

Harmonization of *Bordetella pertussis* Real-Time PCR Diagnostics in the United States in 2012

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Real-time PCR (rt-PCR) is an important diagnostic tool for the identification of *Bordetella pertussis*, *Bordetella holmesii*, and *Bordetella parapertussis*. Most U.S. public health laboratories (USPHLs) target IS481, present in 218 to 238 copies in the *B. pertussis* genome and 32 to 65 copies in *B. holmesii*. The CDC developed a multitarget PCR assay to differentiate *B. pertussis*, *B. holmesii*, and *B. parapertussis* and provided protocols and training to 19 USPHLs. The 2012 performance exercise (PE) assessed the capability of USPHLs to detect these three *Bordetella* species in clinical samples. Laboratories were recruited by the Wisconsin State Proficiency Testing program through the Association of Public Health Laboratories, in partnership with the CDC. Spring and fall PE panels contained 12 samples each of viable *Bordetella* and non-*Bordetella* species in saline. Fifty and 53 USPHLs participated in the spring and fall PEs, respectively, using a variety of nucleic acid extraction methods, PCR platforms, and assays. Ninety-six percent and 94% of laboratories targeted IS481 in spring and fall, respectively, in either singleplex or multiplex assays. In spring and fall, respectively, 72% and 79% of USPHLs differentiated *B. pertussis* and *B. holmesii* and 68% and 72% identified *B. parapertussis*. IS481 cycle threshold (C_T) values for *B. pertussis* samples had coefficients of variation (CV) ranging from 10% to 28%. Of the USPHLs that differentiated *B. pertussis* and *B. holmesii*, sensitivity was 96% and specificity was 95% for the combined panels. The 2012 PE demonstrated increased harmonization of rt-PCR *Bordetella* diagnostic protocols in USPHLs compared to that of the previous survey.

Clinical laboratories increasingly depend on real-time PCR (rt-PCR) to diagnose pertussis, a respiratory disease caused by *Bordetella pertussis*. Similar disease symptoms are also caused by *Bordetella parapertussis* and sometimes by *Bordetella holmesii* or *Bordetella bronchiseptica*. Many rt-PCR assays target an insertion element, IS481, present in high copy number (218 to 238 copies) in *B. pertussis* (1, 2), low copy number (32 to 65 copies) in *B. holmesii* (3), and some strains of *B. bronchiseptica* (4). If IS481 is the sole target, the laboratory cannot report a specific result for *B. pertussis* and will miss *B. parapertussis* infections. The CDC multitarget rt-PCR assay to detect and identify *B. pertussis*, *B. holmesii*, and *B. parapertussis* is a multiplex assay targeting IS481, IS1001 in *B. parapertussis* (pIS1001), and the IS1001-like element in *B. holmesii* (hIS1001) (5). As confirmation of *B. pertussis*, the assay also includes a singleplex rt-PCR for *ptxS1*, which encodes the first subunit of the pertussis toxin. PCR inhibition is identified with a singleplex assay for human *rnaseP*. After CDC laboratory validation with isolates and clinical specimens, the multitarget assay was offered to U.S. public health laboratories (USPHLs) along with a series of onsite trainings, webinars, and additional communications.

With the increase in rt-PCR diagnostics, public health, hospital, and commercial laboratories have adopted many protocols that differ in DNA extraction methods, rt-PCR platforms, and protocols. It is important, therefore, to monitor and assess the ability of laboratories to demonstrate consistency in diagnostic accuracy and precision. Several surveys and performance exercises have been performed in Europe and one of each in the United States (6–9). For instance, eight hospital laboratories in France in 2006 to 2007 participated in a performance evaluation provided by the French National Centre of Reference of Pertussis and other *Bordetellales* (6). All eight laboratories used rt-PCR targeting IS481, with resulting sensitivity and specificity of 92.2% and 94.3%, respectively. Some laboratories also included targets to

