Towards Improved Accuracy of *Bordetella pertussis* Nucleic Acid Amplification Tests

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In many clinical microbiology laboratories, nucleic acid amplification tests such as PCR have become the routine methods for the diagnosis of pertussis. While PCR has greatly increased the ability of laboratories to detect *Bordetella pertussis* infections, it has also been associated with false-positive results that can, given the tendency of *B. pertussis* to cause outbreaks, result in unnecessary and costly control measures. The species specificity of *Bordetella* gene targets and their number of copies per genome greatly impact the performance characteristics of nucleic acid amplification tests for *B. pertussis*. It is crucial that laboratorians recognize these characteristics, to limit false-positive test results and prevent pseudo-outbreaks.

> **"Have no fear of perfection—you'll never reach it."**
> —Salvador Dali

The laboratory diagnosis of pertussis has gone through fundamental changes in the last 20 years. Culture and direct fluorescent-antibody (DFA) staining of nasopharyngeal secretions have been largely replaced by nucleic acid amplification tests (NAATs) such as PCR. PCR has undergone substantial changes as a clinical diagnostic tool during this time, from conventional PCR with agarose gel or microwell plate detection of amplicons, to real-time amplification and detection. A number of *Bordetella* gene targets have been described and applied to diagnostic PCR tests. These include the single-copy pertussis toxin operon and multiple-copy insertion sequences. The extent to which these gene targets are shared among *Bordetella* species and their copy number per genome greatly affect the performance characteristics of NAATs. Herein I provide an update on NAATs for the diagnosis of pertussis, with an emphasis on gene targets and their impact on test accuracy, as well as the "real world" performance of PCR, including its role in pseudo-outbreaks.

EPIDEMIOLOGY

Whooping cough, or pertussis, is a highly contagious respiratory disease caused by *Bordetella pertussis*. In spite of widespread childhood vaccination, over 27,000 cases were reported in the United States in 2010 (http://www.cdc.gov/pertussis/outbreaks.html, last accessed 26 February 2012). These reported cases may represent the "tip of an iceberg" that consists of 800,000 to 3.3 million cases in the United States every year (3). Worldwide, an estimated 50 million cases and 300,000 deaths occur annually (http://www.who.int/immunization _monitoring/diseases/pertussis_surveillance/en/index.html, last accessed 26 February 2012). Epidemics of pertussis often occur in 3- to 5-year cycles, interspersed with local outbreaks. Humans are the sole reservoir of *B. pertussis*. Adolescent and adults, in whom immunity wanes several years after prior infection or vaccination, transmit the organism to susceptible infants. Pertussis in older children and adults is generally characterized by prolonged cough without the inspiratory whoop or posttussive vomiting typically observed in infants. While the incidence of pertussis in the United States is highest among children under 1 year of age, the incidence among older age groups has increased substantially during the past decade (http://www.cdc.gov /pertussis/surv-reporting.html, last accessed 26 February 2012). In

response, acellular vaccines are now recommended as a booster for adolescents and adults.

MICROBIOLOGY AND CLINICAL SIGNIFICANCE

The genus *Bordetella* is composed of eight species, four of which are responsible for respiratory disease in humans: *B. bronchiseptica*, *B. holmesii*, *B. parapertussis*, and *B. pertussis*. *B. parapertussis* may be responsible for up to 20% of pertussis-like disease, more often in young children (20). Illness is generally milder than that caused by *B. pertussis*, but young patients frequently present with the symptoms of classic pertussis (20). *B. bronchiseptica* is an infrequent cause of respiratory disease in humans, usually occurring in persons with underlying immunodeficiency diseases. Cases often involve exposure to mammals—usually farm animals or pets (38). *B. holmesii* is the most recently identified species associated with pertussis-like illness in humans. Yih et al. were the first to report a pertussis-like illness in patients with *B. holmesii* infections, isolating the organism from less than 1% of nasopharyngeal specimens collected from patients with suspected pertussis between 1994 and 1998 (39). A study using PCR failed to detect *B. holmesii* DNA in any nasopharyngeal swabs collected from patients (no age information provided) in Finland and The Netherlands with suspected pertussis between 1992 and 2003 (1). However, another studying using PCR detected *B. holmesii* DNA in 20% of the samples collected from adolescents and adults with suspected pertussis in France from 2009 to 2011 (21). In total, the role that these "nonpertussis" *Bordetella* species play in human respiratory disease is not insignificant; they should be considered when designing a nucleic acid amplification-based diagnostic test, whether that test is *B. pertussis* specific or a multispecies assay. Perhaps more than any other *Bordetella* species, *B. bronchiseptica* has been shown to possess a number of gene targets initially thought to be specific to *B. pertussis*. Some of these gene targets may be present in few isolates of *B. bronchiseptica*—as will be discussed later in this review—necessitating the analysis of a large

