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SHORT COMMUNICATION

Isolation and characterization of *Bordetella pseudohinzii* in mice in China

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Abstract

We report on the first detection and isolation of *B. pseudohinzii* (*Bordetella pseudohinzii*) in laboratory mice in China. Forty-one *B. pseudohinzii* strains were isolated from 3094 mice in 33 different laboratory animal facilities in southern China. The isolates were identified through culture and genome sequenceing. Phylogenetic analysis based on the sequences of 16S rRNA and OmpA genes demonstrated that these strains were on the same clade as other *B. pseudohinzii* strains isolated from mice. Experimental infected mice presented an asymptomatic infection. *B. pseudohinzii* replicated in both the respiratory tract and the digestive tract. Most importantly *B. pseudohinzii* shed via feces and infected a group of sentinel mice in a separate cage via cage padding contaminated with *B. pseudohinzii*-positive feces, indicating that *B. pseudohinzii* could transmit efficiently among mice and contaminate environmental facilities. Our study highlights the importance of routine monitoring of the pathogen in laboratory mice and provides vital insights into the transmission of Brodetellae in rodents and human.

KEYWORDS

Bordetella pseudohinzii, isolation, laboratory mice, transmission

1 | INTRODUCTION

The genus *Bordetella* consists of nine species that could infect a variety of hosts, including humans, birds and rodents.[1-4] *B. pertussis*, the agent of whooping cough in humans, together with *B. parapertussis* and *B. bronchiseptica*, are commonly classified as "classical" *Bordetella*.[5] The other six species (*Bordetella holmesii*, *Bordetella trematum*, *Bordetella avium*, *Bordetella petrii*, *Bordetella hinzii* and *Bordetella pseudohinzii*) are classified as "non-classical" *Bordetella*.[1,6]

Recently, *B. pseudohinzii* has been identified and isolated from laboratory-raised mice.[7,8] Whole-genome analysis has demonstrated that *B. pseudohinzii* shares high similarity with *B. hinzii*, which is a causuative agent of respiratory disease in poultry and is associated with several cases of human infection.[3,8-10] There are 3206 genes present in the genomes of both *B. pseudohinzii* and *B. hinzii*, with 570 genes being specific to *B. hinzii*, and 390 genes being specific to *B. pseudohinzii*. Therefore, routine diagnostic tests were unable to distinguish between them. It has been reported that *B. hinzii* has been detected in experimental facilities and is associated with histopathological changes in the lung.[4,11,12] In light of the similarity between the two species, it has been suggested that the pathogen observed in these studies was actually *B. pseudohinzii*.[4,8]

B. pseudohinzii has been detected and isolated in laboratory mice facilities across the world, including the United States, Japan,

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Malaysia and Germany.[6,7,12,13] This pathogen will cause immunocompetent mice to have a subclinical infection and immunodeficient mice to have rhinitis or pneumonia respiratory symptoms. The current FELASA (Federation for Laboratory Animal Science Associations) recommendations for the health monitoring of mouse colonies in experimental units do not list *B. pseudohinzii* in their health report form,[14] but the The American Association for Laboratory Animal Science (AALAS)/FELASA working group refers to *B. hinzii* as an “exotic agent” that “should be mentioned when found”.[15] Studies using mice as the animal model in China are increasing, but the infection status of *B. pseudohinzii* in the laboratory animal facilities in China had never been investigated. In the present study, *B. pseudohinzii* was screened from a total of 3094 mice from 33 facilities and isolated. The characterization of *B. pseudohinzii* was also studied.

2 | MATERIALS AND METHODS

2.1 | Sample collection and bacterial isolation

A total of 3094 respiratory secretions were collected from mice from 33 different laboratory animal facilities in Southern China from 2015 to 2018. The specimens were transported on ice to the laboratory within 24 hours of sampling. The samples were inoculated on trypticase soy agar supplemented with 5% sheep blood and cultured under anaerobic conditions at 37°C for 24-48 hours. The isolates were gram stained, examined by microscope and identified using the ATB Expression microbe identification system (BioMérieux).

