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RESEARCH ARTICLE

False-Positive Diagnostics of *Bordetella Pertussis* using IS481 PCR is Limited in Danish Patients

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Abstract:

Background:

Bordetella pertussis is routinely detected using real-time PCR based on the multicopy insertion sequence IS481, which is not specific for *Bordetella pertussis*.

Objective:

The aim of this retrospective study was to evaluate the proportion of other *Bordetella* species misidentified as *Bordetella pertussis* using IS481-targeted real-time PCR.

Methods:

Clinical specimens from 228 Danish patients (median age 15 years, 0 to 90 years old) formerly identified as positive for *Bordetella pertussis* (IS481+) by routine PCR in 2011-2015, were subjected to real-time PCR targeting the insertion sequences IS1002 and IS1001.

Results:

The results showed that 2.3% of the samples were false-positive for *Bordetella pertussis*.

Conclusion:

In conclusion, we found that misidentification of *Bordetella pertussis* using IS481 PCR is limited in Danish patients.

Keywords: *Bordetella pertussis*, Real-time PCR, IS481, IS1002, IS1001, False-positive.

Article History

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1. INTRODUCTION

Whooping cough is a highly contagious respiratory disease. It causes both local outbreaks and epidemics despite being vaccine-preventable. Whooping cough continues to be a significant cause of morbidity and infant mortality worldwide. Ten different species compose the genus *Bordetella*, four of which are known to infect humans: *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella holmesii* and *Bordetella bronchiseptica*. Humans are the sole reservoir for *B. pertussis*, which is the primary cause of whooping cough or pertussis. *B. parapertussis* may be responsible for up to 20% of the cases of pertussis-like disease, but the illness is often less severe than the one caused by *B. pertussis* [1]. *B. holmesii* is associated

with bacteremia, infective endocarditis, and respiratory illness, particularly in functionally or anatomically asplenic patients. However, *B. holmesii* has also been isolated from nasopharyngeal specimens of immunocompetent individuals with pertussis-like illness. *B. bronchiseptica* is primarily an animal pathogen, but will occasionally infect humans and, like *B. holmesii*, *B. bronchiseptica*, may cause invasive disease. Known cases describe bacteremia, peritonitis and respiratory tract infection in immunocompromised patients [2, 3].

PCR is the prevailing method used for detection of *Bordetella pertussis* in clinical samples, being fast, sensitive and less laborious compared to culturing. Molecular detection of *B. pertussis* is commonly based on insertion sequence IS481, which is present in multiple copies (>50 copies) in the *B. pertussis* genome, increasing the sensitivity of the PCR [4]. A high sensitivity of the assay is necessary in order to

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document the high incidence of pertussis, continuously found in the local population [5]. However, the target sequence IS481 is also present in the genomes of *B. holmesii* (about 8-10 copies), and of some *B. bronchiseptica* [1]. In this study, a method described by Roorda *et al.* [6] involving a PCR assay targeting IS1001 and IS1002 as well as IS481 is used for specific detection of *B. pertussis*. IS1002 is present in the genomes of *B. pertussis* and *B. parapertussis*, while IS1001 is found in all *B. parapertussis* and in a few *B. bronchiseptica*. Thus, a specimen containing *B. pertussis* will be positive for IS481 and IS1002 and negative for IS1001 Table 1 [1].

Table 1. *Bordetella* species and presence of insertion sequences.

Presence/No. of Copies per Genome			
Species	IS481	IS1001	IS1002
<i>B. pertussis</i>	+/>50	-	+/4-10
<i>B. parapertussis</i>	-	+/-20	+/9
<i>B. holmesii</i>	+/8-10	-	-
<i>B. bronchiseptica</i>	- ^a	- ^a /1-7	-

^a Isolates of *B. bronchiseptica* can sometimes contain few copies of IS481 and/or IS1001.

The objective of this study was to retrospectively evaluate the proportion of other *Bordetella* species potentially misidentified as *B. pertussis* in patient samples collected between 2011 and 2015 in our region using IS481 based real-time PCR (rt-PCR).

2. MATERIALS AND METHODS

Clinical specimens were sputum and throat swabs collected from 228 Danish patients identified as positive for *B. pertussis* by routine PCR in 2011-2015. Stored extracted DNA (NucliSENS[®] easyMAG[®], bioMérieux) previously positive for IS481 was reanalyzed by rt-PCR targeting the insertion sequences IS1002 and IS1001. Samples had been kept for quality control and were anonymized, therefore patients did not have to provide informed written consent.

Target sequences of the PCR were IS481, IS1001, and IS1002 Table 2. Phocine Herpes Virus (PhHV) was added to the samples as internal control in order to monitor the DNA

extraction as well as the PCR amplification efficiency. Furthermore, negative control, as well as positive target controls were included in each run.

PCR assays for detection of IS481 were performed in 25 µl reaction volume containing 1× Lightcycler[®] 480 Probes master (Roche), 0.5 µM of IS481 forward and reverse primers, 0.2 µM of the IS481 probe and 5 µl extracted DNA. The amplification of IS1001, IS1002 and PhHV were run in separate reaction mixes containing 1× Lightcycler[®] 480 Probes master (Roche), 1.0 µM of each primer, 0.2 µM of the probe and 5 µl of extracted DNA. Amplification was carried out on a Lightcycler[®] 480 (Roche). The temperature profile included initial denaturation of 10 min at 95°C followed by 45 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 1 sec. The Crossing Point (Cp) values were determined by the 2nd Derivative Max method using the Lightcycler[®] 480 software. A Cp value <40 combined with an acceptable curve was considered positive.