TABLE 1 *Bordetella* insertion sequences

Insertion sequence	Presence/no. of copies per genome[a]				References
	B. pertussis	*B. parapertussis*	*B. holmesii*	*B. bronchiseptica*[b]	
IS*481*	+/>50	−/NA	+/8-10	(+)[c]/ND	11, 28, 31
IS*1001*	−/NA	+/~20	−/NA	(+)[d]/1-7	29, 31, 34
hIS*1001*	−/NA	−/NA	+/3-5	−/NA	1, 31
IS*1002*	+/4-8	+/9	−/NA	−[e]/1	29, 34

[a] Symbols and abbreviations: +, present in all isolates; (+), present in some isolates; −, absent from all isolates; NA, not applicable; ND, not determined.
[b] Human-derived *B. bronchiseptica* isolates only.
[c] One of 73 human-derived isolates was positive (31).
[d] Four of 73 human-derived isolates were positive (31).
[e] Found in rare animal-derived isolates (34).

number of diverse isolates to rule out cross-reactivity with *B. pertussis*-specific PCR assays.

LABORATORY DIAGNOSIS

DFA staining and culture. Because of their increased sensitivity, NAATs such as PCR have replaced DFA and culture for the detection of *B. pertussis* in many laboratories. DFA staining of nasopharyngeal secretions for *B. pertussis* has both poor sensitivity and poor specificity; a positive result is no longer part of the U.S. Centers for Disease Control and Prevention (CDC) pertussis case definition (http://www.cdc.gov/vaccines/pubs/surv-manual/chpt10-pertussis.html, last accessed 26 February 2012). Culture remains the gold standard for laboratory diagnosis of pertussis due to a specificity of essentially 100%. The CDC recommends that culture be performed during a suspected outbreak to confirm positive PCR results. However, culture is usually much less sensitive than NAATs, in part because the organism is highly labile and does not survive well during specimen transport. Rapid specimen transport and the use of a suitable transport medium are critical for optimal culture sensitivity.

Serology. Serology can be a useful diagnostic method, but with important caveats. The presence of IgG antibodies as a result of vaccination or past infection limits the specificity and confounds interpretation of IgG-based tests. Repeated infections over a person's lifetime can result in a muted IgM response and poor sensitivity of tests that detect this class of antibody. Detection of IgA antibodies provides a specific diagnosis, but infants often have inconsistent IgA responses (20), limiting the sensitivity of IgA-based tests in this important age group. The most accurate serologic diagnosis of pertussis is retrospective and requires two serum specimens collected several weeks apart to demonstrate a 4-fold or greater change in the IgG titer. In the United States, serologic tests for *B. pertussis* are usually performed in public health and commercial reference laboratories. At the time that this minireview was written, there were no U.S. Food and Drug Administration (FDA)-cleared serology-based tests for *B. pertussis*.

Nucleic acid amplification. PCR tests for the detection of *B. pertussis* in clinical specimens were first described in 1989 and targeted the pertussis toxin operon and an insertion element (11). Early PCR assays relied on agarose gels or microwell plates for the detection of amplified DNA. Real-time PCR for the detection of *B. pertussis* was first described in 2001 (15, 28). Almost immediately upon its initial description as a diagnostic tool, PCR was shown to be substantially more sensitive than culture and DFA (7, 10, 18). Not unexpectedly, PCR remains positive longer during the course of disease and after initiation of antibiotic therapy than culture (5,

33). PCR sensitivity decreases substantially with increasing patient age and duration of symptoms (33). Some individuals who were exposed to pertussis cases but never developed symptoms were positive by PCR (10). These asymptomatic transient infections are likely due to full or partial immunity.

