

RESEARCH ARTICLE

# Improving specificity of *Bordetella pertussis* detection using a four target real-time PCR

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## Abstract

The incidence of whooping cough, a contagious respiratory disease caused by *Bordetella pertussis*, is on the rise despite existing vaccination programmes. Similar, though usually milder, respiratory symptoms may be caused by other members of the *Bordetella* genus: *B. parapertussis*, *B. holmesii*, and *B. bronchiseptica*. Pertussis diagnosis is mostly done using PCR, but the use of multiple targets is necessary in order to differentiate the different *Bordetella* spp. with sufficient sensitivity and specificity. In this study we evaluate a multiplex PCR assay for the differentiation of *B. pertussis* from other *Bordetella* spp., using the targets IS481, IS1001, IS1002, and *recA*. Moreover, we retrospectively explore the epidemiology of *Bordetella* spp. infections in Belgium, using the aforementioned assay over a three-year period, from 2013 until 2015.

## Introduction

Whooping cough or pertussis is a contagious, acute respiratory illness caused by the Gram-negative coccobacillus *Bordetella pertussis* [1]. It is characterised by a paroxysmal cough, although symptoms can be atypical in infants younger than five months, and even fatal [2]. Pertussis in adolescents and adults is typically milder and presents with less severe symptoms [3], but is still of particular concern due to the associated risk of contagion [4]. Despite existing vaccination programmes, the number of whooping cough cases is still rising in Europe [5, 6] and North America [7].

Besides *B. pertussis*, three other members of the *Bordetella* genus are associated with respiratory infection in humans: *B. parapertussis*, *B. holmesii*, and to a lesser degree *B. bronchiseptica* [8]. These *Bordetella* species can cause respiratory disease with nonspecific symptoms as well as symptoms similar to those caused by *B. pertussis*, but milder [9]. However, pertussis vaccines do not provide cross-protection against these related *Bordetella* spp. [10, 11] Therefore, the differentiation of *B. pertussis* from other *Bordetella* spp. in respiratory illness is important for the evaluation of the vaccines' efficacy, as misdiagnosis can lead to misinterpretation of the

vaccines' failure rate. Moreover, accurate identification of *B. pertussis* is needed to determine the necessity of prophylactic antibiotic treatment for the index cases and their contacts, as its utility in *B. parapertussis* and *B. holmesii* cases is still unclear [12, 13, 14].

As culture is difficult and lacks sensitivity, the diagnosis of *Bordetella* spp. infections often involves real-time polymerase chain reaction (PCR) of nasopharyngeal specimens, which is much more sensitive than bacterial culturing [15, 16, 17]. Segments of insertion sequences (IS) are often used as targets, particularly IS481 and IS1001 for *B. pertussis* and *B. parapertussis* respectively. Tests using these targets are sensitive because the IS are present in numerous copies in the bacterial genomes, but they are not species-specific. IS481 is found in *B. pertussis*, *B. holmesii*, and some *B. bronchiseptica* [16]. IS1001 is found in *B. parapertussis* and some *B. bronchiseptica* [17]. While hIS1001, which is found in *B. holmesii*, is highly homologous to IS1001, there are some sequence differences [17]. Thus, IS481 and IS1001 can be used as targets for screening, but confirmation with other targets will improve specificity.

IS1002 and *recA* have been used previously as secondary targets [18, 19]. IS1002 is found in *B. pertussis*, *B. parapertussis*, and some *B. bronchiseptica*, while *recA* is found in *B. holmesii*. Other *Bordetella* spp. also carry *recA*, but several polymorphisms allow for specific targeting [18]. When combined, the targets IS481, IS1001, IS1002, and *recA* can be used to differentiate *Bordetella* spp. in respiratory illness. IS481 and IS1001 can be used to screen for *B. pertussis* and *B. parapertussis* respectively, while IS1002 can confirm a positive screen, or alternatively, absence of IS1002 and presence of *recA* confirms a case of *B. holmesii*.

Most commercially available diagnostic PCR assays for pertussis use fewer targets and do not differentiate between *B. pertussis* and other *Bordetella* spp. such as *B. holmesii*. In order to improve specificity, we evaluate the use of the four PCR targets IS481, IS1001, IS1002, and *recA*, in the differentiation of *B. pertussis* from other *Bordetella* spp. that cause respiratory illness. Furthermore, we describe the incidence of respiratory infections caused by *Bordetella* spp. in Belgium from 2013 until 2015 using the described assay.

## Materials and methods

### Clinical sample preparation

Prior to nucleic acid (NA) extraction, viscous samples were pre-treated with Sputasol (Oxoid Ltd., Basingstoke, England) in a 1:1 dilution. 100  $\mu$ l of each sample was used for NA extraction using NucliSENS easyMag (bioMérieux, Grenoble, France) according to the manufacturer's instructions. Phocine herpesvirus (PhHV) was added as an internal control to monitor the extraction and the amplification's efficiency [20].

