

Molecular and antimicrobial analyses of non-classical *Bordetella* isolated from a laboratory mouse

Shih Keng LOONG¹), Nur Hidayana MAHFODZ¹), Haryanti Azura Mohamad WALI²), Siti Aisyah A. TALIB²), Siti Noraisah Ahmad NASRAH³), Pooi Fong WONG^{2,4}) and Sazaly ABUBAKAR^{1,3})*

¹Tropical Infectious Diseases Research & Education Centre, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

²Animal Experimental Unit, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

³Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

⁴Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

(Received 13 August 2015/Accepted 13 December 2015/Published online in J-STAGE 17 January 2016)

ABSTRACT. Accurate identification and separation of non-classical *Bordetella* species is very difficult. These species have been implicated in animal infections. *B. hinzii*, a non-classical *Bordetella*, has been isolated from mice in experimental facilities recently. We isolated and characterized one non-classical *Bordetella* isolate from the trachea and lung of an ICR mouse. Isolate BH370 was initially identified as *B. hinzii* by 16S ribosomal DNA and *ompA* sequencing. Additionally, isolate BH370 also displayed antimicrobial sensitivity profiles similar to *B. hinzii*. However, analyses of *nrdA* sequences determined its identity as *Bordetella* genogroup 16. The isolation of BH370 from a healthy mouse suggests the possibility of it being a commensal. The *nrdA* gene was demonstrated to possess greater phylogenetic resolution as compared with 16S ribosomal DNA and *ompA* for the discrimination of non-classical *Bordetella* species.

KEY WORDS: *Bordetella*, infectious disease, laboratory mouse, Malaysia

doi: 10.1292/jvms.15-0472; *J. Vet. Med. Sci.* 78(4): 715–717, 2016

The genus *Bordetella* comprises gram-negative, rod-shaped bacteria that can cause respiratory diseases in humans and animals [7]. Classical *Bordetella* species (*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*) are usually associated with serious human respiratory infections [3, 14]. Differentiation of non-classical *Bordetella* species that rarely cause human infection is difficult [3]. The most common diagnostic assays include real-time PCR [9] in combination with examination of growth properties, biochemical tests, serotyping and partial 16S rRNA gene sequencing [14]. However, these methods are laborious, and it is still arduous to specifically differentiate non-classical *Bordetella* species. Recently, researchers have isolated *B. hinzii* from mice bred in experimental facilities in Japan [5]. The same group also proposed *B. hinzii* as a possible pathogen in mice [6], potentially interfering with animal research and possibly serving as a source of infection for other mice in the experimental facility. Here, we report the isolation and characterization of a non-classical *Bordetella* isolate from the trachea and lung of a healthy laboratory mouse.

The Animal Experimental Unit, Faculty of Medicine, University of Malaya, has an established health monitoring program for rodents housed in the facility to ensure the animals are free of specific pathogens that could interfere with scientific experiments. Rodents submitted to the facility for

health screening were quarantined in individually ventilated cages (Tecniplast, Via Maggio, Italy). All cages, animal feed, beddings and surgical tools were sterilized before use. The animals were maintained at a room temperature of 19 to 21°C and relative humidity of 54 to 65% under a 12:12 hr light:dark cycle. The current study and the animal handling procedures were approved by the Institutional Animal Care and Use Committee, University of Malaya (Reference no. 2013-11-12/AEU/B/WPF). Healthy mice that were between 6 and 12 weeks old and had no observed signs of illness were randomly chosen and anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) prior to blood collection through cardiac puncture. Blood collected from mice was tested by serology (SMART-SPOT Sentinel Panel Test, Mouse Sera, Biotech Trading Partners, Encinitas, CA, U.S.A.) for the following agents; epizootic diarrhea of infant mice, mouse hepatitis virus, *Mycoplasma pulmonis*, mouse parvovirus, minute virus of mice, pneumonia virus of mice, respiratory enteric orphan virus, Sendai virus and Theiler's murine encephalomyelitis virus. Due to the small volume of circulating blood in mice, no euthanasia was performed, as the mice would experience hypovolemia after cardiac puncture, which would result in death. All works were performed in a dedicated procedure room away from breeding facilities. Necropsy and harvesting of selected organs (trachea, lungs, liver and the gastrointestinal tract) were performed inside a biosafety cabinet in the procedure room. The organs were subjected to microbiology culture for *Corynebacterium kutscheri*, *Citrobacter rodentium*, *Salmonella* spp., *Helicobacter* spp. and *Clostridium piliformis*. The trachea and lungs of the euthanized rodents were transferred into tubes with ceramic beads, and the specimens were homogenized using a Minilys tissue homogenizer (Bertin Technologies, Montigny-le-