While PCR is the NAAT most frequently used for the detection of *B. pertussis*, an assay based on loop-mediated isothermal amplification has been reported in the literature (13). At the time that this minireview was written, there were no FDA-cleared NAATs for *B. pertussis* or other *Bordetella* species. There are several Conformité Européenne-marked real-time PCR kits for the detection of *B. pertussis* and *B. parapertussis*, all targeting insertion sequences, either IS*481* alone or both IS*481* and IS*1001*.

GENE TARGETS IN *BORDETELLA* NAATS

Insertion sequences (Table 1). Most[1] PCR tests are based on the detection of insertion sequences—transposable DNA elements approximately 1,000 bp in length that can be inserted into multiple sites on the same chromosome. Insertion sequences are usually present in multiple copies per genome, increasing the sensitivity of PCR tests. The insertion sequence IS*481* is perhaps the most well-described and validated target for *B. pertussis*. There are more than 50 copies of IS*481* per *B. pertussis* genome (28). However, IS*481* is also present in *B. holmesii* at 8 to 10 copies per genome (28) and in rare human isolates of *B. bronchiseptica* (1 of 73 isolates tested) (31). While *B. holmesii* has been associated with pertussis-like disease, it has not been identified as a cause of outbreaks. Therefore, NAATs based on IS*481* alone have limited clinical value, particularly when used in outbreak settings. This will be discussed in more detail later in this minireview. IS*1001* is found in *B. parapertussis* at approximately 20 copies per genome (35) and has been used in multiplex PCR with IS*481* to detect and differentiate *B. pertussis* and *B. parapertussis* (14, 16, 29, 31). IS*1001* is also present in isolates of *B. bronchiseptica* (4 of 73 human-derived isolates tested by Tatti et al. [31] and 26 of 38 animal-derived isolates tested by van der Zee et al. [34]) at 1 to 7 copies per genome (34). IS*1001* has not been reported in *B. holmesii*. Therefore, multiplex assays based on IS*481* and IS*1001* will not differentiate *B. pertussis* and *B. holmesii*. An IS*1001*-like element, hIS*1001*, is found in *B. holmesii* at 3 to 5 copies per genome (1). Tatti et al. did not detect hIS*1001* in any isolates of *B. pertussis*, *B. parapertussis*, or *B. bronchiseptica* (31). Consequently, multiplex PCR assays or testing algorithms that include hIS*1001* in addition to IS*481* and IS*1001* would differentiate *B. pertussis* and *B. holmesii*. IS*1002* is found in *B. pertussis* (4 to 8 copies per genome) and *B. parapertussis* (9 copies per genome) (34) but apparently not in *B. holmesii* or *B.*

TABLE 2 Proteins encoded by single-copy *Bordetella* PCR gene targets[a]

Protein encoded by target gene	Reference(s)
B. pertussis specific	
BP283 (thiolase)	24
BP485	24
Shared among *Bordetella* species	
Adenylate cyclase	4
BP3385	9, 26
Filamentous hemagglutinin[b]	14
Flagellin[b]	12
Pertactin	27, 36
Pertussis toxin[b]	11, 22, 31, 32
Porin[b]	6
RecA[b]	1, 8, 25

[a] With the exception of the gene for pertussis toxin, PCR assays targeting the genes that encode these proteins have not been well validated.
[b] Species-specific primers or PCR assays using postamplification differentiation of species have been described.

bronchiseptica (the number of isolates tested was not provided) (29). van der Zee et al. found IS*1002* in 1 of 38 animal-derived *B. bronchiseptica* isolates tested (34). Therefore, a multiplex PCR mixture containing IS*1002*, IS*481*, and IS*1001* may allow the detection and differentiation of *B. pertussis*, *B. parapertussis*, and *B. holmesii*. A PCR that detects IS*1002* alone would detect both *B. pertussis* and *B. parapertussis* without differentiation (which may not be important). The IS*1002* sequence identity of *B. pertussis* and *B. parapertussis* is 93% (34), which may allow the differentiation of these species by distinct melting temperature peaks. To my knowledge, this has not been reported in the literature. An IS*1002* PCR may not cross-react with *B. holmesii* or *B. bronchiseptica*, but analysis of a large number of diverse isolates is necessary to confirm this.

