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Short communication

Virulence and molecular aspects of *Bordetella avium* isolated from cockatiel chicks (*Nymphicus hollandicus*) in Brazil

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ABSTRACT

Bordetella avium is an opportunistic pathogen that presents tropism for ciliated epithelia, leading to upper respiratory tract disease in turkeys. This agent has also been associated with Lockjaw Syndrome in psittacine birds, but literatures describing the importance of this agent in such species are rare. The purpose of the present study was to report the first outbreak of *B. avium* infection in juvenile cockatiels demonstrating the Lockjaw Syndrome in Brazil and to investigate the antimicrobial resistance profile and phenotypic and genotypic characteristics of these strains. Surprising, the strains obtained from five infected cockatiel chicks from three different breeders from different Brazilian states showed a clonal relationship using the Pulsed Field Gel Electrophoresis and Single Enzyme Amplified Fragment Length Polymorphism techniques. The virulence potentials of the *B. avium* strains were assessed using tracheal adherence and cytotoxic effects on a VERO cell monolayer.

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1. Introduction

Bordetella avium is the etiological agent for bordetellosis or bird rinotracheitis, a disease that has been described in many avian species, including Muscovy ducks, domesticated geese, partridges, turkeys, chickens, ostriches, cockatoos, conures, macaws, parrot finches and cockatiels (Hinz and Glunder, 1985; Clubb et al., 1994; Raffel et al., 2002). The widespread dissemination of this pathogen in both wild and domesticated birds has been demonstrated by a serum prevalence survey, showing that 41 of the 61 species analyzed presented antibodies against *B. avium* (Raffel et al., 2002).

The first description of a bordetellosis outbreak in cockatiel chicks was reported by Clubb et al. from Canada in 1994. The affected birds presented rhinitis, sinusitis and temporomandibular joint rigidity (Lockjaw Syndrome). The clinical signs of *B. avium* infection in cockatiels include anorexia, sneezing, coughing, serous nasal discharge, crop and intestinal dilatation, emaciation, dehydration, a swollen infra-orbital sinus, glassy eyes, vocal alterations and jaw stiffness.

The adhesion and multiplication of *B. avium* in the upper respiratory tract promotes the chronic active inflammation of the nasal cavity, infra-orbital sinuses and lachrymal glands, with hyperplasia of the mucous membrane, and a loss of cilia and squamous metaplasia. Heterophilic tracheitis might occur, and, in some birds, the

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infection may be extended to the lungs, leading to bronchitis and pneumonia (Clubb et al., 1994; Scott et al., 2001). The inflammation of the skeletal muscle and submandibular edema (myositis) associated with perineuritis and osteomyelitis affect the jaw muscle and cranial bones, leading to ''lockjaw'' (Scott et al., 2001).

No previous studies reporting the occurrence of this agent in Brazilian wild or exotic psittacine birds have been reported. In the present study, the strains of *B. avium* from three outbreaks of bordetellosis in juvenile cockatiels in Brazil were characterized by means of phenotypic and genotypic methods.

2. Materials and methods

2.1. Birds

Five cockatiel chicks (*Nymphicus hollandicus*) originating from three different breeders in Brazil were examined. Birds 1 and 2 were from breeder A (São Paulo), bird 3 was from breeder B (Mato Grosso), and birds 4 and 5 were from breeder C (Mato Grosso). The birds died after exhibiting the clinical signs and were subjected to necropsy and the collection of material for a bacteriological examination. The birds presented temporomandibular rigidity (''Lockjaw Syndrome''), anorexia, sneezing, coughing, serous nasal discharge, emaciation, dehydration and swollen infraorbital sinuses. Swabs from the air sacs, trachea and coana were collected and maintained in Amies transport media until processing.

The LSS BA 2509 *B. avium* strain isolated from turkeys (*Meleagris gala pavo*) with airsacculitis, from the Swine Health Laboratory (FMVZ-USP) collection, was used as a positive control in all of the tests.

2.2. Culture and identification of B. avium

The swabs collected were cultured in blood agar base with 5% of defibrinated sheep blood, MacConkey agar and Smith-Baskerville agar (Difco/BBL, Detroit, MI, USA), at 37 °C under aerobic conditions for 24–48 h. After the incubation period, two colonies showing a suggestive morphology from each bird examined were subjected to biochemical tests and the polymerase chain reaction (PCR) technique. All of the isolates were stored at −80 °C until further analysis.