### Bacterial strain preparation

Reference strains as well as previously well-defined strains from clinical isolates were stored at  $-80^{\circ}\text{C}$  prior to use, then grown on laboratory-prepared Regan-Lowe agar plates (Oxoid Ltd., Basingstoke, England) at  $35^{\circ}\text{C}$ . One colony of each strain was suspended in 200  $\mu$ l Tris-EDTA (pH 8) and boiled to obtain the template NA.

### Real-time PCR

Primers and probes for IS481, IS1001, and IS1002 were based on Roorda et al. (2011) [19], those for *recA* were based on Guthrie et al. (2010) [18], and those used for internal control were based on van Doornum et al. (2003) [21] (sequences in Table 1). Primers and probes were manufactured by Eurogentec (Liège, Belgium), MGB probes were manufactured by Applied Biosystems (Warrington, UK).

**Table 1. Sequences and labelling of real-time PCR primers and probes.**

Target	Primer/probe	Sequence (5'-3') and labels
IS481	Forward primer	GCCGGATGAACACCCATAAG [19]
	Reverse primer	GCGATCAATTGCTGGACCAT [19]
	Probe	6FAM-CGATTGACCTTCTTACGTC-MGBNFQ [19]
IS1001	Forward primer	AATTGCTGCAAGCCAACCA [19]
	Reverse primer	CCAGAGCCGTTTGTGTTTCGT [19]
	Probe	VIC-ACATAGACCGTCAGCAG-MGBNFQ [19]
IS1002	Forward primer	CTAGGTCGAGCCCTTCTTGTAAAC [19]
	Reverse primer	GCGGGCAAGCCACTTGTGA [19]
	Probe	HEX-CATCGTCCAGTTCTGTGCATCACCC-BHQ1 [19]
recA	Forward primer	CGGTTGCTGGGTCTCG [18]
	Reverse primer	CCCGCGGCAGACCAC [18]
	Probe	6FAM-CATCGCATTGGGCG-MGBNFQ [18]
PhHV	Forward primer	GGGCGAATCACAGATTGAATC [21]
	Reverse primer	GCGGTTCCAAACGTACCAA [21]
	Probe	CY5-TTTTATGTGTCGCCACCATCTGGATC-BHQ2 [21]

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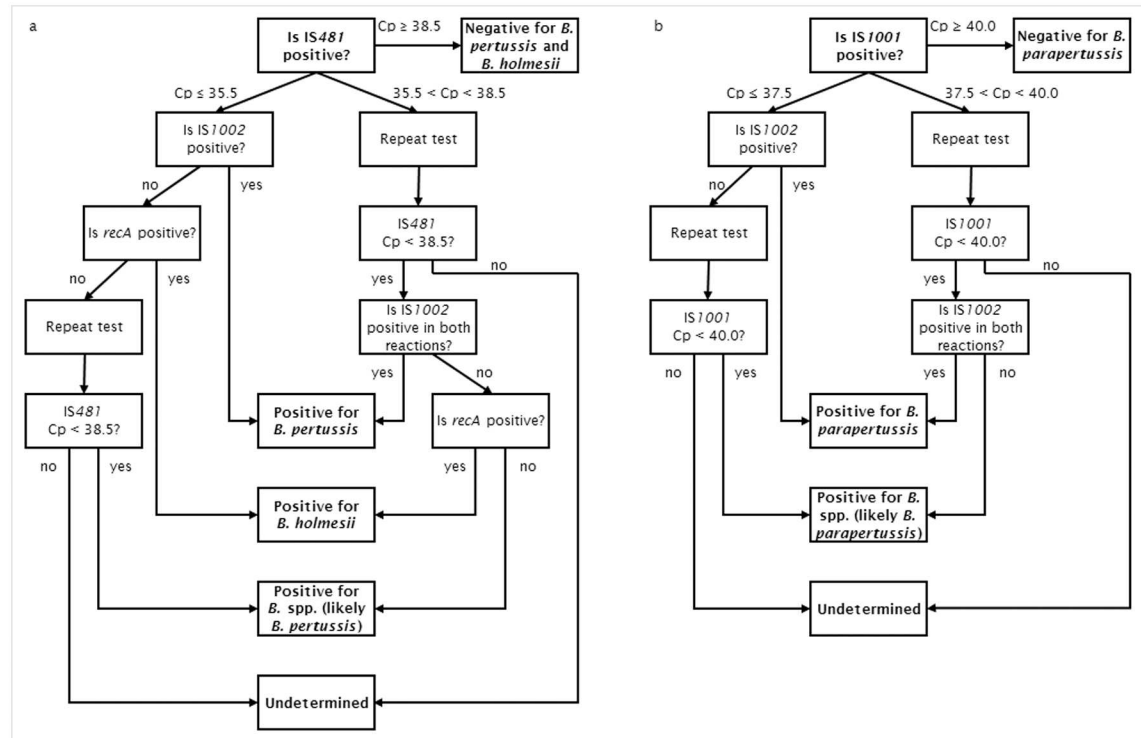
Two different multiplex PCR's were performed, one for the detection of IS481 and IS1001, and one for the detection of *recA* and IS1002. A 50 µl reaction mixture consisting of 0.3 µM of each of the primers, 0.2 µM of each of the probes, iQ Multiplex Powermix (Bio-Rad Laboratories, Temse, Belgium), and 5.0 µl of the extracted NA was used in each PCR. Amplification was performed on the LightCycler 480 II PCR system (Roche Diagnostics, Mannheim, Germany). The PCR thermal profile consisted of a 3-minute cycle at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. Acquisition of the fluorescence signal was set during each cycle. A final cooling step of 30 seconds at 40°C was included. The crossing point (Cp) values were determined automatically using the “second derivative maximum” method present in the Roche LightCycler 480 Software, version 1.5. An algorithm with cut-off values was developed and used for the interpretation of the real-time PCR results (Fig 1).

