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REVIEW

# Optimizing polymerase chain reaction testing for the diagnosis of pertussis: current perspectives



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**Abstract:** Nucleic acid testing has revolutionized the diagnosis of pertussis in the clinical microbiology laboratory and has become the main avenue of testing for pertussis infection. Real-time polymerase chain reaction (RT-PCR) is an important tool for timely diagnosis of pertussis and is more sensitive than culture. The most commonly amplified targets are the insertion-sequence (IS) genes, which are found in multiple copies in the genome of *Bordetella* species. Some strains of *Bordetella pertussis* have more than 200 copies of IS*481* in their genome. This high number of repeats allows RT-PCR assays to be very sensitive and makes nucleic acid testing two to three times more sensitive than culture. Despite these advantages, RT-PCR can give inaccurate results due to contamination or lack of specificity. Contamination can easily happen during specimen collection, DNA extraction, or nucleic acid amplification steps. To avoid contamination, laboratories need to have quality controls and good workflows in place. The poor specificity of the nucleic acid assays amplifying the IS genes is because they are found in various *Bordetella* species and, thus, not unique to a specific species. *Bordetella holmesii*, a more recently described *Bordetella* species found to be responsible for respiratory symptoms similar to pertussis in adolescents and adults, can be misidentified as *B. pertussis* in RT-PCR assays that amplify only the IS*481* target. Use of multiple targets may improve specificity of RT-PCR assays for pertussis. In the past few years, the US Food and Drug Administration has cleared three commercial assays for the detection of *B. pertussis* in respiratory specimens. Several commercial assays and analyte-specific reagents, which are not US Food and Drug Administration cleared, are available for the detection of one or more *Bordetella* species by nucleic acid testing. Because of the diversity of nucleic acid amplification assays used, pertussis testing is not standardized across clinical laboratories.

**Keywords:** nucleic acid testing, real-time PCR, *Bordetella pertussis*, *Bordetella* species

## Introduction

Pertussis is a disease of the respiratory tract caused by the fastidious Gram-negative rod bacteria *Bordetella pertussis*, and a pertussis-like illness can be caused by *Bordetella parapertussis*. Pertussis can cause serious illness in infants, children, and adults. Pertussis is still prevalent worldwide and is an important cause of death among neonates and infants too young to have completed their primary vaccination series. WHO estimates that, in 2008, 16 million pertussis cases occurred worldwide and that approximately 195,000 children died from the disease, representing 2% of the global causes of death in children aged 1–59 months.[1,2] Approximately half of infants younger than 1 year of age who develop the disease are hospitalized.[3] Before vaccination, pertussis was one of the most common childhood diseases worldwide. Pertussis continues to be a public concern even in countries with high vaccination coverage.

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In the USA, from 1940 through 1945, before the introduction of the pertussis vaccine, the incidence of pertussis was approximately 150 cases per 100,000 population. Following the implementation of the whole-cell pertussis vaccine in the late 1940s, pertussis gradually declined to approximately eight cases per 100,000 population in 1960 and reached a low of approximately one case per 100,000 population during 1980–1990. Since then, the number of reported cases of pertussis has begun to increase gradually, and in 2014, more than 28,000 cases were reported nationwide with an incidence of 9.1 cases per 100,000 population (CDC Surveillance report).[4,5] While the incidence of pertussis in the USA is still highest among infants younger than 1 year of age, the incidence among older children and adults has increased substantially during the past decade. The reasons for the increase, despite high vaccination coverage, are not fully understood, but several factors have been suggested, including genetic changes in *B. pertussis*, decreased effectiveness of vaccine, waning of immunity from vaccination over time, increasing awareness by clinicians, and better laboratory detection.[6,7]