2.3. DNA isolation and PCR amplification

Purified DNA was recovered according to the Boom et al. (1990) protocol of DNA extraction and stored at −20 °C.

The strains were confirmed as *B. avium* through PCR with the primers N-avium (GCGGCGTCAACACATACTCTT-GAT) and C-avium (AGGGAGGTCAGATAGCTCTAGAAT), as described by Savelkoul et al. (1993).

The amplification was performed in a 50 μL reaction mixture, containing 20 ng DNA template, 1.5 mM/L $MgCl_2$, 0.2 mM/L each dNTP, 0.4 μM each primer, 1 U Taq DNA polymerase (LGC Biotecnologia, São Paulo, Brazil), 1× PCR buffer, and ultrapure water. The PCR was performed in 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. The amplified products were subjected to electrophoresis through 1.5% agarose gels, stained with BlueGreen® (LGC Biotecnologia, São Paulo, Brazil), and identified by means of a 100 bp DNA ladder. The strains presenting the 520 bp band were confirmed as *B. avium*.

2.4. Pulsed-field gel electrophoresis (PFGE)

The extraction of the genomic DNA was performed according to Chang and Chui (1998). The digestion of the genomic DNA was performed using 30 U of restriction endonuclease *Xba*II at 30 °C for 24 h. The electrophoresis was performed with 1% Seakem Gold Agarose (Cambrex Bio Science Rockland, Inc., NJ, USA) using the CHEF-DR III System (Bio-Rad Laboratories, CA, USA) in 0.5× TBE at 12 °C. The DNA fragments were separated at 6 V/cm, with pulse times from 0.5 to 40 s for 24 h. The gel was stained with 1× Sybr®Safe (Invitrogen Corporation, CA, USA) for 40 min and photographed under UV transillumination. The DNA fragments were identified by means of the Lambda DNA-PFGE marker (New England BioLabs Inc., Ipswich, MA, USA).

2.5. Single-enzyme amplified length polymorphism typing (SE-AFLP)

A single enzyme AFLP protocol was performed according to McLauchlin et al. (2000), and the electrophoresis was conducted through 1.5% agarose gels at 22 V for 24 h. The amplified products were stained with Blue Green® (LGC Biotecnologia, São Paulo, Brazil) and then compared to a 100 bp DNA ladder.

2.6. Antimicrobial susceptibility

Antimicrobial susceptibility tests were performed employing the Kirby Bauer technique, compliant with the standardized protocol established in the M31-A3 Document (CLSI, 2009). The antimicrobial agents tested were ampicillin (AMP), amoxicillin (AMO), ceftiofur (CFT), cotrimoxazole (COT), enrofloxacin (ENO), norfloxacin (NOR), ciprofloxacin (CIP), erythromycin (CIP), florfenicol (FLOR), lincomycin (LIN), penicillin (PEN), sulfadimethoxine (SUL), and tetracycline (TET). The reference strains tested as the quality controls for the panels were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213.

2.7. Statistical analyses

The levels of relatedness of the isolates were determined using the Dice coefficient with Bionumerics 6.6 software (Applied Maths NV, Sint-Martens-Latem, Belgium). For the PFGE analysis, the isolates were considered as part of different pulsotypes when differing by four or more bands. For the SE-AFLP, the different subtypes had to show less than 90% similarity (Van Belkum et al., 2007).

2.8. Hemagglutination assay

A hemagglutination assay using guinea pig erythrocytes was conducted as described by Temple et al. (1998), but the strains were cultured on Colonization Factor Agar (CFA) at 37 °C for 24 h as described by Knöbl et al. (2006).

2.9. Tracheal attachment assay

A tracheal attachment assay was conducted as described by Knöbl et al. (2006) using the tracheal rings (transverse 2 mm fragments) obtained from one euthanized cockatiel chick (*N. hollandicus*). The bacterial strains were grown overnight on Smith-Baskerville at 37 °C for 24 h.

The adherence test was performed by depositing 100 μL of a bacterial suspension (2×10^7 UFC/mL) into each well of 24-well microplates (Corning, MA, USA); the plates were incubated at 37 °C for 2 h. The rings were washed six times with 50 mM PBS (pH 7.4) and incubated overnight. The washed rings were formalin-fixed, paraffin-embedded and stained with Giemsa for examination by light microscopy.