### Bacterial culture

For culture, clinical specimens were inoculated onto laboratory-prepared Regan-Lowe agar upon arrival at the facilities. Plates were incubated at 35°C in a humidified aerobic atmosphere and examined daily for suspect colonies for up to twelve days.

Suspect colonies were identified using the microflex LT MALDI-TOF platform and MALDI Biotyper 3.0 software (Bruker Daltonics GmbH, Leipzig, Germany) [22, 23]. Identification to the species level was accepted when all matches with a log score above 1.7 belonged to the same species, or when the best match had a log score both above 2.0 and more than 0.200 higher than that of the other matched species.

When MALDI-TOF results were inconclusive, the species was determined based on biochemical characteristics: growth or absence of growth on charcoal agar, Haemophilus agar and MacConkey agar, presence of oxidase and presence of urease. Positive results for all characteristics indicated *Bordetella bronchiseptica*. Growth on charcoal agar and Haemophilus agar and presence of urease but not oxidase indicated *Bordetella parapertussis*. Growth on charcoal agar only, combined with the presence of oxidase but not urease indicated *Bordetella pertussis* [24].



**Fig 1. Algorithm for the interpretation of the four-target real-time PCR assay for the detection of *Bordetella* for samples positive for IS481 (a) and IS1001 (b).**

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### Accuracy of real-time PCR

Accuracy was verified using a group of selected, previously well characterised strains derived from clinical samples, as well as reference strains (ATCC9340, ATCC9797, LMG15945, LMG15946), samples from Quality Control for Molecular Diagnostics (QCMD) 2011, and the extracted NA of different strains from a EUpert-labnet network panel (<http://www.ecdc.europa.eu/en/publications/Publications/20120906-TER-EQA-pertussis.pdf>). These samples included twenty-seven *B. pertussis* samples, nineteen *B. parapertussis* samples, seven *B. holmesii* samples, four samples of other *Bordetella* spp. and five non-*Bordetella* samples.

### Analytical specificity of real-time PCR

The specificity of the primers and probes was first checked by performing a BLAST search on their sequences (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Specificity was further tested by performing the previously described real-time PCR on twelve well-defined *B. bronchiseptica* strains and seven *B. holmesii* strains, as well as other *Bordetella* spp. (*B. avium* (n = 2), *B. petrii* (n = 2), *B. hinzii* (n = 4), *B. trematum* (n = 2), *B. ansorpii* (n = 2)). The following bacteria of different genera were also tested: *Acinetobacter baumani* (n = 1), *A. iwoffii* (n = 1), different *Burkholderia* spp. (n = 20), *Enterobacter aerogenes* (n = 2), *Haemophilus influenzae* (n = 2), *Klebsiella pneumoniae* (n = 1), *Moraxella catarrhalis* (n = 1), *Neisseria meningitidis* (n = 1), *Pseudomonas aeruginosa* (n = 2), *P. putida* (n = 2), *Staphylococcus aureus* (n = 2), *Stenotrophomonas maltophilia* (n = 1), and different *Streptococcus* spp. (n = 8).

## Analytical sensitivity of real-time PCR

Analytical sensitivity was determined using three reference strains: ATCC9797 (*B. pertussis*), ATCC15311 (*B. parapertussis*), and LMG15945 (*B. holmesii*). They were suspended in physiological saline at a theoretical concentration of  $1.5 \times 10^8$  CFU/ml (0.5 McFarland). Serial dilutions were made from these suspensions, and 100- $\mu$ l aliquots of each dilution were extracted and tested with real-time PCR. Tests were performed ten to twelve times for each strain. The analytical sensitivity was determined by calculating the limit of detection (LOD 95%), i.e. the lowest concentration that is detected 95% of the time, using probit regression analysis in SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA).

## Precision of real-time PCR

Replication experiments were performed to determine precision, using three dilutions (strong positive, positive and weak positive) of the reference strains in physiological water. These samples were tested at least ten times in five different runs over the course of five days. The intra-run and inter-run coefficients of variance (CV) were calculated based on the  $C_p$  value of the assay using SPSS Statistics 20.0.