## *Bordetella* species

The genus *Bordetella* contains eight species, four of which are known to cause respiratory disease in humans: *B. bronchiseptica*, *B. holmesii*, *B. parapertussis*, and *B. pertussis*. *B. holmesii* is a novel organism that was first described in 1995 as a cause of bacteremia in patients with comorbidities such as asplenia, but since has been shown to cause respiratory symptoms in otherwise healthy individuals.[8] *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are closely related species, but *B. parapertussis* and *B. bronchiseptica* do not produce the pertussis toxin due to a mutation in the promoter region of the genes encoding this toxin.[9] *B. holmesii* does not produce the virulence factors produced by the other three species. *B. parapertussis* is found in sheep and humans, and *B. pertussis* is thought to be a strictly human pathogen.[10] *B. bronchiseptica* can cause respiratory infections in many animal species and, infrequently, also in humans. An increasing number of pertussis-like cases are attributed to the emergent pathogen *B. holmesii*.[8] It is still unknown whether this species is truly pathogenic for humans or only an opportunistic organism, but microbiologic diagnosis has confirmed the presence of *B. holmesii* in human respiratory samples.[8]

## Laboratory diagnosis of pertussis

There are two different approaches to the diagnosis of pertussis in the laboratory: the direct approach, which consists of identifying the microorganism responsible for the disease, either by culture, direct fluorescent antibody (DFA) testing, or real-time polymerase chain reaction (RT-PCR), and the indirect approach which consists of detecting specific antibodies in the serum of infected individuals.

### Culture

Isolation and identification of *B. pertussis* is highly specific, but the recovery is low and declines with age, if the patient has received prior antibiotic therapy effective against *B. pertussis*, if the collection of the specimen has been delayed beyond the first 2 weeks of illness, and if the patient has been vaccinated.[11] However, culture is particularly useful for confirming pertussis diagnosis when an outbreak is suspected and allows for strain identification and antimicrobial resistance testing.

### DFA testing

DFA staining of nasopharyngeal swabs or nasopharyngeal aspirates is rapid and simple, but because of its low sensitivity and variable specificity, it is not accepted as proof of infection.[10]

### Real-time polymerase chain reaction

RT-PCR is more sensitive than culture and has been accepted as evidence of laboratory confirmation of pertussis in different countries (examples: USA and Canada). It is still recommended to send specimens for culture, particularly during outbreaks, as bacterial isolates may be required for evaluation of antibacterial resistance or epidemiologic studies. RT-PCR is able to detect the organism at a later stage of the infection, when the ability to culture the organism declines.

### Serology

Serology consists of detecting specific anti-pertussis toxin (PT) antibodies in the serum of infected individuals after 2–3 weeks of the cough. Serology testing should not be used in infants, as their immune systems are immature and susceptible to interference by maternal antibodies, or in patients who have been vaccinated within 1 year since it does not differentiate between antibodies due to the vaccine and natural infection. Generally, serologic tests are more useful for diagnosis in later phases of the disease when it is too late to perform culture or nucleic acid amplification testing (NAAT). Other antibodies to pertussis antigens are not routinely available but are usually included in pertussis vaccine trials (eg, anti-filamentous hemagglutinin, anti-fimbriae,

anti-pertactin). A battery of antibody tests may be advantageous rather than just anti-PT.

## RT-PCR testing for the diagnosis of pertussis

RT-PCR has revolutionized the way clinical laboratories diagnose *B. pertussis*. NAAT is a rapid and sensitive way to detect *B. pertussis* from upper respiratory samples. It does not require viable bacteria for detection. This permits the use of more diverse collection devices and simplifies the transport and storage of specimens for pertussis testing.

RT-PCR has an optimal sensitivity during the first 3 weeks of illness when bacterial DNA is still present in the nasopharynx.[12] One study showed that the bacterial DNA could be detected by RT-PCR up to 58 days into illness even after antimicrobial therapy.[13] Nevertheless, after the fourth week of cough, the amount of bacterial DNA rapidly diminishes and might be too low to be detected by NAAT, and thus, serology (IgA or IgG anti-PT or other antibody testing) is recommended.