2.10. Cell culture assay

The bacterial culture supernatant was obtained from the bacterial strains growing in Luria Bertani Broth (Difco-BBL, Detroit, MI, USA) at 37 °C for 18 h with shaking. The culture was centrifuged at $8800 \times g$ for 30 min, and the supernatant was filtered through a 0.22 μm pore polyvinylidenedifluoride membrane (Millipore, Barueri, Brazil).

The test was conducted after the inoculation of 50 μL into the microplate wells where VERO cells monolayer (monkey kidney cell) had been cultivated. The morphological changes of cells were examined using a phase-contrast microscope after the addition of the culture supernatant (Ohnishi et al., 2008).

3. Results

All ten of the tested strains were positive for the *B. avium*-specific PCR, confirming the biochemical tests results. Using PFGE, all of the cockatiel chick strains showed the same pulsotype, with 20 bands and DNA fragment sizes varying from 10 to 250 kb. By using SE-AFLP, it was also possible to observe only one genotype: the SE-AFLP revealed six to nine bands, with sizes varying from 400 to 1300 bp. Despite the clonal relationship observed among the *B. avium* strains from the cockatiel chicks, the turkey strain LSS BA 2509 showed less than 70% similarity when compared with these strains, as determined using PFGE and SE-AFLP (Fig. 1).

All of the strains from the cockatiel chicks were sensitive to ampicillin, amoxicillin, penicillin, ceftiofur, enrofloxacin, norfloxacin, ciprofloxacin, erythromycin, florfenicol and cotrimoxazole. The resistance to lincomycin and sulfadimethoxine were common to all of the isolates, and four strains showed resistance to tetracycline. The resistance profile of the turkey isolate is presented in Fig. 1.

The phenotypic assays showed that all of the strains presented guinea pig hemagglutination and were also able to adhere to the tracheal rings, binding to the cilia and non-ciliated cells (mainly connective tissue). Some of the tracheal tissue had marked damage after the incubation period, with the disruption of the mucosal architecture. The morphological changes observed in the VERO cells after the addition of the supernatant culture between 18 and 24 h after inoculation suggest that all of the strains produce a cytotoxin (Fig. 2).

4. Discussion

This is the first report of *B. avium* infection in Brazil and the first use of the PFGE and SE-AFLP methods to characterize this species genotypically. Previously, Sacco et al. (2000) and Register et al. (2003) have described the genotypic characterization of *B. avium* strains from turkeys using ribotyping and REA.

Although the cities of São Paulo and Cuiaba are separated by a distance of 1600 km, the results of the PFGE and SE-AFLP analyses showed a clonal relationship among the strains studied, suggesting a common origin. As these birds are exotic, the matrices of different breeders may have the same origin, which could explain the clonal relation of the strains and the introduction of the bacteria to flocks. Unfortunately, the service of animal health protection in Brazil includes only notifiable diseases, such as Newcastle disease, Influenza virus, *Salmonella* and *Mycoplasma* infections.

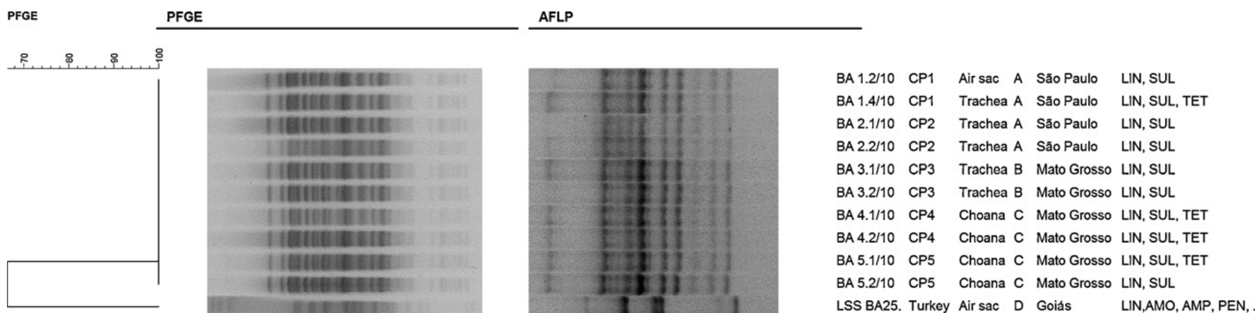

Fig. 1. Dendrogram showing the PFGE and SE-AFLP analyses of *B. avium* isolated from cockatiel chicks (*Nymphicus hollandicus*) the origin data and resistance profile.