2.2 | 16S rRNA gene and OmpA gene sequencing and phylogenetic analysis

Seven clinical isolates from different experimental animal facilities were randomly selected for genetic analysis. The genomic DNA was extracted using the bacterial genomic DNA extraction kit (Tiangen Bio-chemical Technology) according to the manufacturer's protocol. The 16S rRNA gene was amplified using universal primers (forward primer: 5'-AGAGTTTGATCMTGGCTCAG-3', reverse primer: 5'-TACGGYTACCTTGTTACGACTT-3'). The partial *B. pseudohinzii* outer membrane protein A (OmpA) gene was amplified using primer pairs as previously described.[6] All the primers were synthesized by Sangon Biotech. The PCR conditions were set up in a 50 μL volume containing 5 μL of genomic DNA, 0.4 μmol/L of each primer, 25 μL of Premix ExTaq (Takara, Japan) and 19.4 μL of distilled water as follows: pre-denaturation at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 30 seconds; annealing at 58°C for 30 seconds, extension at 72°C for 60 seconds; and finally 72°C for 10 minutes. Then 8 μL of the PCR product was subjected to electrophoresis on a 2% agarose gel. The PCR product was recovered using a DNA purification kit and ligated to the pMDl9-T vector (Takara, Japan). The positive clones were sequenced. A phylogenetic tree was generated by Mega5 software using the neighbor-joining method. The robustness of the hypothesis was tested with 1000 nonparametric bootstrap analyses.

2.3 | Experimental infection of *B. pseudohinzii* in mice

The sequences of the OmpA gene in the seven clinical isolates were 100% identical with each other. So one isolate of the seven *B. pseudohinzii* strains was randomly selected for the experimental infection. The animal infection experiment was approved by the Institutional Animal Care and Use Committee of Guangdong Laboratory Animals Monitoring Institute. Eight-week-old SPF female SPF ICR mice were obtained from a commercial supplier (Guangdong Medical Laboratory Animal Center). All the mice were determined to be *B. pseudohinzii*-negative by PCR. The mice were randomly divided into 4 groups: inoculated, sentinel, cohabiting, and control. Twenty-one mice served as the inoculated group. Each mouse in this group was inoculated with 30 μL bacterial suspension at a concentration of 3.0×10^8 CFU/mL. The mice in the control group were inoculated with 30 μL PBS. The animals were observed daily for clinical signs including oral and nasal discharge and action state. To determine the replication of *B. pseudohinzii* in mice, three animals in the inoculated group were euthanized on each of days 2, 5, 9, 12, 16, 19 and 21 post inoculation (PI). Immediately following euthanasia, a nasal swab, trachea, lung, and cecal contents were collected from each animal. Samples of the corncob padding contaminated with feces from the animals' caging, and swabs of cage inner walls and outlets were also collected at the same time. All the samples were prepared as homogenates with PBS and the presence of *B. pseudohinzii* was determined using PCR. When the dirty padding from the inoculated group was identified as positive for *B. pseudohinzii* by PCR, a single sample of 50 mL of the dirty padding was moved to the sentinel group cage containing 15 mice, and another 15 SPF mice were move into the inoculated group as the cohabiting group. Three mice from each group were euthanized on each of days 7, 14, 21, 28 and 35 post inoculation (PI). The day on which the dirty padding from the inoculated group was identified as *B. pseudohinzii* positive by PCR was considered as the time of inoculation for the sentinel and cohabiting groups. PI tissue samples were harvested and PCR assays for sequencing the OmpA gene were conducted as above. Two control mice were sacrified on each of days 14, 21, 28 and 35 post inoculation (PI) and tested for *B. pseudohinzii* by PCR.