### Amplification targets

Several *Bordetella* gene targets have been selected for the amplification and detection of *Bordetella* species by NAAT. The most commonly used targets are the insertion-sequence (IS) genes because they are usually found in multiple copies in the bacterial genome and, thus, increase the sensitivity of the nucleic acid assay. However, they are usually found in more than one species of *Bordetella* and, thus, not limited to a single *Bordetella* species. IS*481* is found in 50 to more than 200 copies in the genome of *B. pertussis*, 8–65 copies in the genome of *B. holmesii*, and less than five copies in the genome of some strains of *B. bronchiseptica*.[14–16] IS*1001* is present in approximately 20 copies in the genome of *B. parapertussis* and 1–7 copies in the genome of *B. bronchiseptica*. IS*1002* is found as 1–9 copies in the genome of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*.[15–17] hIS*1001* is only found in the genome of *B. holmesii* and in only 3–21 copies (Table 1).[18]

The insertion sequences, IS*481* and IS*1001*, are the most commonly used RT-PCR targets for the diagnosis of *B. pertussis* and *B. parapertussis*, respectively. Since both targets are also present in more than one species of *Bordetella*, these RT-PCR assays potentially can misidentify *B. holmesii* and *B. bronchiseptica* as *B. pertussis* or *B. parapertussis*, respectively.[19]

NAAT assays that use a single gene-copy target like the pertussis toxin S1 promoter *ptxA*-pr gene also known as *ptxP*, which is only found in *B. pertussis*, and the housekeeping gene $recA_{BH}$ that is only found in *B. holmesii* are very specific but, in general, have poor sensitivity.[20–23] Their sensitivity is comparable to culture, in contrast to RT-PCR assays that use multicopy targets like IS*481*, which are approximately 2–3 times more sensitive than culture.[24–26]

Some laboratories have developed multiplex RT-PCR assays that incorporate the *B. holmesii*-specific $recA_{BH}$ primers with the IS*481* primers to differentiate between *B. pertussis* and *B. holmesii*.[19,21,23]

Because pertussis NAATs are not standardized across clinical laboratories, the results may not be comparable from one laboratory to another. Different testing methods, amplification targets, and result interpretation are used to determine positive, indeterminate, or negative results.

### Collecting devices

Dacron and rayon swabs are the recommended swabs for the collection of nasopharyngeal specimens to be tested by RT-PCR and also culture. The calcium-alginate swabs are not recommended as they inhibit the polymerase chain reaction.[27] Recently flocked swabs (eg, ESwab; Copan Diagnostics, Inc., Corona, CA, USA) have been shown

**Table 1** Targets that are most commonly used for the detection of *Bordetella pertussis* by nucleic acid amplification

| | Insertion-sequence target | | | Single-gene target | | |
|---|---|---|---|---|---|---|
| | IS*481*[14–16] (copy number per genome) | IS*1001*[15,16] (copy number per genome) | IS*1002*[15,16] (copy number per genome) | hIS*1001*[a,14,15] (copy number per genome) | *ptxA*-pr[b,20,22,23] (copy number per genome) | $recA_{BH}$[19,21,23] (copy number per genome) |
| *Bordetella pertussis* | 50 to >200 | 0 | 4–8 | 0 | 1 | 0 |
| *Bordetella parapertussis* | 0 | 20 | 9 | 0 | 0 | 0 |
| *Bordetella bronchiseptica* | 5 (not present in all strains)[16] | 1–7 (not present in all strains)[16] | 1 (not present in all strains)[16] | 0 | 0 | 0 |
| *Bordetella holmesii* | 8–65 | 0 | 0 | 3–21 | 0 | 1 |

**Notes:** [a]Holmesii IS1001; [b]pertussis toxin S1 promotor, also known as *ptxP*.
**Abbreviation:** IS, insertion-sequence.

to be acceptable collecting devices for the collection of nasopharyngeal specimens for detection of pertussis by RT-PCR testing.[28]

## US Food and Drug Administration cleared commercial assays

Until a few years ago, there were no US Food and Drug Administration (FDA) cleared NAATs for *B. pertussis* or other *Bordetella* species. In the last 4 years, the FDA approved three commercial NAATs for rapid *B. pertussis* testing, but there is still no FDA-cleared assay for the detection of any other *Bordetella* species (Table 2).