## Laboratory-based surveillance of pertussis

Surveillance data on circulating *B. pertussis*, *B. parapertussis* and *B. holmesii* in Belgium, based on the results of the real-time PCR for IS481, IS1001, *recA*, and IS1002, were gathered retrospectively from 11919 respiratory samples collected from January 2013 until December 2015.

## Results

### Accuracy of real-time PCR

All clinical strains and reference strains were identified correctly: all *B. pertussis* strains were positive for IS481 and IS1002, and negative for *recA* and IS1001. All *B. parapertussis* strains were positive for IS1001 and IS1002, and negative for *recA* and IS481. All *B. holmesii* strains were positive for IS481 and *recA*, and negative for IS1001 and IS1002.

Out of the twelve QCMD samples, all but one sample tested correctly: four out of five *B. pertussis* strains were positive for IS481 and IS1002, and negative for *recA* and IS1001, only one very weak positive (10 CFU/ml) *B. pertussis* sample was negative for all targets. The *B. parapertussis* strain was positive for IS1001 and IS1002, and negative for *recA* and IS481. The *B. holmesii* strain was positive for IS481 and *recA*, and negative for IS1001 and IS1002. The *B. bronchiseptica*, *B. hinzii*, and non-*Bordetella* samples tested negative for all targets.

Finally, out of ten NA samples from the EUpert-labnet network panel, eight were identified correctly. One *B. pertussis* NA sample with a low concentration of 0.02 pg/ $\mu$ l was negative for IS1002, but positive for IS481. One *B. holmesii* sample was positive for IS1001 as well as for IS481 and *recA*. Using the algorithm developed for our assay, these results did not lead to an incorrect identification (S1 Table).

### Analytical specificity of real-time PCR

BLAST analysis showed that IS481 and IS1001 may cross-react with *B. bronchiseptica* and *B. holmesii*. IS1002 was specific for *B. pertussis* and *B. parapertussis*. Finally, *recA* showed cross-reactivity with some *Burkholderia* spp.

Following BLAST analysis the assay was performed on different strains. Six out of seven *B. holmesii* strains were negative for both IS1001 and IS1002, one was positive for IS1001. Out of twelve tested *B. bronchiseptica* strains, four were positive for IS481 and two for IS1001. One

IS481-positive strain was also positive for IS1002, and as such could have been misinterpreted as *B. pertussis*. No cross-reaction was detected in other *Bordetella* spp.

As BLAST analysis suggested possible cross-reactivity of *recA* with some *Burkholderia* spp., this target was tested in twenty *Burkholderia* strains, belonging to eleven different species. Cross-reaction was detected in one *Burkholderia stabilis* strain and one *Burkholderia multivorans* strain. No cross-reaction with any of the other targets was found.

Finally, the assay was tested with various other bacteria, including bacteria that cause respiratory infections as well as commensals. Minor cross-reactivity was found in *Stenotrophomonas maltophilia* for IS481 and in *Enterobacter aerogenes* for IS1001, however, the other targets remained negative (S1 Table).

### Analytical sensitivity of real-time PCR

The limit of detection (LOD 95%) for IS481 was 145 CFU/ml for *B. pertussis* and 152 CFU/ml for *B. holmesii*. For IS1001, the LOD 95% was 242 CFU/ml for *B. parapertussis*. The *recA* assay had an LOD 95% of 2577 CFU/ml for *B. holmesii*. Finally, for IS1002 the LOD 95% was 1954 CFU/ml for *B. pertussis* and 1725 CFU/ml for *B. parapertussis* (S1 Table).

### Precision of real-time PCR

The coefficients of variance (CV) for the detection of IS481 ranged from 1.1% to 8.4% intra-run, and from 1.8 to 5.4% inter-run. For IS1001, they ranged from 1.7% to 3.7% intra-run, and from 0.8% to 2.0% inter-run. The intra-run CV for *recA* ranged from 1.8% to 2.4%, and the inter-run CV ranged from 0.8% to 1.2%. Finally, for IS1002, the intra-run and inter-run CV ranged from 1.9% to 4.0% and from 0.3% to 1.9% respectively for *B. pertussis*, and from 2.3% to 6.2% and from 1.3% to 3.8% respectively for *B. parapertussis* (S1 Table).

### Laboratory-based surveillance of pertussis

The Belgian National Reference Centre (NRC) for *B. pertussis* performed real-time PCR on 11919 respiratory samples between 2013 and 2015. These data were interpreted using the algorithm presented above (Fig 1). Among these samples, 1189 (10.0%) were found positive for *B. pertussis*. 872 (73.3%) of these were positive for both IS481 and IS1002 and reported “positive for *B. pertussis*”, while 317 (26.7%) were positive for IS481 only and reported as “positive for *Bordetella* spp., probably *B. pertussis*”. 151 samples (1.3%) were reported positive for *B. parapertussis*, of which 112 (74.2%) were positive for both IS1001 and IS1002 (reported “positive for *B. parapertussis*”), and 39 (25.8%) positive for IS1001 only (reported as “positive for *Bordetella* spp., probably *B. parapertussis*”). Furthermore, 15 samples (0.1%) were found positive for *B. holmesii*, and 425 samples (3.6%) were reported as “undetermined”, i.e. they tested positive for IS481 or IS1001 but negative for IS1002 and *recA*, then negative for all targets upon repeat testing (S2 Table).