confirm *B. pertussis* or other species, including *B. parapertussis*. In a 2010 survey of pertussis diagnostics in 27 European countries, 74% of countries reported using serology, followed by 67% using PCR, and 63% using culture (7). This contrasts with a previous survey in the United States in which 123 public health, commercial, and hospital laboratories reported that 7% perform serologic diagnostics, 54% perform PCR, and 71% perform culture (8). Along with the survey, 41 laboratories participated in a performance exercise that included 13 public health laboratories. Laboratories employing various extraction and rt-PCR methods reported *B. pertussis* with a sensitivity of 92% (8). In a 2011 European PCR performance exercise involving 24 national reference laboratories from 19 countries, 100% of laboratories detected *B. pertussis* in high concentrations within the samples of extracted DNA provided to them. However, 14 out of 24 laboratories (58%) misidentified *B. holmesii* as *B. pertussis* because they were targeting IS481 only, with no target to distinguish *B. holmesii* from *B. pertussis* (9).

With a high dependence on PCR for pertussis diagnostics and evidence that extraction, PCR platform, protocol, and PCR targets

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TABLE 1 Fall and spring panels, each containing 12 samples of *Bordetella pertussis*, *B. holmesii*, *B. parapertussis*, and non-*Bordetella* species

Panel and sample no.	Organism	Sample bacterial concn (CFU/ml)
Spring		
BP12-1-1	<i>Bordetella pertussis</i>	1.0×10^6
BP12-1-2	<i>Pseudomonas aeruginosa</i>	1.0×10^5
BP12-1-3	<i>B. parapertussis</i>	1.0×10^3
BP12-1-4	<i>B. holmesii</i>	1.0×10^3
BP12-1-5	<i>B. pertussis</i>	1.0×10^3
BP12-1-6	<i>B. holmesii</i>	1.0×10^6
BP12-1-7	<i>Haemophilus influenzae</i>	1.0×10^5
BP12-1-8	<i>B. parapertussis</i>	1.0×10^4
BP12-1-9	<i>B. pertussis</i>	1.0×10^5
BP12-1-10	<i>B. pertussis</i>	1.0×10^3
BP12-1-11	<i>B. holmesii</i>	1.0×10^3
BP12-1-12	<i>B. parapertussis</i>	1.0×10^4
Fall		
BP12-2-1	<i>B. pertussis</i>	1.0×10^5
BP12-2-2	<i>B. parapertussis</i>	1.0×10^4
BP12-2-3	<i>B. holmesii</i>	1.0×10^6
BP12-2-4	<i>B. parapertussis</i>	1.0×10^6
BP12-2-5	<i>B. pertussis</i>	1.0×10^4
BP12-2-6	<i>B. holmesii</i>	1.0×10^6
BP12-2-7	<i>B. parapertussis</i>	1.0×10^6
BP12-2-8	<i>B. pertussis</i>	1.0×10^7
BP12-2-9	<i>P. aeruginosa</i>	1.0×10^5
BP12-2-10	<i>B. holmesii</i>	1.0×10^5
BP12-2-11	<i>B. pertussis</i>	1.0×10^5
BP12-2-12	<i>H. influenzae</i>	1.0×10^5

vary among laboratories (7, 8), it is important to provide external assessment through performance exercises to analyze the consistency in PCR results across laboratories. We report here the results of the 2012 rt-PCR performance exercise for USPHLs for the detection of *B. pertussis*, *B. parapertussis*, and *B. holmesii*.

MATERIALS AND METHODS

Laboratory recruitment, testing, and reporting. State and local public health laboratories were recruited by the Wisconsin State Laboratory of Hygiene (WSLH) Proficiency Testing program through the Association of Public Health Laboratories (APHL), in partnership with the CDC. Two panels of bacteria were sent in the spring and fall of 2012 that contained 12 samples each of *Bordetella* and non-*Bordetella* species in saline at 1×10^3 to 1×10^6 CFU/ml in the spring and 1×10^4 to 1×10^7 CFU/ml in the fall (Table 1). DNA from human A549 cells was included in all the samples at a concentration that would result in a crossing cycle threshold (C_T) value of 30 to 34 when amplifying *rnaseP*. Samples were aliquoted and stored frozen at -80°C until shipped on wet ice to participating laboratories. Laboratories were instructed to use their routine nucleic acid extraction and PCR procedures and to complete a worksheet to record when samples were received, thawed, and analyzed. The extraction method, rt-PCR platform, PCR targets, and number of replicates were also reported. Results included C_T values, melting point (MP) value (if applicable), target interpretation, and a final interpretation for the sample. Participants entered answers of positive, negative, equivocal, indeterminate, or not tested for *B. pertussis*, *B. parapertussis*, *B. holmesii*, and *B. pertussis/holmesii* undifferentiated (BpBh) for each sample. The BpBh response choice was intended for laboratories that performed a single target assay, i.e., IS481, that prevented them from distinguishing between *B. pertussis* and *B. holmesii*. When laboratories misunderstood the directions to complete the work-