In May 2012, Idaho Technology, Inc., was the first to receive FDA clearance for RT-PCR testing of *B. pertussis* for its FilmArray Respiratory Panel. It targets the single-copy promoter region of the pertussis toxin gene that is specific for *B. pertussis*. The product insert reports a sensitivity of 4,000 CFU/mL (~450 CFU/reaction).

In March 2014, Meridian Bioscience, Inc., Cincinnati, OH, USA, received FDA clearance for its new molecular diagnostic

**Table 2** Available commercial assays/analyte-specific reagents (ASRs) for the detection of *Bordetella* species by nucleic acid testing

| Commercial kits | Target(s) amplify | Technology | Detect | FDA cleared (year) | Available in the USA |
|---|---|---|---|---|---|
| FilmArray Respiratory Panel, Idaho Technology, Inc. | Promoter region of the *B. pertussis* toxin gene | Real-time PCR | *B. pertussis* | Yes (2012) | Yes |
| *illumi*gene *Pertussis*, Meridian Bioscience, Inc. | IS*481* | Loop-mediated isothermal DNA amplification technology | *B. pertussis* | Yes (2014) | Yes |
| AmpliVue *Bordetella*, Quidel Corporation | IS*481* | Isothermal amplification technology | *B. pertussis* | Yes (2014) | Yes |
| Verigene® Respiratory Pathogens Flex Test (RP Flex), Nanosphere Inc. | Not specified | Nanosphere's unique gold nanoparticle probe technology | *Bordetella* group (comprised of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*), *B. holmesii, B. pertussis* | No | Yes |
| MGB Alert® *B. pertussis*/*parapertussis* ASRs, Elitech Biosciences | Not specified | Real-time PCR | *B. pertussis*, *B. parapertussis* | No | Yes[30] |
| MultiCode® *Bordetella pertussis*/*parapertussis* ASRs, EraGen Biosciences, Luminex | Not specified | MultiCode-RTx technology | *B. pertussis*, *B. parapertussis* | No | Yes[30] |
| BORTEDELLA R-gene® and *Bordetella parapertussis* r-gene, BioMerieux Inc. | IS*481*/IS*1001* | Real-time PCR | *B. pertussis*, *B. parapertussis* | No | No |
| Simplexa *Bordetella pertussis*/*parapertussis* ASRs, Focus Diagnostics | IS*481*/IS*1001* | Real-time PCR | *B. pertussis*, *B. parapertussis* | No | Yes[30] |
| SmartCycler *Bordetella pertussis*/*parapertussis* ASRs, Cepheid | IS*481*/IS*1001* | Real-time PCR | *B. pertussis*, *B. parapertussis* | No | Yes[31] |
| *DIAGENODE Bordetella pertussis* and *parapertussis*, Becton, Dickinson and Company | IS*481*/IS*1001* | Real-time PCR | *B. pertussis*, *B. parapertussis* | No | No |
| GenoQuick® *Bordetella*, Hain Lifescience GmbH | IS*481*/IS*1001* | DNA STRIP technology | *B. pertussis*, *B. parapertussis* | No | No |
| GeneProof *Bordetella pertussis*/*parapertussis* PCR, GeneProof a.s. | IS*1001*/IS*1002* | Real-time PCR | *B. pertussis*, *B. parapertussis* | No | No |
| LightMix® Kit *Bordetella pertussis* and *parapertussis*, TIB MOLBIOL GmbH, Roche Diagnostics GmbH | IS*481*/IS*1001* | Real-time PCR | *B. pertussis*, *B. parapertussis* | No | Yes |

**Abbreviations:** FDA, US Food and Drug Administration; *B. pertussis*, *Bordetella pertussis*; PCR, polymerase chain reaction; IS, insertion sequence; *B. parapertussis*, *Bordetella parapertussis*; *B.* bronchiseptica, *Bordetella* bronchiseptica; *B. holmesii*, *Bordetella holmesii*.

test for *B. pertussis*, on the *illumi*gene® molecular platform. The *illumi*gene *Pertussis* assay utilizes loop-mediated isothermal DNA amplification technology to detect *B. pertussis* by targeting the IS*481* insertional element of the *B. pertussis* genome. It claims a sensitivity of 3,265 CFU/mL (1.48 CFU/test). The product insert includes this disclaimer "Respiratory infection with *B. pertussis*, *B. holmesii*, or *B. bronchiseptica* may yield positive test results in IS*481* assays".