Of the samples found positive for *B. pertussis* with real-time PCR, 28.8% were also positive in culture. For *B. parapertussis*, this percentage was similar at 29.8%. Only two of the samples reported as “positive for *Bordetella* spp., probably *B. pertussis*” and none of the samples reported as “positive for *Bordetella* spp., probably *B. parapertussis*” were recovered in culture. These samples were associated with higher Cp values. The association between Cp value and culture recovery is presented in Table 2. Out of the fifteen *B. holmesii* samples, five were found positive in culture.

A few samples were found positive for more than two targets. Seven samples were positive for IS481, IS1001 and IS1002, and were interpreted as co-infection of *B. pertussis* and *B. parapertussis*. In culture, two of these were found positive for *B. pertussis* and three for *B.*

**Table 2. Association between Cp value and recovery in culture, for samples positive for IS481 (a) and IS1001 (b).**

a	Cp value IS481	PCR positive	Culture positive ( <i>B. pertussis</i> )	Recovery culture/PCR
	Cp ≤ 20	165	133	80.6%
	20 < Cp ≤ 25	122	81	66.4%
	25 < Cp ≤ 30	227	89	39.2%
	Cp > 30	675	39	5.8%
	Total	1189	342	28.8%
b	Cp value IS1001	PCR positive	Culture positive ( <i>B. parapertussis</i> )	Recovery culture/PCR
	Cp ≤ 20	7	7	100.0%
	20 < Cp ≤ 25	16	13	81.3%
	25 < Cp ≤ 30	22	14	63.6%
	Cp > 30	105	11	10.5%
	Total	151	45	29.8%

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*parapertussis*. Two other samples were positive for IS481, *recA* and IS1002 and were interpreted as co-infection of *B. pertussis* and *B. holmesii*. These samples were negative in culture.

## Discussion

In this study, the use of a multiplex real-time PCR assay for the detection of *B. pertussis*, *B. parapertussis*, and *B. holmesii* was investigated. By combining a sensitive screening method with a confirmation assay, the risk of false positive results is reduced and the specificity increased. The strength of our study lies in the extensive testing of the real-time PCR targets, during validation and during a three-year study with clinical samples.

Our real-time PCR assay uses the targets IS481/IS1001 for screening and *recA*/IS1002 for confirmation. We showed that this method is accurate, sensitive, fairly specific, and precise. *B. pertussis*, *B. parapertussis*, and *B. holmesii* reference and clinical strains were identified correctly. The detection limit of our method was in line with that of other assays described for IS481 and IS1001, ranging from 145 to 242 CFU/ml for *B. pertussis*, *B. parapertussis* and *B. holmesii* [19, 25, 26, 27]. We also found that the sensitivity of the PCR for *recA*/IS1002 is around ten times lower than that of the PCR for IS481/IS1001. This lower sensitivity can be explained by the lower copy numbers of *recA* and IS1002 genes.

The targets used in this study were fairly specific. The targets of the screening assay, IS481 and IS1001, do not show cross-reactivity with other respiratory pathogens. However, they do show cross-reactivity with *B. holmesii* and sometimes with *B. bronchiseptica*. IS481, IS1001 and IS1002 have all been previously detected in some *B. bronchiseptica* strains [19]. Therefore the possibility of cross-reactivity should be taken into account when using these targets. This problem is limited by the use of target combinations: both IS481 (screening) and IS1002 (confirmation) need to be positive to identify *B. pertussis*, both IS1001 and IS1002 need to be positive to identify *B. parapertussis*.

Using our method, about a fourth of the cases positive for IS481 remained negative for IS1002 (and *recA*). These cases are reported as “positive for *Bordetella* spp., probably *B. pertussis*”. Samples positive for IS1001 but negative for IS1002 (and *recA*) are reported as “positive for *Bordetella* spp., probably *B. parapertussis*”. Cases such as these occur relatively frequently due to the difference in sensitivity between the screening and the confirmation targets, the latter being present in much lower copy numbers.

For weak positive results (i.e. high Cp values) in the screening assay, cross-reactivity, in particular with *B. bronchiseptica*, cannot be ruled out. However, *B. bronchiseptica* is rarely found

in respiratory samples from patients displaying symptoms of whooping cough: in over 2000 tested samples only one cross-reacting *B. bronchiseptica* strain was cultured [28]. Therefore the probability of *B. bronchiseptica* causing a false positive result may be considered negligible. Because of the low occurrence of *B. holmesii* and *B. bronchiseptica* in patients displaying pertussis symptoms, it is acceptable to consider cases with a Cp lower than 38.5 for IS481 (or lower than 40 for IS1001) as probable cases of *B. pertussis* (or *B. parapertussis*), even if they are negative for IS1002 and *recA*.