sheet and filled in answers for the *B. pertussis* or *B. holmesii* species response in addition to the BpBh choice, only one answer was chosen based on the PCR targets that the lab reported, to avoid counting the laboratory's answers twice.

Statistics. For each panel and sample, lab-reported results were compared with previously determined results to establish the accuracy and precision of the tests. Statistical summaries of performance were tabulated for completeness of testing and reporting and for performance metrics. Sensitivity and specificity were calculated using known positive and negative samples. Positive samples for each species included all respective species-positive samples in each panel. Since negative samples must be included to compute meaningful specificity estimates, and since negative samples for a given species have no concentration, samples that were positive for some other species at the same respective concentration were included as the negative samples. Thus, a uniform set of positive and negative samples was identified for each species at each concentration, allowing for consistent computations of sensitivity and specificity at each concentration and across all concentrations. For those computations, responses of indeterminate and equivocal were counted as positive.

For counts of tests reported and for counts of positive and negative detections, all relevant tests were used. For example, a given lab might have tested all specimens in a given panel for *B. pertussis* but only some of the specimens for *B. parapertussis*. Similarly, a lab might have distinguished between *B. holmesii* and *B. pertussis* in some specimens but not in others. Finally, a lab might have reported or tested the spring and fall panels differently. In such cases, the number of tests for the reported statistics may be different for different pathogens and may be greater than the number of labs. Other differences between counting tests and counting labs appear in the figure and tables and are designated where not apparent from context.

In addition to the qualitative positive/negative analyses which produced estimates of sensitivity and specificity, a quantitative analysis of the C_T s was performed. Pearson's correlation coefficients were calculated relating C_T values of frequently reported rt-PCR targets to sample bacterial concentrations (CFU/ml).

RESULTS

Fifty USPHLs representing 41 states participated in the spring PE, and 53 USPHLs from 42 states took part in the fall PE. USPHLs included state and local public health laboratories, resulting in participation by multiple laboratories in some states.

Three methods for nucleic acid extraction were reported. The most common method was automated magnetic-based extraction, reported by 44% and 53% of laboratories in the spring and fall, respectively. The MagNA Pure platform (Roche Applied Science, Indianapolis, IN) was the most frequently reported automated system, employed by 38% and 45% of USPHLs in the spring and fall, respectively, followed by NucliSENS EasyMAG (bioMérieux, Durham, NC). The other methods employed were extraction with a manual spin column kit (Qiagen, Valencia, CA), reported by 30% and 28% of laboratories, and heat lysis, reported by 16% and 9%, respectively. Five laboratories from each panel did not provide their extraction methods.

Laboratories reported five rt-PCR platforms. The majority of laboratories employed the AB7500 fast machine (Applied Biosystems, Foster City, CA), reported by 58% and 53% in the spring and fall, respectively. The other platforms reported were the SmartCycler (Cepheid, Sunnyvale, CA), employed by 26% and 24% USPHLs; LightCycler (Roche Applied Science), reported by 10% and 17%; iCycler iQ (Bio-Rad, Hercules, CA), employed by 4% in each panel; and the FilmArray (BioFire Diagnostics, Salt Lake City, UT), employed by 2% (one laboratory) in each panel.