*CORRESPONDENCE TO: ABUBAKAR, S., Tropical Infectious Diseases Research & Education Centre, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. e-mail: sazaly@um.edu.my
©2016 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

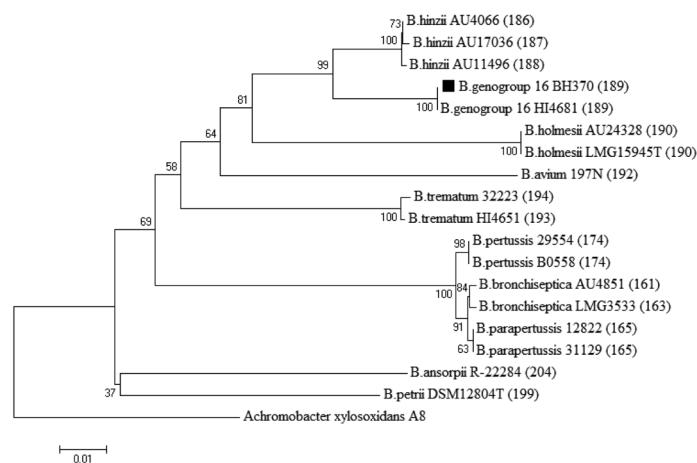


Fig. 1. Neighbor-joining phylogenetic tree of *Bordetella* spp. 765-bp *nrdA* loci. Isolate BH370 is indicated by a black-filled square symbol before its species name. *nrdA* locus numbers are indicated in parentheses. *Achromobacter xylosoxidans* was used as an outgroup. Numbers at nodes indicate bootstrap values (%) for 1,000 replicates. The scale bar indicates nucleotide substitutions per site.

Bretonneux, France). A small portion of the homogenate was inoculated onto Columbia agar with 5% sheep blood and incubated at 37°C under ambient conditions for 24 hr. Cultured bacterial isolates were Gram stained, and a lactose fermentation test using phenol red lactose broth was performed. An antimicrobial sensitivity profile was determined using a disc diffusion assay on Mueller-Hinton agar incubated at 37°C for 24 hr. The antibiotic discs (BBL™ Sensi-Disc™, Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) used in the disc diffusion assay were amikacin (30 µg), amoxicillin-clavulanic acid (20/10 µg), ampicillin (10 µg), cefoperazone (75 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), gentamicin (10 µg), imipenem (10 µg) and meropenem (10 µg). Results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute [1].

The trachea and lung specimens from an 8-week-old male ICR mouse with no sign of illness grew small, non-lactose fermenting Gram-negative rods (isolate BH370). Serological assays suggested that the mouse was free of the 9 regularly monitored common pathogens. Nucleic acid amplification and sequencing of the 16S ribosomal DNA gene, as described previously [11], were performed to identify the isolate. PCR fragments were purified, sequenced and compared against available sequences in GenBank. BLAST results of the partial 16S ribosomal DNA gene from BH370 (Accession no. LN836016) showed a 100% sequence identity match with *B. hinzii* strain 3224 (Accession no. AB371725), which had been isolated previously from a sick mouse. However, the isolate was also closely related to other *Bordetella* species (*B. avium*, *B. holmesii*, *B. parapertussis* and *B. bronchiseptica*), as their sequences showed as high as a 99% 16S ribosomal DNA sequence identity match. A separate PCR specifically amplifying the *ompA* gene of *B. hinzii* [12] to ascertain the identity of the isolate was performed. The resulting PCR fragment from BH370 was sequenced