In December 2014, Quidel Corporation received clearance from the FDA for its AmpliVue *Bordetella* assay for the detection of *B. pertussis* nucleic acids isolated from nasopharyngeal swab specimens. The assay utilizes an isothermal amplification technology of helicase-dependent amplification of the insertion sequence IS*481*. The assay limit of detection for *B. pertussis* is 2,358 CFU/mL (3.93 CFU/assay). The product insert mentions cross-reactivity with *B. bronchiseptica* and *B. holmesii*.

## Laboratory developed assays

Many commercial assays and analyte-specific reagents are available in the market for *B. pertussis* and/or *B. parapertussis* detection in clinical specimens, but they are not FDA cleared. Review of the literature comparing the performances of some of the commercial assays to laboratory developed assays shows that the performance of commercial assays varies, some performing better than others.[29,30] Any commercial assays or laboratory-developed assays need to be fully validated to demonstrate that the performance of the assay is acceptable for patient testing.[31]

Automation has become part of molecular testing, and many laboratories are using automated platforms to extract bacterial DNA from respiratory samples, but manual extraction using extraction kits or simple hit lysis are still performed. Not all extraction methods have the same efficiency to extract bacterial DNA, and a comparison between the different methods has yet to be performed to assess the efficiency of each method.[32] Manual extraction increases the possibility of DNA cross-contamination. Hit lysis is a very efficient technique to release DNA but does not remove potential PCR inhibitors, and an internal control needs to be added to the RT-PCR reaction to monitor the presence of any PCR inhibitor.

## Advantages of NAAT

The use of RT-PCR to diagnose pertussis has improved the sensitivity of diagnosis and decreased the turnaround time to diagnosis, permitting faster treatment and prophylaxis of contacts. It has extended the window period in which testing for pertussis is still reliable up to 58 days into illness.[13] Antibiotics do not affect the result of the RT-PCR assays. RT-PCR testing has given us a new understanding of the epidemiology of pertussis in the adolescent and adult population and, thus, has provided better control and prevention of outbreaks.

## Drawbacks of NAAT

Contamination is a major problem in testing for pertussis by NAAT. Pseudo outbreaks have been reported with false-positive RT-PCR results due to contamination.[33] The contaminant can be introduced during specimen collection or during processing and set up of the RT-PCR assay. RT-PCR assays that use repetitive IS target genes, like the IS*481*, are more susceptible to contamination than single-gene target assays because of the very high quantity of repeats present in the bacterial genome. To avoid such events, it is important that strategies like unidirectional workflow and quality control (negative and positive controls) be put in place. The other issue in using IS targets is the interpretative dilemma of results where high cycle threshold (Ct) values of more than 35 cycles may represent concentrations of DNA target of less than one bacterial genome per assay reaction.[34] The clinical significance of such a low level of bacteria is not clear and clinical correlation is mandatory. A high Ct value could represent a true infection in somebody who had been previously vaccinated, or someone who had received antimicrobial therapy, or it could be due to an inadequate specimen collection in a truly infected person or contamination in performing the assay.[35] Laboratories need to define which Ct values they will call a positive, indeterminate, or negative result. High Ct values in NAATs that used IS targets need to be interpreted with caution as they might represent false-positive results and should be analyzed in conjunction with patients' signs and symptoms and epidemiological information. A false-positive result can mislead patient management and treatment and possibly miss a major diagnosis. It will also have an impact on contact prophylaxis.[15]

The consequence of miscalling a *Bordetella* species has not been fully investigated. The significance and the health impact of the detection of *B. holmesii* in healthy individuals with pertussis-like symptoms are not certain. Controversy as to whether the bacterium is a mere colonizer with minimal pathogenic implication or a true respiratory pathogen is still debated.[8,19] The true prevalence of *B. holmesii* in the population is not known. Its prevalence is difficult to evaluate because the majority of clinical laboratories are

using RT-PCR assays that target only the IS*481* region that does not discriminate between *B. pertussis* and *B. holmesii*. The laboratories that use RT-PCR assays which are species-specific are not attracted to identify *B. holmesii* because of its unknown significance.