For both panels, 94% of laboratories targeted IS481 either as a

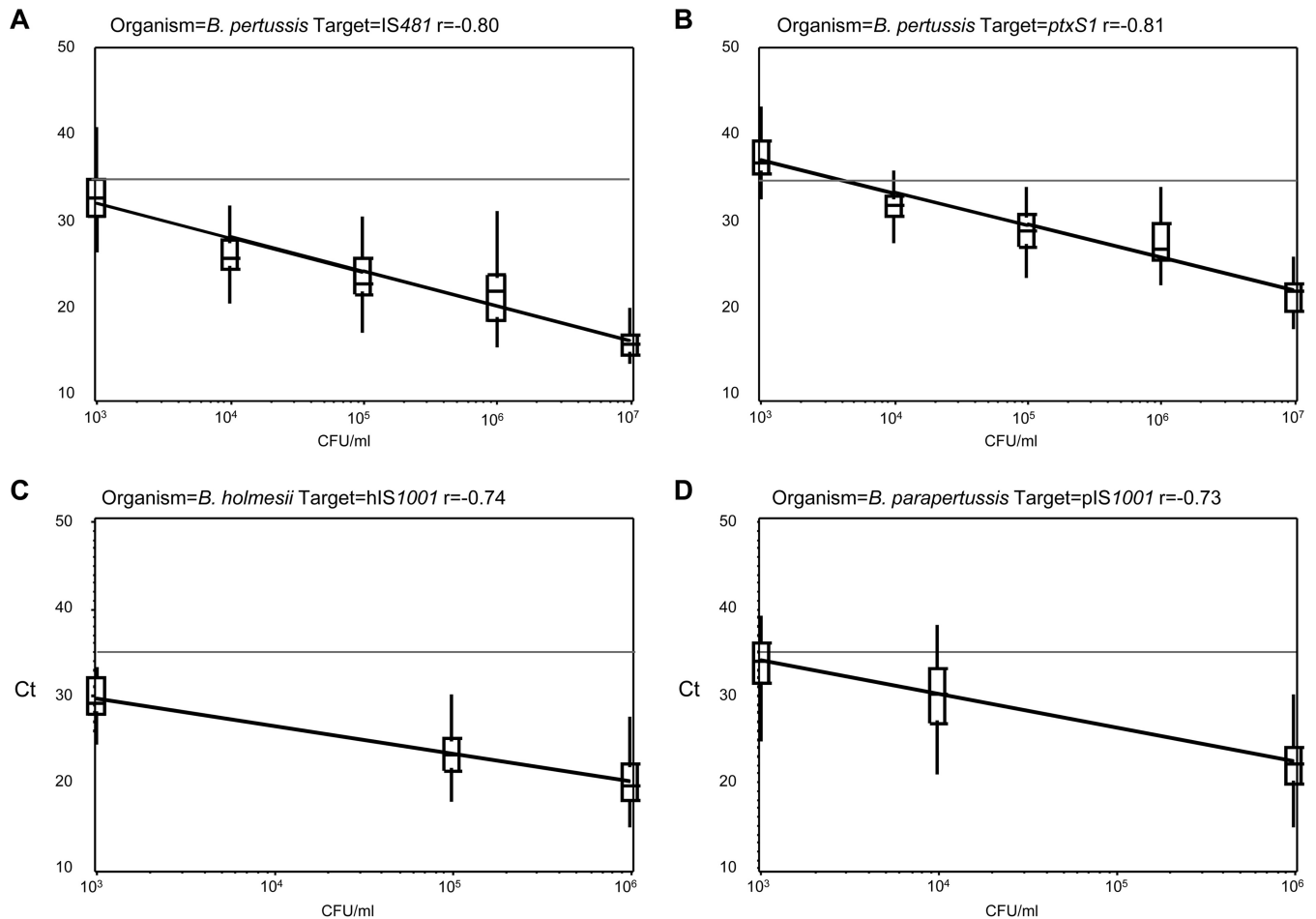


FIG 1 Correlation of cycle threshold (C_T) values with sample concentrations for four real-time PCR targets, combining results from the spring and fall performance panels. Pearson's correlation coefficient (r) is shown above each plot. (A) Eight *B. pertussis* samples, IS481 target ($n = 386$). (B) Eight *B. pertussis* samples, *ptxS1* target ($n = 177$). (C) Six *B. holmesii* samples, hIS1001 target ($n = 147$). (D) Six *B. paraptussis* samples, pIS1001 target ($n = 198$). Each horizontal line indicates the positive cutoff value for IS481 in the CDC multitarget assay ($C_T < 35$).