(Accession no. LN836017), and BLAST identified BH370 as *B. hinzii* with 98% similarity. Sequencing of *ompA* substantiates the confirmation of non-classical *Bordetella* species due to its greater sequence variation as compared with the 16S ribosomal DNA gene [12]. Nevertheless, the GenBank public database contained only three *B. hinzii ompA* entries, which we felt was not quite sufficient to strongly support the identification. The multilocus sequence typing scheme for *Bordetella* [2] failed to amplify PCR products for DNA sequencing. To resolve the identity ambiguity of isolate BH370, we employed an additional method that has been shown to reliably differentiate species within the genus *Bordetella* by analyzing the trimmed 765-bp sequences of *nrdA* [14], which encodes the ribonucleoside-diphosphate reductase alpha chain. At this gene locus, BH370 was distinctively identified as belonging to *Bordetella* genogroup 16, as shown in Fig. 1, and its nucleotide sequence can be accessed at the PubMLST website (<http://pubmlst.org/bordetella>) [8]. Resistance to amoxicillin-clavulanic acid, ampicillin, cefuroxime, ceftriaxone and cefotaxime was revealed for BH370 (Table 1).

The differentiation of non-classical *Bordetella* species is difficult and labor intensive [14]. Commercial biochemical identification systems, such as API 20NE strips, currently only incorporate two *Bordetella* species, *B. avium* and *B. bronchiseptica*, in their libraries [3]. Even the application of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was shown to classify a *Bordetella* isolate only down to the genus level [7]. Yet confirmation of species is important for the determination of antibiotic treatment and appropriate infection control measures. Since 16S ribosomal DNA and *ompA* sequences could not provide definite resolution to specifically identify and separate non-classical *Bordetella* species, we employed a novel genotypic identification scheme based on the *nrdA* sequences [14]. BH370 was defined as *Bordetella* genogroup

Table 1. Antimicrobial sensitivity results for *Bordetella* genogroup 16 isolate BH370

Antibiotic	Sensitivity testing results by disc diffusion assay ^{a)} on isolate BH370
Amikacin	S (19 mm)
Amoxicillin/clavulanic acid	R (11 mm)
Ampicillin	R (11 mm)
Cefoperazone	S (26 mm)
Ceftazidime	S (27 mm)
Ciprofloxacin	S (22 mm)
Cefuroxime	R (12 mm)
Ceftriaxone	R (19 mm)
Cefotaxime	R (20 mm)
Gentamicin	S (18 mm)
Imipenem	S (25 mm)
Meropenem	S (25 mm)

a) Zone diameter values are in parentheses. R, resistant; S, sensitive. Results were interpreted according to CLSI breakpoints for *Enterobacteriaceae*.

16 bearing *nrdA* locus 189. The high sequence similarity of *B. hinzii* strain 3224 to our isolate suggests the possibility that strain 3224 was misidentified as *B. hinzii*, since its identification was done only with 16S ribosomal DNA sequencing. Another isolate, which was isolated from a cystic fibrosis mouse, carrying an identical *nrdA* locus was found in the PubMLST database [8]. The phylogenetic tree (Fig. 1), built using *nrdA* loci of representative *Bordetella* isolates clearly showed that *Bordetella* genogroup 16 and *B. hinzii* share a very close phylogenetic relationship. However, the reservoir and transmission routes for *B. hinzii* are currently unknown [7]. *B. hinzii* has been reported to cause infection in humans [3, 4] and rodents [5, 7], and laboratory mice have also been discovered to harbor *B. hinzii* in Japan [5, 6]. We suggest that rodents could be the reservoir for *Bordetella* genogroup 16, even though human infection has not been reported thus far.

Antimicrobial sensitivity assays revealed that isolate BH370 was resistant to amoxicillin/clavulanic acid, ampicillin, cefuroxime, ceftriaxone and cefotaxime, but sensitive to amikacin, cefoperazone, ceftazidime, gentamicin, imipenem and meropenem. Other publications have shown that *B. hinzii* isolated from clinical cases [3, 4, 13] was resistant or showed intermediate resistance to ampicillin, cefuroxime, ceftriaxone, cefotaxime and ciprofloxacin, which was similar to the antimicrobial sensitivity of BH370, excluding ciprofloxacin. The largely β -lactam resistance profile of isolate BH370 mirrors those observed by others for *B. hinzii* [3, 4, 13] and *B. bronchiseptica* [10], reinforcing the close association between *Bordetella* genogroup 16 and other *Bordetella* species. In the case of BH370, however, the discovery of this organism in a mouse with the absence of any clinical symptoms could suggest that it exists as a commensal. It may rarely cause noticeable infection in mice, as observed in *B. hinzii*-positive mice in Japan [5], or it could be an opportunistic pathogen in sick mice, as in the case of *B. hinzii* strain 3224 [6]. Hence, we suggest that screening for this organism should be considered in routine health

monitoring programs for laboratory rodents.