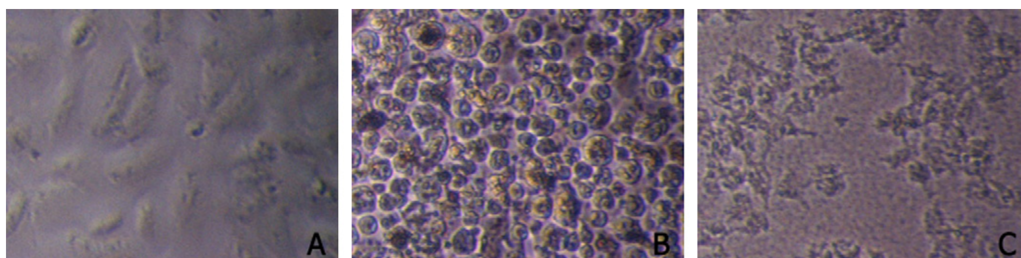

Fig. 2. VERO cells under the effects of *Bordetella avium* culture supernatants. (A) Negative control (uninoculated Luria Bertani broth): unchanged monolayer after 18 h. (B) Monolayer after an 18-h challenge: the cells became round shaped with pyknotic-like nuclei. (C) Monolayer after a 24-h challenge: the cytopathogenic effect (magnification: 100×).

The strains analyzed in this study were susceptible to most of the antimicrobial agents tested, with the exception of tetracycline, lincomycin and sulfadimethoxine. These data are in accordance with the results obtained by Mortensen et al. (1989) who evaluated the antimicrobial susceptibility of *B. avium* and *B. bronchiseptica* isolates. The turkey isolate showed a wide spectrum of antimicrobial resistance that was comparable to that of the cockatiels isolates, most likely due to the greater selective pressure from the antimicrobial use in commercial flocks.

The virulence factors for *B. avium* included fimbriae and the presence of hemagglutinin and tracheal cytotoxin, dermonecrotic toxin (DNT), and osteotoxin production (Gentry-Weeks et al., 1993). Temple et al. (1998) described certain *B. avium* virulence factors *in vivo* and *in vitro*, concluding that the ability of a strain to cause an *in vivo* disease is correlated with its ability to adhere to the ciliated tracheal cells *in vitro*. Mutants that were DNT negative and hemagglutination negative were avirulent in turkey poults. In this study, all of the strains presented a hemagglutination activity after being cultured in CFA and were also positive for the tracheal ring cells assay. Although this experiment did not involved any experimental animal inoculation, the phenotypic test results suggest the potential pathogenicity of these strains.

Gentry-Weeks et al. (1988) examined *B. avium* for the virulence factors common to *Bordetella pertussis*, including the pertussis toxin, filamentous hemagglutinin, adenylate cyclase, dermonecrotic toxin, and tracheal cytotoxin, and the authors concluded that a dermonecrotic toxin and tracheal cytotoxin are the putative virulence factors for *B. avium*. Dermonecrotic toxin (DNT) and tracheal cytotoxin are present in many *Bordetella* species and were involved in the damage of tracheal tissue.

There is a high similarity between the DNTs from *B. pertussis, B. parapertussis* and *B. bronchiseptica*, but the DNTs from *B. avium* present some genetic and biological differences (Walker and Weiss, 1994). Comparing the genomic sequence of *B. avium* and other *Bordetella* species, Sebaihia et al. (2006) described that the gene encoding the dermonecrotic toxin (*dnt*) is present in the *B. avium* genome but at a different location than in those of the previously sequenced species. The morphological changes observed in this study were very similar to the changes observed by Ohnishi et al. (2008) who evaluated *B. pertussis* strains, yet new *in vitro* and *in vivo* studies are required to characterize the action of the toxins produced by the *B. avium* strains isolated from cockatiels.

B. avium could be considered a rare opportunistic human pathogen. The first report of human disease associated with *B. avium* was described by Spilker et al. (2008) in patients with cystic fibrosis. Employing 16S rRNA gene sequencing, Harrington et al. (2009) identified two isolates from human pneumonia that presented 98 and 100% sequence similarity to the *B. avium* ATCC 35086 strain. The zoonotic concern should taken into consideration because cockatiels are a very common pet bird in Brazil.

5. Ethics

The animal experiment was conducted with the approval of the School of Veterinary Medicine and Animal Science, São Paulo University, Ethics Committee, project number 1802/2009.

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