single target or as part of a multitarget assay. In the spring and fall, respectively, 76% and 83% of USPHLs tested multiple targets, including 46% and 47% that performed the CDC assay. Laboratories were considered to have performed the CDC assay if they included the multitarget assay, IS481, pIS1001, and hIS1001, with or without *ptxS1* confirmation or internal control. In the spring and fall, respectively, 46% and 53% included *ptxS1* as a target to confirm *B. pertussis*. When considering all targets and protocols, 72% and 79% of USPHLs detected *B. pertussis* (note that these percentages are not sensitivities because they include in the denominator opportunities for testing when no test was reported or, presumably, performed), 52% and 57% detected *B. holmesii*, and 68% and 72% detected *B. paraptussis*, reporting results at least once per panel. Those reporting in only the BpBh category decreased slightly from 18% in the spring to 16% in the fall. Nine *Bordetella* targets were reported: IS481, hIS1001, pIS1001, IS1663, *ptxS1*, *ptxP*, BP485, *recA*, and *prn*. One laboratory did not provide target identities.

A total of 84% and 83% of USPHLs in the spring and fall, respectively, included an internal control. Although various targets were included as internal controls, human *rnaseP* was the most prevalent, utilized by 73.8% and 75% of the laboratories.

Other targets included the gene encoding beta-actin, pCB1, the 16S rRNA gene, QC-DNA SSC from a commercial kit (Cepheid), *recA*, and the beta-globin gene.

In both spring and fall, one laboratory reported either MP or categorical data (i.e., positive or negative) only. All other USPHLs provided C_T values. Two USPHLs in the spring and four in the fall included MP data along with C_T values.

Figure 1 demonstrates that C_T values correlated with concentrations across all labs and both panels (spring and fall combined) for four targets: IS481 ($n = 386$) and *ptxS1* ($n = 177$) for eight *B. pertussis* samples, hIS1001 ($n = 147$) for six *B. holmesii* samples, and pIS1001 ($n = 198$) for six *B. paraptussis* samples. The coefficient of variation (CV) values varied from 8.3% to 28.0% across concentrations for these four targets (**Table 2**). All but one value was $<25\%$. For target IS481, the results for the *B. pertussis* 1×10^7 -CFU/ml sample included one false-negative value listed with an assigned nominal-negative C_T value of 45, skewing the CV to 28%.

From the 50 labs for spring and 53 labs for fall combined, 330 tests of undifferentiated *B. pertussis* or *B. holmesii* were reported, including tests of positive and negative samples; 907 tests of *B. pertussis*, specifically, and 844 tests of *B. holmesii* were reported.

TABLE 2 Coefficient of variation for PCR targets IS481 and *ptxS1* (*B. pertussis* samples), *hIS1001* (*B. holmesii* samples), and *pIS1001* (*B. parapertussis* samples), fall and spring samples combined

PCR target	CV (%) for indicated target at a concn (CFU/ml) of:				
	1×10^{3a}	1×10^4	1×10^5	1×10^6	1×10^7
IS481	12.0	10.2	14.2	17.2	28.0
<i>ptxS1</i>	8.4	8.3	11.8	12.8	11.9
<i>hIS1001</i>	10.8	— ^a	14.3	20.7	—
<i>pIS1001</i>	10.0	13.6	—	19.2	—

^a —, samples not provided at this concentration.

Results are also reported by concentration; for 1×10^3 CFU/ml of any pathogen, for example, 170 tests were reported for *B. pertussis*. For sample BP12-1-5, 36 USPHLs reported tests for *B. pertussis* specifically, and 32 tests were positive. Thirty-seven USPHLs reported *B. pertussis* tests for sample BP12-1-10, and 31 tests were positive. Counts for all combinations of pathogens and concentrations, along with sensitivities and specificities, are reported in Table 3.