3 | RESULTS AND DISCUSSION

Bacteriologic cultures of respiratory secretions collected from 3094 mice yielded various kinds of colonies with different morphologies. Hundreds of colonies were isolated from the samples after 24 hours of incubation on trypticase soy agar supplemented with 5% sheep blood. One type of colony displayed a special morphology that was different from that of the common bacteria. These white, round colonies were apparent after 24 hours of incubation under aerobic conditions. They were of medium sized (1-2 mm), translucent, smooth, convex, and without hemolysis (Figure 1A). Microscopic examination demonstrated that the colonies were gram-negative, short

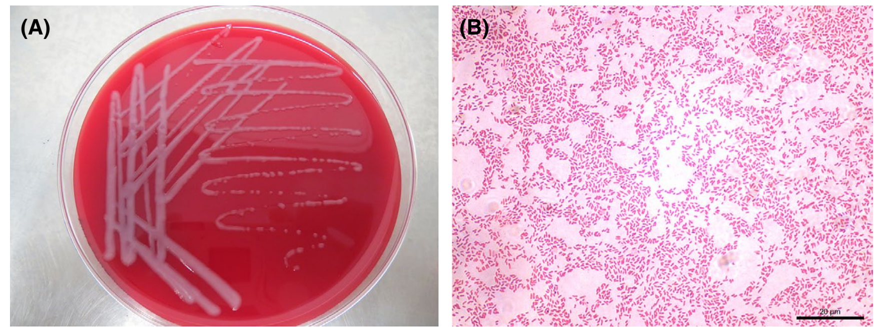

FIGURE 1 Colony morphology and microscopic examination. A, Colony morphology of one isolated strain cultured on 5% sheep blood tryptone soybean agar medium. B, Gram staining and microscopic examination

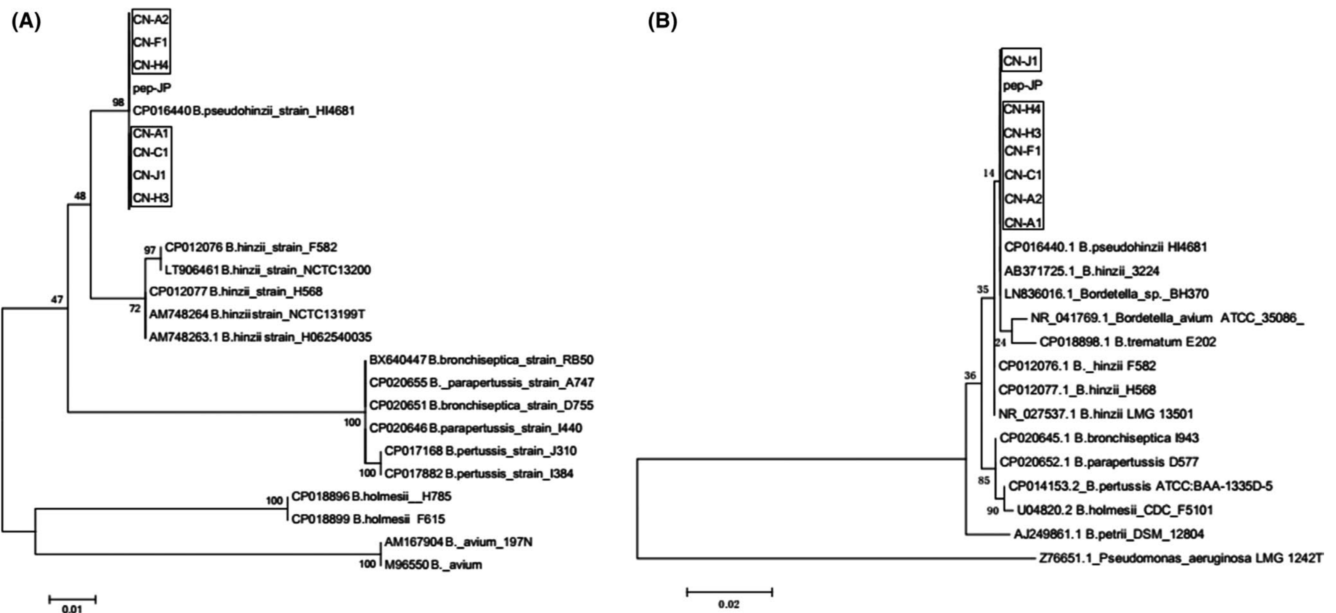

FIGURE 2 Phylogenetic analysis (neighbor-joining) based on the outer membrane protein A (OmpA) gene sequence alignment (A) and 16S rRNA gene sequences (B) of seven isolates. Seven *Bordetella pseudohinzii* strains A1, A2, C1, F1, H3, H4 and J1 were deposited in GenBank under the accession numbers MK072953 to MK072959, respectively

rod-shaped bacteria with densely stained poles (Figure 1B). The API 20NE commercial identification kit (bioMérieux) gave the numerical code 0000067, suggesting with a high level of confidence (96.7%) that the bacteria were *Bordetella avium*. A total of 41 isolates with the same morphology were recovered from tracheal swabs as the dominant organism. All the 41 isolated strains exhibited a consistent profile: code 0000067 on API 20NE.