Single-copy targets (Table 2). Pertussis toxin, the major virulence factor of *B. pertussis*, is a multisubunit complex whose genes are organized as an operon. The promoter region of the pertussis toxin operon is well characterized and an often used target in diagnostic PCR assays (11, 25, 31, 32). The pertussis toxin operon is also present in *B. parapertussis* and *B. bronchiseptica* but due to mutations in the promoter region is not expressed by these species. The presence of these mutations also allows the differentiation of PCR amplicons produced by *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* by size (22) or melting temperature in real-time PCR (25). Without postamplification analysis, pertussis toxin-based PCR assays cannot distinguish among *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. Since the pertussis toxin promoter is not found in *B. holmesii*, primers targeting it have been combined with IS*481* to differentiate *B. pertussis* and *B. holmesii* (32). Pertactin is a major virulence factor produced by *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* associated with adherence to ciliated epithelial cells of the respiratory tract. A real-time PCR assay designed to detect pertactin gene sequences of *B. pertussis* reportedly did not detect *B. parapertussis*, *B. bronchiseptica*, or *B. holmesii* (36). However, the number of isolates tested was not provided. A later study of a large number of human- and animal-derived *B. bronchiseptica* isolates demonstrated cross-reactivity (27). Filamentous hemagglutinin is a major virulence factor of *B. pertussis* that mediates adherence to ciliated epithelial cells. It is also produced by *B. parapertussis* and *B. bronchiseptica*. A real-

time PCR assay that detects these three *Bordetella* species, followed by postamplification differentiation of filamentous hemagglutinin amplicons by melting curve analysis, was recently described (14). A PCR assay targeting the *Bordetella* adenylate cyclase gene was described by Douglas et al. (4). *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were detected by the assay (*B. holmesii* was not tested). Farrell et al. described a PCR assay based on the detection of the outer membrane porin gene and differentiation of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* using species-specific probes (6). None of the probes detected *B. holmesii*. However, few *B. bronchiseptica* and *B. holmesii* isolates were tested. RecA is a protein involved in recombinational DNA repair. Depending on the design of PCR primers and probes, real-time PCR assays targeting the *recA* gene detected *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* but not *B. holmesii* (25) or detected *B. holmesii* only (1, 8). The *B. holmesii*-specific *recA* primers have been incorporated with IS*481* primers into an assay to differentiate *B. pertussis* and *B. holmesii* (8). The flagellin gene was the target of an early PCR assay. PCR primers amplified *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, but *B. pertussis* produced an amplicon of a different size that was distinguished in agarose gels (12). *B. holmesii* was not evaluated. BP3385, a gene of unknown function, was the target of a *B. pertussis*-specific real-time PCR assay that did not detect *B. parapertussis*, *B. hinzii*, *B. holmesii*, or *B. bronchiseptica* (9). However, the number of isolates of each species was not provided. A later study with a large number of isolates found that BP3385 is present in some strains of *B. hinzii* and *B. bronchiseptica* (26). A duplex real-time PCR assay targeting the thiolase gene BP283 and undefined gene BP485 was described by Probert et al. (24). The assay did not detect isolates of *B. holmesii*, *B. parapertussis*, or *B. bronchiseptica*. However, only five *B. bronchiseptica* isolates were tested. With the exception of the pertussis toxin gene, PCR assays based on single-copy targets have not been extensively evaluated and require further analytical and clinical validation.

In addition to the multiplex assays described above, a two-tiered or reflex testing approach can provide adequate species specificity when using an IS*481*-based PCR. In this approach, IS*481* PCR serves as a highly sensitive screening assay. A specimen positive by IS*481* PCR would require further testing using an assay that detects pertussis toxin or another target specific for *B. pertussis* (or conversely, a *B. holmesii*-specific assay to rule it out as the cause of the positive IS*481* PCR result). If unable to verify the positive IS*481* result, the laboratory could report either a negative or an indeterminate result for *B. pertussis*. This approach has been used retrospectively by the CDC to investigate pertussis outbreaks (2) but could also be used prospectively in the diagnostic laboratory. A two-tiered testing algorithm would likely miss some true pertussis infections, but the data in the literature suggest that these false negatives would be relatively low in number (25). In my opinion, these few false negatives from an overall highly sensitive assay would be heavily outweighed by the benefit of fewer false positives and pseudo-outbreaks.