The USPHLs reported >95% sensitivities and specificities for all four diagnostic categories, *B. pertussis*, *B. parapertussis*, *B. holmesii*, and BpBh (Table 3), calculated for the subset of USPHLs that provided results in each category. Eight laboratories (three in the spring, two in the fall, and three in both panels) filled in answers for the *B. pertussis* or *B. holmesii* categories in addition to BpBh, and only one category was counted. Another eight laboratories reported a false-positive result for *B. pertussis* in at least one *B. holmesii* sample (four in the spring, two in the fall, and two in both panels; data not shown). Since negative results for samples of the same concentration were included in the specificity calculation, false-positive results caused by misidentification of *B. holmesii* as *B. pertussis* were a major contributor to reduced *B. pertussis* specificity. For example, *B. pertussis* specificity was highest in the 1×10^4 samples, and the panels did not contain a *B. holmesii* sample at 1×10^4 . *B. pertussis* specificity was lowest in the 1×10^3 and 1×10^6 samples, concentrations at which two and six *B. holmesii* samples, respectively, were provided (Tables 1 and 3). Few USPHLs reported false-positive *B. pertussis* identification for samples other than *B. holmesii*: one laboratory misidentified *Pseudomonas aeruginosa* as *B. pertussis* in the spring panel (2% of laboratories), and three other laboratories each reported one false-positive *B. pertussis* for *B. parapertussis* (two laboratories) or *P. aeruginosa* (one laboratory) in the fall (5.7% of laboratories).

Examining the effect of the DNA extraction method on the results, sensitivity ranged from 94.3% to 100%, and specificity ranged from 88.8% to 100% in *B. pertussis*, *B. parapertussis*, *B. holmesii*, and BpBh results (Table 4). USPHLs that extracted DNA with the EasyMAG reported results with sensitivity and specificity higher than results obtained after DNA extraction with the MagNA Pure system, except for equivalent specificity for *B. parapertussis* samples and lower specificity for *B. holmesii* samples. However, very few USPHLs used the EasyMAG, making comparison difficult. No other trend emerged for a particular DNA extraction method.

DISCUSSION

The 2012 pertussis rt-PCR performance exercise for USPHLs demonstrated improvement over the previous exercise in several

TABLE 3 Sensitivity and specificity for the subset of tests performed by USPHLs in *B. pertussis*, *B. parapertussis*, *B. holmesii*, and *B. pertussis/B. holmesii* (undifferentiated)

Organism	Sample bacterial concn (CFU/ml)	<i>n</i> ^a	Sensitivity (%)	Specificity (%)
			[95% CI]	[95% CI]
<i>B. pertussis</i>	All	907	96.1 (93.2–97.9)	95.1 (93.2–96.8)
	1×10^3	170	88.4 (78.4–94.9)	89.1 (81.3–94.4)
	1×10^4	154	97.6 (87.1–99.9)	99.1 (95.2–100)
	1×10^5	312	97.4 (92.7–99.5)	96.9 (93.4–98.9)
	1×10^6	230	100 (90.3–100)	94.3 (90.1–97.1)
	1×10^7	41	100 (91.4–100)	— ^b
<i>B. parapertussis</i>	All	844	98.6 (95.9–99.7)	99.7 (98.9–100)
	1×10^3	170	97.1 (84.7–99.9)	99.3 (96.0–100)
	1×10^4	141	99.0 (94.8–100)	100 (90.3–100)
	1×10^5	284	—	99.6 (98.1–100)
	1×10^6	213	98.6 (92.7–100)	100 (97.4–100)
	1×10^7	36	—	100 (90.3–100)
<i>B. holmesii</i>	All	666	98.2 (94.7–99.6)	97.8 (96.1–98.9)
	1×10^3	130	96.2 (86.8–99.5)	98.7 (93.1–100)
	1×10^4	111	—	96.4 (91.0–99.0)
	1×10^5	227	100 (88.1–100)	97.5 (94.2–99.2)
	1×10^6	168	98.8 (93.5–100)	98.8 (93.6–100)
	1×10^7	30	—	100 (88.4–100)
<i>B. pertussis/B. holmesii</i>	All	330	97.6 (94.4–99.2)	96.0 (90.9–98.7)
	1×10^3	78	93.7 (84.5–98.2)	100 (78.2–100)
	1×10^4	52	100 (73.5–100)	95.0 (83.1–99.4)
	1×10^5	105	100 (93.5–100)	100 (92.9–100)
	1×10^6	83	100 (94.3–100)	85.0 (62.1–96.8)
	1×10^7	12	91.7 (61.5–99.8)	—

^a *n*, number of tests reported for each organism, including tests of positive and negative samples.