To further determine the characteristics of the clinical isolates, seven isolates were randomly selected for genome sequencing and analysis. The sequences of the OmpA genes were 100% identical with that of the previously reported *B. pseudohinzii* derived from mouse colonies in different countries.[4,7] In addition, the OmpA gene detected in this study had 98% nucleotide sequence homology to *B. hinzii* F582 and H568 isolated from humans, 97% homology to *B. avium*, 93% homology to pertussis, and 92% homology to bronchiseptica and parapertussis. A phylogenetic tree based on the sequences characterized all the seven clinical strains as "*B. pseudohinzii*". All the isolates formed the same cluster in the OmpA phylogenetic tree (Figure 2A). To confirm the OmpA gene analysis, a phylogenetic analysis based on 16S rRNA sequences was performed and demonstrated that the seven isolates also formed the same group with another *B. pseudohinzii* strain H4681 and BH370 (Figure 2B). It also indicated that *Bordetella hinzii* strain 3224 was in the same cluster as *B. pseudohinzii*. Given the similarity between the two species, it has been suggested that *Bordetella hinzii* identified in previous reports was actually *B. pseudohinzii*.[4,8] The phylogenetic analysis performed in the present study also supports that conclusion. The 16S rRNA sequences of seven *Bordetella pseudohinzii* strains A1, A2, C1, F1, H3, H4 and J1 were deposited in GenBank under accession numbers MK072953 to MK072959, respectively.

To investigate the replication of *B. pseudohinzii* in mice, the tissues of the experimentally infected mice were examined for the presence of *B. pseudohinzii* using PCR. The detection results were summarized in Table 1. Experimental infected mice presented an asymptomatic infection. In the inoculation group, *B. pseudohinzii* could be detected as early as day 2 PI in the trachea and cecal contents and was consistently present in theses tissues at all the sampling time points throughout the study. The dirty padding was found to

TABLE 1 Pathogen detection in mice in three groups

Days PI	Inoculated group							Sentinel group					Cohabiting group				
Samples	**2**	**5**	**9**	**12**	**16**	**19**	**21**	**7[a]**	**14**	**21**	**28**	**35**	**7[a]**	**14**	**21**	**28**	**35**
Nasal swab	−	−	+	+	+	+	+	−	−	−	+	+	−	−	−	+	+
Tracheal	+	+	+	+	+	+	+	−	−	+	+	+	−	−	−	+	+
Lung	−	−	−	+	+	+	+	−	−	−	+	+	−	−	−	−	+
Cecal contents	+	+	+	+	+	+	+	−	−	−	+	+	−	−	−	−	+
Dirty padding	−	−	−	+	+	+	+	−	−	−	+	+	−	−	−	−	+
Cage inner wall	−	−	−	+	+	+	+	/	/	/	/	/	/	/	/	/	/
Outlet	−	−	−	−	−	−	−	/	/	/	/	/	/	/	/	/	/

Dirty padding included feces. +: positive, −: negative, /: not determined.
[a]When the dirty padding in the inoculated group was positive for *B. pseudohinzii* by PCR on day 12 PI, a sample of padding was moved to the cage containing the sentinel mice. The day that dirty padding in the inoculated group was determined as positive was considered to be the day of inoculation for the sentinel and cohabiting groups.