3. RESULTS

The Cp values obtained from the IS481 PCR-positive samples were on average 5.3 Cp values lower than the Cp values obtained from the IS1002 PCR, presumably due to differences in copy number of the target sequence (approximately >50 and 10 copies, respectively). Of the 228 clinical samples positive for *B. pertussis* (IS481+) by routine PCR, we therefore only included samples with a Cp value of <32 in the IS481 PCR in order to rule out that any negative result in the IS1002 PCR was due to lower sensitivity. 131 samples obtained a Cp value <32 in the IS481 PCR. 128 of these were also positive in the IS1002 PCR confirming the species identity as *B. pertussis* (IS481+ and IS1002+). Hence the three IS1002 negative samples were subjected to PCR targeting IS1001; all three were found negative (IS481+, IS1002- and IS1001-) and therefore did not contain *B. parapertussis* or IS1001 positive *B. bronchiseptica* DNA. Thus, three samples (2.3%) had been misidentified as *B. pertussis*. Presumably, these samples contained either *B. holmesii* or, less likely, *B. bronchiseptica*.

Patients had a median age of 15 years (0-90 years-old) with 59% of females.

Table 2. Real-time PCR assays targeting the three IS elements and the internal control Phocine Herpes virus (PhHV).

PCR Assay	Label	Sequence (5'-3')	References
IS481	Forward primer	CGG ATG AAC ACC CAT AAG CAT	[14]
	Reverse primer	CGA TCA ATT GCT GGA CCA TTT	
	Probe	FAM-CCC GAT TGA CCT TCC TAC GTC GAC TC-BHQ1	
IS1001	Forward primer	AAT TGC TGC AAG CCA ACC A	[6]
	Reverse primer	CCA GAG CCG TTT GAG TTC GT	
	Probe	FAM-ACA TAG ACC GTC AGC AG-MGB	
IS1002	Forward primer	CTA GGT CGA GCC CTT CTT GTT AAC	[6]
	Reverse primer	GCG GGC AAG CCA CTT GTA	
	Probe	FAM-CAT CGT CCA GTT CTG TTG CAT CAC CC-BHQ1	
PhHV	Forward primer	GGG CGA ATC ACA GAT TGA ATC	[15]
	Reverse primer	GCG GTT CCA AAC GTA CCA A	
	Probe	FAM-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-BHQ1	

4. DISCUSSION

In this retrospective study, we found that in 131 samples identified with routine non-discriminating *B. pertussis* IS481 rt-PCR, the bacterium had been misidentified in three samples. The three samples all originated from male adolescents (ages 14, 15 and 21 years-old) and presumably contained *B. holmesii*. This is consistent with observations from France and the Netherlands of *B. holmesii* in nasopharyngeal-samples from adolescents and young adults [7, 8]. We found a false-positive rate of 2.3%. Other studies have reported a much higher incidence of false-positive *B. pertussis*. Njamkepo *et al.* [7] found that the proportion of *B. holmesii* falsely identified as *B. pertussis* with IS481 PCR was 20.3% of samples collected from French adolescents and adults (n=59). From Chilean patients with suspected pertussis Miranda *et al.* [9] detected *B. holmesii* DNA in 11.1% of IS481-positive samples (n=99). In Norway, Reinton *et al.* [10] detected *B. holmesii* DNA in 5.8% of *B. pertussis* positive samples (n=87) and no *B. bronchiseptica* DNA in 375 other *B. pertussis* positive samples. In other European countries, no *B. holmesii* DNA was detected in nasopharyngeal samples from Finnish (n=2804), Dutch (n=6903) and Belgian (n=1493) patients with pertussis-like illness [3, 11]. Moreover, in Swiss patients, no *B. holmesii* DNA was detected in 194 *B. pertussis* positive samples [2].

The implications of misidentification of *B. pertussis* are currently unclear. Still, correct identification of *B. pertussis* is considered important for several reasons. Firstly, *in vitro* studies suggest that macrolides used to treat *B. pertussis* infections are less effective against *B. holmesii* and probably also against *B. bronchiseptica*. Secondly, a false-positive *B. pertussis* may cause prescription of unnecessary post-exposure prophylaxis, and thirdly, *B. holmesii* or *B. bronchiseptica* misdiagnosed as *B. pertussis* may suggest vaccine failure and lead to excessive public health interventions [1 - 3, 12].

It has been suggested to use multiple target PCR assays for detection of *B. holmesii* and *B. bronchiseptica* in concordance with IS481 to rule out false-positive *B. pertussis* or to use confirmatory PCR assays for specific detection of *B. pertussis* [13 - 15]. However, this will significantly increase workload, cost and turnaround time. As the confirmatory assays are less sensitive than the IS481 PCR, they may come out negative in samples with low amounts of *B. pertussis*, resulting in false-negative reports. In a clinical setting, sensitivity, as well as rapid diagnostics must be prioritized to ensure appropriate treatment and prevention of further transmission.

CONCLUSION

We have shown that false-positive diagnostics of *B. pertussis* using IS481 PCR is limited in Danish patients. This is consistent with the results from other studies [2, 3, 11, 12]. Our priority remains to diagnose *B. pertussis* infection. Thus, the rt-PCR assay targeting IS481 combines high sensitivity with an acceptable false-positive rate of 2.3% if the Cp is set to <32. Supplementary assays should not be necessary on a routine basis but may be applied in situations with clinical suspicion of false-positive results.

ABBREVIATIONS

IS	=	Insertion Sequence
PhHV	=	Phocine Herpes Virus
Cp	=	Crossing Point

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors have no conflict of interests.

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Declared none.

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