Pathology (April 2018) **50(3)**, pp. 322–326

MICROBIOLOGY

Detection and incidence of *Bordetella holmesii* in respiratory specimens from patients with pertussis-like symptoms in New South Wales, Australia

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Summary

Bordetella pertussis, the aetiological agent of whooping cough is routinely diagnosed by polymerase chain reaction (PCR) directed at *IS481*, an insertion sequence target also found in *Bordetella holmesii*. Recent reports have suggested that *B. holmesii* infections can be misdiagnosed as pertussis, which can have a significant impact on public health surveillance. This study investigated the presence of *B. holmesii* in *B. pertussis* positive clinical samples, in order to determine the incidence of *B. holmesii*. Clinical cases of pertussis diagnosed by *IS481*-specific PCR between October 2008 and March 2016 in New South Wales were included. *Bordetella holmesii* was detected through the simultaneous amplification of *IS481* and *B. holmesii* specific insertions sequence, *hIS1001*. A total of 46 of 802 patients were identified to be positive for *B. holmesii* rather than *B. pertussis*, suggesting an incidence rate of 6.5% in 2009, 16.8% in 2010, 7.6% during 2013 and 8.1% during 2015. *Bordetella holmesii* infections were diagnosed during and between pertussis epidemics, however cases of *B. holmesii* and *B. pertussis* co-infections were not found. The predominant age group of *B. holmesii* infection was 11–18 years old, which was significantly different to the mean age of *B. pertussis* infections (0–6 years, $p = 0.023$). These findings revealed that *B. holmesii* was co-circulating alongside the *B. pertussis* epidemic for seven years, hidden from view, as *B. holmesii* infections have been diagnosed as *B. pertussis*. Confirmatory testing of *B. pertussis* positive samples for the presence of *B. holmesii,* especially during pertussis epidemics, should improve the quality of laboratory diagnosis and laboratory surveillance for pertussis. The presence of *B. holmesii* in Australia highlights the importance of testing for this pathogen and ongoing molecular surveillance that can guide the control of whooping cough.

Key words: Pertussis; *Bordetella holmesii*; PCR; *Bordetella pertussis*.

Received 9 May, revised 30 August, accepted 4 October 2017
Available online 15 February 2018

BACKGROUND

Pertussis, or whooping cough, is an acute and highly transmissible respiratory infection, associated with prolonged coughing episodes and mortality in children under five years.[1,2] While symptoms can be mild in adults, the infection is life-threatening in infants.[2] *Bordetella pertussis* is the main aetiological agent of whooping cough, is highly infectious and is re-emerging as a significant pathogen globally.[3] The disease is primarily controlled by immunisation but despite a sustained reduction in childhood mortality by vaccination programs, pertussis continues to circulate.[4] The reasons for this apparent decrease in efficacy include waning immunity over time, and possible 'vaccine escape' from mutating *B. pertussis* strains, which may avoid vaccine associated immune response.[5–8] However, vaccination remains the best method of control, as antibiotic therapy is only effective during the early stage of disease.[9] Surveillance of currently circulating strains is paramount to public health control both for tracing transmission of *B. pertussis* in the community and providing much needed information for future vaccine development.

Bordetella pertussis is primarily detected in clinical samples by polymerase chain reaction (PCR) amplification of the insertion sequence *IS481*;[10] however, *IS481*-based assays are unable to distinguish between *B. pertussis* and *B. holmesii*, an emerging infection that can cause pertussis-like symptoms.[11] As a result, cases caused by *B. holmesii* can be reported as *B. pertussis* positive. This misidentification of pertussis cases may affect the quality of public health laboratory surveillance and assessment of vaccine efficacy for whooping cough. The switch from culture-based to PCR-based laboratory diagnostics and notification of pertussis may have contributed to *B. holmesii* detection, given that both species carry the PCR target *IS481*. Furthermore, the use of cephalexin in selective media for culture of *B. pertussis* may have inhibited growth of *B. holmesii* which appears to be susceptible to first generation cephalosporins.[12]

Despite recent recognition of *B. holmesii* as a significant respiratory pathogen with potential to cause community outbreaks, incidence of *B. holmesii* is unknown and our understanding of its epidemiology remains limited.[13] The few studies around the world have identified *B. holmesii* in up to 29% of upper respiratory tract samples from patients with pertussis-like illnesses (Table 1).[14–21] Interestingly, the 2010 Ohio study showed that almost half of the affected patients were adolescents.[20] Emerging evidence indicates that determining the significance of *B. holmesii* infections[22] within

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2017.10.014

Table 1 Worldwide incidence summary of *B. holmesii* from previous international studies

Country	*B.holmesii* incidence	Year(s)	Evidence of patient-to-patient transmission
Finland and Netherlands[14]	0%	1992–2003	No
Massachusetts, USA[21]	3.5%	1994–1998	No
France[19]	6.8%	2009–2010	No
Chile[16,17]	5.4–11.1%	2010–2011	No
Japan[15]	17%	2010–2011	No
Ohio, USA[20]	29%	2010–2011	Yes
South Africa[18]	4.1%	2012–2013	No

Included are country of study, percentage of incidence, year of study periods, and whether evidence of patient-to-patient transmission is provided in the study.

B. pertussis epidemics is crucial in assessing whether *B. holmesii* is a public health concern. This provides the rationale for our study which aimed to examine the incidence of *B. holmesii* infections in New South Wales, Australia, over an 8 year period.