Based on the extensive validation of the real-time PCR, an algorithm was developed for its interpretation (Fig 1). Using high copy number targets such as IS481 and IS1001 increases the sensitivity of the assay at the cost of an increased risk of false positives. This risk can be reduced by combining multiple targets, as demonstrated in this study.

The implementation of cut-off values will also reduce false positivity, as shown previously in a quality control study [29], and is recommended by the CDC (<http://www.cdc.gov/pertussis/clinical/diagnostic-testing/diagnosis-pcr-bestpractices.html>).

In this study, a cut-off value based on the LOD 95%, determined during validation of the assay, was used. Positive results with a Cp value higher than this value should be repeated, and discordant results should be reported to the requesting physician as “undetermined”, to be considered negative or investigated further based on clinical presentation and serology. A second cut-off value to reduce false positivity was implemented as well. Samples with Cp values higher than this cut-off are immediately reported as negative.

During the study period, *B. pertussis* was diagnosed about ten times as much as *B. parapertussis*, which was diagnosed about ten times as much as *B. holmesii*. Less than four percent of the clinical samples could not be reliably identified using our assay (“undetermined”).

By including the target *recA* in the assay, *B. holmesii* can be differentiated from *B. pertussis*. This is important in routine diagnosis as *B. holmesii* is increasingly reported in Europe, and has often been misidentified as *B. pertussis* in the past [13, 14, 28]. Using our assay, we found fifteen *B. holmesii* strains during the study period, suggesting that *B. holmesii* might indeed occur more frequently than previously reported in Belgium [28].

The algorithm was not developed for the interpretation of co-infections, and they are difficult to identify with the presented method. However, co-infection is likely if the assay is positive for more than two targets. Several possible co-infections were detected during the study that could not be confirmed by culture. Based on the large amount of samples, it seems that the likelihood of co-infections is limited (less than 0.1%).

We demonstrated in this study that there is a high concordance between the Cp value of a sample and the probability of recovery in culture. This finding is consistent with previous results [30]. As culturing of *B. pertussis* is no longer performed diagnostically, but rather for the acquiring of strains to be used in epidemiologic typing and research, it may be worthwhile to try to culture only samples with low Cp values. For *B. pertussis*, culturing has a high success rate for Cp values up to 25. For Cp values between 25 and 30, this rate drops to less than 50%. Culturing in these cases should still be considered for laboratories with a particular interest in obtaining as many strains as possible for epidemiological and virulence studies. When Cp values are higher than 30 the success rate becomes negligible (less than 6%) and culturing is not advisable.

Several PCR targets specific to *B. pertussis* have been described in literature, such as the pertussis toxin promoter (*ptxP*), recommended by the Pertussis PCR Consensus Group [31]. However, the sensitivity of real-time PCR with *ptxP* as a target is considerably lower than with IS481 [32]. Other possible targets for *B. pertussis*, such as the pertactin genes, BP283, BP485, and *ptxS1* have also been described, but often show cross-reactivity with other *Bordetella* spp. [33, 34] Recently an assay with a porin protein as target was described as specific for *B. pertussis*, but a larger specificity study would be needed to confirm this [32].



In conclusion, multiplex real-time PCR with targets IS481/IS1001 can be used for screening, followed by confirmation with targets *recA*/IS1002, in order to differentiate *B. pertussis* from other *Bordetella* spp. that cause respiratory infection or colonisation.

## Supporting information

**S1 Table. Results used to determine accuracy, specificity, analytical sensitivity and precision.**

(XLSX)

**S2 Table. Clinical results from January 2013 until December 2015.**

(XLSX)

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**Conceptualization:** HM LD OS EY DP.

**Formal analysis:** HM LD EY.

**Funding acquisition:** DP.

**Investigation:** LD.

**Methodology:** LD OS EY.

**Project administration:** OS DP.

**Resources:** DP.

**Supervision:** DP.

**Validation:** LD OS.

**Visualization:** HM LD.

**Writing – original draft:** LD EY.

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## References

1. Snyder J, Fisher D. Pertussis in childhood. *Pediatr Rev.* 2012; 33(9):412–20; quiz 20–1. <https://doi.org/10.1542/pir.33-9-412> PMID: 22942366
2. Crowcroft NS, Booy R, Harrison T, Spicer L, Britto J, Mok Q, et al. Severe and unrecognised: pertussis in UK infants. *Arch Dis Child.* 2003; 88(9):802–6. <https://doi.org/10.1136/adc.88.9.802> PMID: 12937105
3. Barlow RS, Reynolds LE, Cieslak PR, Sullivan AD. Vaccinated children and adolescents with pertussis infections experience reduced illness severity and duration, Oregon, 2010–2012. *Clin Infect Dis.* 2014; 58(11):1523–9. <https://doi.org/10.1093/cid/ciu156> PMID: 24633685
4. Menard A, Lehours P, Sarlangue J, Bebear C, Megraud F, de Barbeyrac B. Development of a real-time PCR for the identification of *Bordetella pertussis* and *Bordetella parapertussis*. *Clin Microbiol Infect.* 2007; 13(4):419–23. <https://doi.org/10.1111/j.1469-0691.2006.01659.x> PMID: 17359327