ACKNOWLEDGMENTS. This study was supported in part by the University of Malaya Research Officer Grant Scheme (BR009-2014) and High Impact Research grant scheme (E000013-20001). The authors want to thank Mdm. Hamidah Hamid from the Animal Experimental Unit, Faculty of Medicine, University of Malaya, for her assistance in specimen processing.

REFERENCES

1. Clinical and Laboratory Standards Institute 2012. Performance Standards for Antimicrobial Susceptibility Testing. *Twenty-Second Informational* (Supplement): M100–S21 Clinical and Laboratory Standards Institute, Wayne.
2. Diavatopoulos, D. A., Cummings, C. A., Schouls, L. M., Brinig, M. M., Relman, D. A. and Mooi, F. R. 2005. *Bordetella pertussis*, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of *B. bronchiseptica*. *PLoS Pathog.* **1**: e45. [Medline] [CrossRef]
3. Fry, N. K., Duncan, J., Edwards, M. T., Tilley, R. E., Chitnavis, D., Harman, R., Hammerton, H. and Dainton, L. 2007. A UK clinical isolate of *Bordetella hinzii* from a patient with myelodysplastic syndrome. *J. Med. Microbiol.* **56**: 1700–1703. [Medline] [CrossRef]
4. Funke, G., Hess, T., von Graevenitz, A. and Vandamme, P. 1996. Characteristics of *Bordetella hinzii* strains isolated from a cystic fibrosis patient over a 3-year period. *J. Clin. Microbiol.* **34**: 966–969. [Medline]
5. Hayashimoto, N., Morita, H., Yasuda, M., Ishida, T., Kameda, S., Takakura, A. and Itoh, T. 2012. Prevalence of *Bordetella hinzii* in mice in experimental facilities in Japan. *Res. Vet. Sci.* **93**: 624–626. [Medline] [CrossRef]
6. Hayashimoto, N., Yasuda, M., Goto, K., Takakura, A. and Itoh, T. 2008. Study of a *Bordetella hinzii* isolate from a laboratory mouse. *Comp. Med.* **58**: 440–446. [Medline]
7. Jiyipong, T., Morand, S., Jittapalpong, S., Raoult, D. and Rolain, J. M. 2013. *Bordetella hinzii* in rodents, Southeast Asia. *Emerg. Infect. Dis.* **19**: 502–503. [Medline] [CrossRef]
8. Jolley, K. A. and Maiden, M. C. 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* **11**: 595. [Medline] [CrossRef]
9. Koidl, C., Bozic, M., Burmeister, A., Hess, M., Marth, E. and Kessler, H. H. 2007. Detection and differentiation of *Bordetella* spp. by real-time PCR. *J. Clin. Microbiol.* **45**: 347–350. [Medline] [CrossRef]
10. Lartigue, M. F., Poirel, L., Fortineau, N. and Nordmann, P. 2005. Chromosome-borne class A BOR-1 beta-Lactamase of *Bordetella bronchiseptica* and *Bordetella parapertussis*. *Antimicrob. Agents Chemother.* **49**: 2565–2567. [Medline] [CrossRef]
11. Misbah, S., Hassan, H., Yusof, M. Y., Hanifah, Y. A. and AbuBakar, S. 2005. Genomic species identification of *Acinetobacter* of clinical isolates by 16S rDNA sequencing. *Singapore Med. J.* **46**: 461–464. [Medline]
12. Register, K. B. 2013. Development of a PCR for identification of *Bordetella hinzii*. *Avian Dis.* **57**: 307–310. [Medline] [CrossRef]
13. Palacián Ruiz, M. P., Vasquez Martinez, M. A. and Lopez Calleja, A. I. 2013. Respiratory infection caused by *Bordetella hinzii*. *Arch. Bronconeumol.* **49**: 409–410. [Medline] [CrossRef]
14. Spilker, T., Leber, A. L., Marcon, M. J., Newton, D. W., Darrach, R., Vandamme, P. and Lipuma, J. J. 2014. A simplified sequence-based identification scheme for *Bordetella* reveals several putative novel species. *J. Clin. Microbiol.* **52**: 674–677. [Medline] [CrossRef]