## Summary and conclusion

The resurgence of pertussis has been attributed to several factors, one of them being that the numbers of reported cases may have been inflated because of greater awareness of pertussis and the use of NAAT for the detection of pertussis. However, it is clear that this cannot be the only reason for the increase of reported pertussis cases, and other factors like the diphtheria–tetanus–acellular pertussis (DTaP) vaccines not being as effective as the whole-cell vaccines and waning of immunity from vaccination over time may be contributory.[36,37] The higher sensitivity of NAAT to detect pertussis has demonstrated that adults who had been vaccinated in childhood were susceptible to pertussis infection. They usually have a milder course of illness and may have an atypical clinical presentation. This suggests waning of immunity to pertussis from vaccination over time. The other age group affected includes children born in the USA after 1997 who received only the acellular vaccine for their primary series and had a faster waning of their pertussis immunity compared to those born in the 1940s to early 1990s who received the whole-cell vaccine.[3,7] These observations demonstrated the need for boosters to maintain immunity and the need of a better vaccine that will confer longer immunity against pertussis. Still, the highest burden of disease is seen in infants less than 12 months, and severe and fatal disease continues to occur almost exclusively in infants who are too young, less than 2 months, to be vaccinated against pertussis. During the 2010 pertussis epidemic, the main strategy to protect neonates was "cocooning", which involved vaccinating all people who are in contact with the child.[3] However, this method was not enough to prevent pertussis infection. This is because cocooning does not provide any direct protection (antibodies) to the infant, and it is difficult to ensure that everyone who is around the neonate has received the pertussis vaccine. In 2011, it was shown that antipertussis antibodies from the mother were efficiently transferred to the fetus through the placenta, conferring protection to the vulnerable infants until they are old enough to receive the primary series of DTaP starting at the age of 2 months.[38] It is now recommended that every pregnant woman receive Tdap during the third trimester of every pregnancy because antibody levels decline between pregnancies.[3,39]

The need for a new vaccine that confers better and longer immunity than the acellular pertussis vaccine and that does not have the adverse reactions associated with the whole-cell vaccine is evident. Understanding the epidemiology of pertussis in vaccinated groups of individuals with waning immunity is important for the design of a better vaccine. The diagnosis of pertussis in this group of individuals is challenging because of the nonspecific clinical symptoms they present with. The increased sensitivity of NAAT has permitted us to detect the bacteria in this group of people for whom culture was not sensitive enough. The problem is lack of standardization of testing and that the accuracy of diagnosis varies among laboratories. Different protocols, DNA extraction methods, nucleic acid amplification techniques, and amplification targets are used by laboratories to detect *Bordetella* species. These factors influence the sensitivity and the accuracy of results.[32] Conformity in the diagnostic approaches to detect pertussis is desirable to have a true knowledge of the prevalence of pertussis in the population. It is imperative that laboratories assess the performance of their nucleic acid amplification assay by participating in external assessments to ensure that their results are comparable to their peers.

To achieve better standardization of pertussis testing by RT-PCR, by adaptation of multiplex RT-PCR assays, one can target the IS genes (eg, IS*481*, IS*1000*) to confer high sensitivity, and single-target genes specific to *Bordetella* species (eg, pertussis toxin S1 promoter *ptxA*-pr or $recA_{BH}$) to achieve high specificity. Multiplex RT-PCR with primers and probes that give high sensitivity and specificity for all four major clinical *Bordetella* species would be ideal.

## Disclosure

The authors report no conflicts of interest in this work.

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