in our study. Though analysis showed some differences, there was high similarity among them. *B. avium* isolates may be allocated into similar clusters with the index of about 0.7, and the isolates that had close genetic relationships were always in the same cluster. By using these criteria, the method produced highly reproducible results and *B. avium* isolates may be consistently classified into well-defined clusters.

The results of the dendrogram analysis showed that the coefficient of similarity among 22 *B. avium* isolates varied from 0.54 to 0.95. In theory, the same isolate should have specific and stable DNA fingerprinting. Based on multiple random primers of different sequences, it may be possible to evaluate and analyze the whole genomic DNA of bacteria without knowing the exact sequences. The coefficient of similarity would always be 1 even using different primers. In our study, single primer amplification resulted in a situation of similarity index being 1. However, the similarity indexes were all less than 1 based on comprehensive analysis of the four primers used in the experiment. The results indicated that all the 22 *B. avium* cultures were isolated from different sources. Thus, RAPD typing must be based on the combination of profiles produced by multiple primers, not rely on polymorphism analysis of a single primer when it was used to distinguish different bacterial isolates.

We analyzed 22 *B. avium* cultures by RAPD and plate-agglutination test (serotyping), and compared the results from these two methods. In contrast, RAPD analysis reflected the genetic diversity and distance of bacterial culture especially when multiple primers were used. However, analysis based on serological method mainly reflected the changes in antigens. *B. avium* isolates Ba11, Ba12 and Ba13 belonged to serovar B when typed with plate-agglutination test. Though isolates of Ba12 and Ba13 were always in the same cluster when typed by RAPD, Ba11 was only found in another cluster separately. The results revealed that RAPD had no ability to distinguish all *B. avium* isolates existing within two serotypes. There was no direct correlation observed between the RAPD and serological method.

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