REAL-WORLD PERFORMANCE OF PCR TESTS FOR *BORDETELLA*: THE GOOD, BAD, AND THE UGLY

The widespread use of PCR to diagnose pertussis has provided many benefits, primarily as a result of its high sensitivity relative to other laboratory methods. These benefits include detection and treatment of additional infections (particularly among adolescents and adults) and, hence, better control and prevention of

outbreaks. The use of PCR has increased our understanding of the effect of patient age and duration of symptoms on the sensitivity of not only PCR but also culture. It has revealed the significance of pertussis in older persons and the important role that these age groups play in disease transmission. As a result, vaccine boosters are now available and recommended for adolescents and adults.

Laboratory testing during pertussis outbreaks is often conducted under such stresses as the demand for a rapid turnaround of test results, a higher-than-normal testing volume (often including specimens from individuals with a low pretest probability of disease), and the cocirculation of other respiratory pathogens. Weaknesses of any diagnostic test are more likely to be revealed under these conditions, and NAATs for *B. pertussis* are no exception. Pseudo-outbreaks of pertussis due to false-positive PCR results have been reported in the literature and have a variety of different root causes. They result in the unnecessary expenditure of considerable resources by health care institutions and public health departments. Cross-reactivity of a *B. pertussis* IS*481* PCR test with *B. holmesii* in a specimen from an emergency department nurse resulted in the contact or evaluation of a number of pediatric patients and their caregivers, additional health care workers, and persons who attended a conference with the index patient (37). Weakly positive results from real-time PCR assays that target IS*481* have contributed to pseudo-outbreaks (2, 19). Many of these weakly positive samples were not verifiable by alternate PCR assays that targeted different genes. Additionally, the majority of PCR-positive cases tested by IgG enzyme-linked immunosorbent assay were negative for anti-pertussis toxin IgG (2). PCR-positive cases with weakly positive results by IS*481* real-time PCR (crossing threshold values of >35) were unlikely to meet the clinical case definition and were more likely to be false positives (9, 23). Environmental contamination in patient clinics and the resulting contamination of specimens have contributed to pseudo-outbreaks (19). Some *B. pertussis* vaccines have been shown to contain genomic DNA, and aerosolization of vaccine likely results in the contamination of the hands of health care providers and environmental surfaces (30). Performance of specimen collection and vaccination in the same room was linked to false-positive PCR results (19). Many of the false-positive patient specimens from the pseudo-outbreak, as well as environmental swabs from the clinics, had high crossing threshold values (>35). Contamination occurring in the laboratory has also contributed to a pseudo-outbreak (17). The contamination was resolved after the laboratory switched to new PCR primers that amplified a different gene target. Details of the PCR method were not provided. However, as this contamination event occurred in the late 1990s, it is likely that a conventional PCR assay was used. Real-time PCR, which does not require the opening of tubes following PCR, greatly reduces the risk of contamination from amplicons.

SUMMARY

NAATs such as PCR are the most sensitive laboratory methods routinely available for the detection of *B. pertussis*. With a rapid turnaround time and a capacity for high throughput, PCR testing can have a substantial positive impact on patient care and the management of outbreaks. The predictive value of positive NAAT results is perhaps the greatest limitation of these methods. IS*481*-based NAATs will detect *B. holmesii*, which for epidemiologic and clinical purposes is considered a false positive. Environmental contamination in patient clinics has been identified as a source of false-positive test results. The positive predictive value of *B. pertussis* NAATs can be improved by amplifying gene targets that are not shared among species, using multiplex assays or a two-tiered testing algorithm to confirm positives, implementing an indeterminate range for assays that target multiple-copy insertion sequences, segregation of "clean" and "dirty" areas both in patient clinics and the diagnostic laboratory, and testing only symptomatic persons. Additional guidance for health care professionals on PCR testing for *B. pertussis*—including specimen collection and interpretation of test results—is available from the CDC (http://www.cdc.gov/pertussis/clinical/diagnostic-testing/diagnosis-pcr-bestpractices.html, last accessed 26 February 2012).

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