be positive on day 12 PI. The bacteria could also be detected in the nasal swabs and lung on days 12 PI, as well as in the trachea and cecal contents. The replication of *B. pseudohinzii* in trachea and lung of the mice has been demonstrated in previous studies.[4,6] Our novel finding in the present study was that the infected mice shed the bacteria in their feces which contaminated the cage padding. We therefore investigated fecal transmission and replication of *B. pseudohinzii* in separately caged sentinel mice that received a composite sample of contaminated bedding (bedding used in the inoculation group that tested positive for *B. pseudohinzii*). After 4 weeks of exposure to the dirty bedding, the sentinel mice were positive to *B. pseudohinzii* in all the samples tested (Table 1). The *B. pseudohinzii*-positive bedding had thus become the new infection source. This result suggested that *B. pseudohinzii* can transmit through feces and infect other mice. The mice in the cohabiting group living in the same cage as the inoculation group tested positive for *Bordetella pseudohinzii* in trachea and nasal swabs at 28 days PI, and in all the samples at 35 days PI. All the samples collected from the control mice were negative for *B. pseudohinzii* throughout the study.

Our study is the first to report a natural infection of *Bordetella pseudohinzii* in captive mouse colonies in China. The infection rate (41/3094) was 1.32%, which is consistent with another study reported in Japan.[11] Furthermore, the bacteria could be detected in almost all the mice species studied, including C57BL/6 mice, BALB/c mice, ICR sentinel mice, immunodeficient mice, and transgenic mice. Ten out of 33 facilities had *B. pseudohinzii* contamination, and one facility tested positive for *B. pseudohinzii* 5 consecutive times in 3 years, indicating that *B. pseudohinzii* can be a long-term contaminant in laboratory animal facilities, which should be addressed as a significant problem.

B. pseudohinzii is a non-classical *Brodetellae* species similar to *B. avium* and *B. hinzzi*, which makes them hard to distinguish.[8] Currently, the identification library of the commercial biochemical identification system contains only two *Bordetella* species profiles, *B. avium* and *B. bronchiseptica*. Thus *B. pseudohinzii* was identified as *B. avium* using the commercial kit. Confirmation of the isolates can be reliably performed by nucleotide sequencing. The phylogeny reconstructed using the sequences of 16S RNA and the OmpA gene characterized the isolates as *B. pseudohinzii* from the *Bordetella* genus.

Previous studies have demonstrated that *B. pseudohinzii* infects the respiratory tract of mice and has a potential negative effect on pulmonary research.[4] *B. pseudohinzii* could be detected in the trachea, lung and cecal contents of the experimentally infected mice in the present study. Our results not only confirmed previous studies showing that *B. pseudohinzii* replicates in the respiratory tract, but also showed that the bacteria could replicate in the digestive tract and shed via feces. Separately caged sentinel mice exposed to the padding contaminated with the feces from the inoculated group tested positive to *B. pseudohinzii* in all the collected samples after 4 weeks of exposure, indicating that *B. pseudohinzii* can transmit efficiently among laboratory mice and contaminate environmental facilities. The detection of *B. pseudohinzii* in one facility 5 consecutive times in 3 years also illustrated that the pathogen can exist for a long time in laboratory animals and facilities. In light of this observation, laboratory animal researchers should pay more attention to *B. pseudohinzii* and enhance the monitoring of this pathogen to guarantee the quality of their laboratory animals.

To date, *B. pseudohinzii* has been isolated not only from laboratory mice, but also from wild rats.[4,16] Although there is no evidence that *B. pseudohinzii* could infect humans, *B. hinzii* which shares high similarity with *B. pseudohinzii* has been isolated from humans and is associated with mutiple diseases.[9,10,17-20] *B. hinzii* has been isolated from wild rodents, but the source of transmission remains elusive.[16,21] The isolation of *B. pseudohinzii* in wild rodents together with our finding that the bacterica can shed through the feces and transmit to other animals raises public health concerns.[16] Wild rodents represent an important reservoir of multiple human pathogens.[22,23] Whether *B. hinzii* can transmit from wild rodents to infect humans requires further investigation in the future. The infection model established in our study may provide important insights into the transmission of Brodetellae in rodents and humans.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

JW designed and coordinated the overall study. LM, SH and YL carried out the experimental work. FM and LF conducted pathological examinations. MC, JP and YZ analyzed the data. All authors discussed the results and wrote the manuscript.

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