METHODS

All available clinical samples from cases diagnosed as positive for *B. pertussis* by in-house PCR at the Centre for Infectious Diseases and Microbiology Laboratory Services (CIDMLS, NSW Health Pathology) between October 2008 and March 2016 were included in the study. Date of received specimen collection, age of individual, postcode of residence and gender were obtained from the laboratory database at CIDMLS. The samples consisted of DNA extracted from nasopharyngeal aspirates, nasopharyngeal swabs or throat swabs, thawed from −20°C to room temperature. The DNA was extracted by the NucliSENSE easyMAG DNA extraction system (BioMérieux, USA). The swab was first cut and incubated in 1 mL of lysis buffer for 10 min at room temperature then 250 µL of the sample was extracted and stored at −20°C. The following extraction was performed as per manufacturer's protocol, resulting in 100 µL of DNA.

Retrospective screening was performed on samples still available in storage, and involved simultaneously retesting for *IS481* and a *B. holmesii* specific insertion sequence *hIS1001* by real-time PCR (rtPCR) in the Roche LightCycler 480 (Roche Diagnostics, Switzerland). The primers utilised in these diagnostic PCRs are outlined in Table 2. *Bordetella pertussis* and *B. holmesii* DNA extracted from isolates from the CIDMLS culture collection were used as positive controls and sterile water as a no-template control. The *IS481* PCR reaction mixture (20 µL) contained QIAGEN HotStarTaq MasterMix (Qiagen, Germany), made up to final concentrations of 1.5 mM $MgCl_2$, 200 µM dNTPs and 1.23 U HotStarTaq DNA polymerase (Qiagen), 300 nM of *IS481* forward and *IS481* reverse primers (Sigma Aldrich, USA) and 120 nM of TaqMan FAM probe (Sigma Aldrich) and 5 µL of DNA extract. The *hIS1001* PCR assay included the following: 400 nM for *hIS1001* forward and *hIS1001* reverse primers each (Sigma Aldrich, USA), 50 nM of TaqMan probe (Sigma Aldrich), 1x Roche LightCycler ProbeMaster (Roche Diagnostics), and molecular-grade PCR water to make a 15 µL mastermix. DNA extract (5 µL) was added resulting in a total reaction volume of 20 µL. A thermocycling profile was designed for the amplification and detection for both the *IS481* and *hIS1001* assays and consisted of a denaturation step of 95°C for 10 min, followed by 45 cycles of amplification at 95°C for 10 s, 55°C for 30 s and 72°C for 15 s, at a ramp rate of 4.4°C/s, and the reaction was allowed to cool at 40°C for 10 min.

Any *B. holmesii* positive samples were investigated further, using an assay which was directed at the *ptxA* gene which is specific to *B. pertussis*, to determine whether the *B. holmesii* cases were co-infections along with *B. pertussis*. The *ptxA* assay was prepared with 800 nM of forward and reverse primers (Sigma Aldrich), 600 nM of TaqMan probe (Sigma Aldrich), 1x Roche LightCycler ProbeMaster, and molecular-grade PCR water to make a 15 µL mastermix reaction volume, with 5 µL of DNA extract added to form a 20 µL total reaction volume. Included were a dilution of *B. pertussis* to act as a positive control, and *B. holmesii* DNA and water as negative and no-template controls, respectively.

All positive samples were retested to eliminate possibility of contamination, and positive samples were indicated by a cut-off of 35 cycles; any samples between 35 and 40 cycles were judged as an indeterminate and retested. If the sample remained positive with a similar CP value, then the sample was deemed positive. A positive *ptxA* result indicated presence of *B. pertussis*, and combined with a positive *B. holmesii* result, suggested a co-infection with both *B. pertussis* and *B. holmesii.* However, as *ptxA* is carried in only one copy on the *B. pertussis* genome, the assay is expected to be less sensitive when compared to the PCR that targets multi-copy *IS481*.

Results were statistically analysed by Excel Mac 2011 (Microsoft, USA) using in-built graph plotting, and BoxplotR (Wellcome Trust, UK). The *p* value was calculated using an unpaired t-test through GraphPad t-test calculator (GraphPad Software, USA).

RESULTS

Bordetella holmesii was detected in 46 of 802 samples tested by rtPCR (Supplementary Table 1, Appendix A), providing an incidence rate of 5.8% over the 8 year time period (Supplementary Fig 1, Appendix A). Specifically, *B. holmesii* incidence was 6.5% in 2009, 16.8% in 2010, 7.6% during 2013 and 8.1% during 2015 (Table 3). *Bordetella holmesii* was detected during low and high pertussis periods, suggesting that it can co-circulate with *B. pertussis.* These samples were based on the positive amplification of *IS481* and *hIS1001* sequences. A total of 75 samples were negative for both assays, indicating that either the concentration of DNA was low or had degraded over time in storage. The remaining 681 samples were positive for *IS481* only,

Table 2 Primers and probes utilised in *Bordetella sp.* identification

Primer	Sequence	Product size	Reference
hIS1001		67 bp	Rodgers *et al.*, 2012
Forward	5′ TCA TCG CGC ATC AGA TAA GC		
Reverse	5′ CGG TAA AGT TGG ACG AGT TGC T		
Probe	5’ [6FAM] TGA GCA AGG GCT GGT TGG CCT G [BHQ-1]		
IS481		62 bp	Glare *et al.*, 1990
Forward	5′ GAT TCA ATA GGT TGT ATG CAT GGT T		
Reverse	5′ AAT TGC TGG ACC ATT TCG AGT CGA CG		
Probe	5’ [6FAM] AT GAA CAC CCA TAA GCA TGC CCG [BHQ-1]		
ptxA		55 bp	Rodgers *et al.*, 2012
Forward	5′ CGC CAG CTC GTA CTT C		
Reverse	5′ GAT ACG GCC GGC ATT		
Probe	5’ [6FAM] AAT ACG TCG ACA CTT ATG GCG A [BHQ-1]		