5. de Melker HE, Schellekens JF, Neppelenbroek SE, Mooi FR, Rumke HC, Conyn-van Spaendonck MA. Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. *Emerg Infect Dis.* 2000; 6(4):348–57. <https://doi.org/10.3201/eid0604.000404> PMID: [10905967](https://pubmed.ncbi.nlm.nih.gov/10905967/)
6. Sizaire V, Garrido-Esteba M, Masa-Calles J, Martinez de Aragon MV. Increase of pertussis incidence in 2010 to 2012 after 12 years of low circulation in Spain. *Euro Surveill.* 2014; 19(32).
7. Cherry JD. Epidemic pertussis in 2012—the resurgence of a vaccine-preventable disease. *N Engl J Med.* 2012; 367(9):785–7. <https://doi.org/10.1056/NEJMp1209051> PMID: [22894554](https://pubmed.ncbi.nlm.nih.gov/22894554/)
8. Mattoo S, Foreman-Wykert AK, Cotter PA, Miller JF. Mechanisms of *Bordetella* pathogenesis. *Front Biosci.* 2001; 6:E168–86. PMID: [11689354](https://pubmed.ncbi.nlm.nih.gov/11689354/)
9. Cherry JD, Seaton BL. Patterns of *Bordetella parapertussis* respiratory illnesses: 2008–2010. *Clin Infect Dis.* 2012; 54(4):534–7. <https://doi.org/10.1093/cid/cir860> PMID: [22156857](https://pubmed.ncbi.nlm.nih.gov/22156857/)
10. Khelef N, Danve B, Quentin-Millet MJ, Guiso N. *Bordetella pertussis* and *Bordetella parapertussis*: two immunologically distinct species. *Infect Immun.* 1993; 61(2):486–90. PMID: [8423077](https://pubmed.ncbi.nlm.nih.gov/8423077/)
11. Zhang X, Weyrich LS, Lavine JS, Karanikas AT, Harvill ET. Lack of cross-protection against *Bordetella holmesii* after pertussis vaccination. *Emerg Infect Dis.* 2012; 18(11):1771–9. <https://doi.org/10.3201/eid1811.111544> PMID: [23092514](https://pubmed.ncbi.nlm.nih.gov/23092514/)
12. Bolding J, Kamat D. Whooping cough caused by *Bordetella parapertussis*. *Infect Med.* 2004; 21(6).
13. Pittet LF, Emonet S, Schrenzel J, Siegrist CA, Posfay-Barbe KM. *Bordetella holmesii*: an under-recognised *Bordetella* species. *Lancet Infect Dis.* 2014; 14(6):510–9. [https://doi.org/10.1016/S1473-3099\(14\)70021-0](https://doi.org/10.1016/S1473-3099(14)70021-0) PMID: [24721229](https://pubmed.ncbi.nlm.nih.gov/24721229/)
14. Pittet LF, Posfay-Barbe KM. *Bordetella holmesii*: Still Emerging and Elusive 20 Years On. *Microbiol Spectr.* 2016; 4(2).
15. Knorr L, Fox JD, Tilley PA, Ahmed-Bentley J. Evaluation of real-time PCR for diagnosis of *Bordetella pertussis* infection. *BMC Infect Dis.* 2006; 6:62. <https://doi.org/10.1186/1471-2334-6-62> PMID: [16556317](https://pubmed.ncbi.nlm.nih.gov/16556317/)
16. Reischl U, Lehn N, Sanden GN, Loeffelholz MJ. Real-time PCR assay targeting IS481 of *Bordetella pertussis* and molecular basis for detecting *Bordetella holmesii*. *J Clin Microbiol.* 2001; 39(5):1963–6. <https://doi.org/10.1128/JCM.39.5.1963-1966.2001> PMID: [11326023](https://pubmed.ncbi.nlm.nih.gov/11326023/)
17. Tatti KM, Sparks KN, Boney KO, Tondella ML. Novel multitarget real-time PCR assay for rapid detection of *Bordetella* species in clinical specimens. *J Clin Microbiol.* 2011; 49(12):4059–66. <https://doi.org/10.1128/JCM.00601-11> PMID: [21940464](https://pubmed.ncbi.nlm.nih.gov/21940464/)
18. Guthrie JL, Robertson AV, Tang P, Jamieson F, Drews SJ. Novel duplex real-time PCR assay detects *Bordetella holmesii* in specimens from patients with Pertussis-like symptoms in Ontario, Canada. *J Clin Microbiol.* 2010; 48(4):1435–7. <https://doi.org/10.1128/JCM.02417-09> PMID: [20181919](https://pubmed.ncbi.nlm.nih.gov/20181919/)
19. Roorda L, Buitenwerf J, Ossewaarde JM, van der Zee A. A real-time PCR assay with improved specificity for detection and discrimination of all clinically relevant *Bordetella* species by the presence and distribution of three Insertion Sequence elements. *BMC Res Notes.* 2011; 4:11. <https://doi.org/10.1186/1756-0500-4-11> PMID: [21255383](https://pubmed.ncbi.nlm.nih.gov/21255383/)
20. Niesters HG. Quantitation of viral load using real-time amplification techniques. *Methods.* 2001; 25(4):419–29. <https://doi.org/10.1006/meth.2001.1264> PMID: [11846611](https://pubmed.ncbi.nlm.nih.gov/11846611/)
21. van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J Clin Microbiol.* 2003; 41(2):576–80. <https://doi.org/10.1128/JCM.41.2.576-580.2003> PMID: [12574249](https://pubmed.ncbi.nlm.nih.gov/12574249/)
22. Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P, et al. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J Clin Microbiol.* 2008; 46(6):1946–54. <https://doi.org/10.1128/JCM.00157-08> PMID: [18400920](https://pubmed.ncbi.nlm.nih.gov/18400920/)
23. Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R, Kostrzewa M, Geider K. Classification and identification of bacteria by mass spectrometry and computational analysis. *PLoS One.* 2008; 3(7):e2843. <https://doi.org/10.1371/journal.pone.0002843> PMID: [18665227](https://pubmed.ncbi.nlm.nih.gov/18665227/)
24. Versalovic J. *Manual of Clinical Microbiology*, 10th edition, chapter 43, ASM Press 2012. 739–750
25. Kusters K, Reischl U, Schmetz J, Riffelmann M, Wirsing von Konig CH. Real-time LightCycler PCR for detection and discrimination of *Bordetella pertussis* and *Bordetella parapertussis*. *J Clin Microbiol.* 2002; 40(5):1719–22. <https://doi.org/10.1128/JCM.40.5.1719-1722.2002> PMID: [11980949](https://pubmed.ncbi.nlm.nih.gov/11980949/)
26. Templeton KE, Scheltinga SA, van der Zee A, Diederer BM, van Kruijssen A, Goossens H, et al. Evaluation of real-time PCR for detection of and discrimination between *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella holmesii* for clinical diagnosis. *J Clin Microbiol.* 2003; 41(9):4121–6. <https://doi.org/10.1128/JCM.41.9.4121-4126.2003> PMID: [12958235](https://pubmed.ncbi.nlm.nih.gov/12958235/)

27. Xu Y, Xu Y, Hou Q, Yang R, Zhang S. Triplex real-time PCR assay for detection and differentiation of *Bordetella pertussis* and *Bordetella parapertussis*. *APMIS*. 2010; 118(9):685–91. <https://doi.org/10.1111/j.1600-0463.2010.02644.x> PMID: 20718721
28. Van den Bossche D, De Bel A, De Smet D, Heylen O, Vekens E, Vandoorslaer K, et al. Prevalence of *Bordetella holmesii* and *Bordetella bronchiseptica* in respiratory tract samples from Belgian patients with pertussis-like symptoms by sensitive culture method and mass spectrometry. *Acta Clin Belg*. 2013; 68(5):341–8. <https://doi.org/10.2143/ACB.3341> PMID: 24579240
29. Tatti KM, Martin SW, Boney KO, Brown K, Clark TA, Tondella ML. Qualitative assessment of pertussis diagnostics in United States laboratories. *Pediatr Infect Dis J*. 2013; 32(9):942–5. <https://doi.org/10.1097/INF.0b013e3182947ef8> PMID: 23587980
30. Vestrheim DF, Steinbakk M, Bjornstad ML, Moghaddam A, Reinton N, Dahl ML, et al. Recovery of *Bordetella pertussis* from PCR-positive nasopharyngeal samples is dependent on bacterial load. *J Clin Microbiol*. 2012; 50(12):4114–5. <https://doi.org/10.1128/JCM.01553-12> PMID: 23035189
31. Riffelmann M, Wirsing von Konig CH, Caro V, Guiso N, Pertussis PCRCG. Nucleic Acid amplification tests for diagnosis of *Bordetella* infections. *J Clin Microbiol*. 2005; 43(10):4925–9. <https://doi.org/10.1128/JCM.43.10.4925-4929.2005> PMID: 16207944
32. Hasan MR, Tan R, Al-Rawahi GN, Thomas E, Tilley P. Evaluation of amplification targets for the specific detection of *Bordetella pertussis* using real-time polymerase chain reaction. *Can J Infect Dis Med Microbiol*. 2014; 25(4):217–21. PMID: 25285127
33. Probert WS, Ely J, Schrader K, Atwell J, Nossoff A, Kwan S. Identification and evaluation of new target sequences for specific detection of *Bordetella pertussis* by real-time PCR. *J Clin Microbiol*. 2008; 46(10):3228–31. <https://doi.org/10.1128/JCM.00386-08> PMID: 18753352
34. Vincart B, De Mendonca R, Rottiers S, Vermeulen F, Struelens MJ, Denis O. A specific real-time PCR assay for the detection of *Bordetella pertussis*. *J Med Microbiol*. 2007; 56(Pt 7):918–20. <https://doi.org/10.1099/jmm.0.46947-0> PMID: 17577056