^b —, in calculable values due to empty cells, e.g., no false negatives with 95% CI.

areas, including increased participation (50 [spring] and 53 [fall] versus 41 total laboratories, including 13 USPHLs, in the 2010 performance exercise) and a higher percentage of laboratories that include multiple targets in their assays (76% versus 61%) (8). Sensitivity of *B. pertussis* detection improved from 92% to 96%. This comparison was made with the understanding that laboratories were recruited differently in the previous exercise and included commercial and hospital laboratories and public health laboratories (8). Qualitative improvement was observed between the 2012 spring and fall panels for the *B. pertussis* results. The majority of *B. holmesii* samples misidentified as *B. pertussis* were reported by six laboratories in the spring, and only two laboratories reported false-positive *B. pertussis* for *B. holmesii* samples in both panels. Among the laboratories in the spring panel, two reported equivocal or indeterminate results for *B. pertussis*, which were converted to positive results in this analysis. This conversion was chosen in case of nucleic acid loss due to shipping. One laboratory added the *hIS1001* target to its protocol for the fall panel and subsequently did not report equivocal or indeterminate results. Two USPHLs that reported *B. pertussis* false-positive results only in the spring made no change to their rt-PCR targets, so improvement must have occurred for other reasons. The four *B. pertussis* false-positive results for *B. parapertussis* or *P. aeruginosa* samples (one in spring and three in fall) may indicate cross-con-

TABLE 4 Sensitivity, specificity, and confidence interval for the subset of tests performed by USPHLs in all diagnostic categories, by DNA extraction method

Organism	DNA extraction method	<i>n</i> ^a	Sensitivity (% [95% CI])	Specificity (% [95% CI])
<i>B. pertussis</i>	All	907	96.1 (93.2–97.9)	95.2 (93.2–96.8)
	Automated, EasyMAG	062	100 (83.2–100)	100 (91.6–100)
	Automated, MagNA Pure	371	94.3 (88.6–97.7)	95.6 (92.2–97.8)
	Crude boil, heat lysis	84	100 (87.7–100)	100 (93.6–100)
	Spin column, Qiagen	270	94.6 (87.9–98.2)	94.9 (90.6–97.6)
	Not specified	120	100 (91.2–100)	88.8 (79.7–94.7)
<i>B. parapertussis</i>	All	844	98.6 (95.9–99.7)	99.7 (98.9–100)
	Automated, EasyMAG	77	100 (83.9–100)	100 (93.6–100)
	Automated, MagNA Pure	348	98.8 (93.8–100)	100.0 (98.6–100)
	Crude boil, heat lysis	84	95.2 (76.2–99.9)	98.4 (91.5–100)
	Spin column, Qiagen	286	98.6 (92.5–100)	99.5 (97.4–100)
	Not specified	49	100 (73.5–100)	100 (90.5–100)
<i>B. holmesii</i>	All	666	98.2 (94.7–99.6)	97.8 (96.1–98.9)
	Automated, EasyMAG	60	100 (78.2–100)	93.3 (81.7–98.6)
	Automated, MagNA Pure	311	97.4 (91.0–99.7)	99.6 (97.6–100)
	Crude boil, heat lysis	72	94.4 (72.7–99.9)	96.3 (87.3–99.5)
	Spin column, Qiagen	175	100 (91.4–100)	97.0 (92.5–99.2)
	Not specified	48	100 (73.5–100)	97.2 (85.5–99.9)
<i>B. pertussis</i> / <i>B. holmesii</i> (undifferentiated)	All	330	97.6 (94.4–99.2)	96.0 (90.9–98.7)
	Automated, EasyMAG	19	100 (76.8–100)	100 (47.8–100)
	Automated, MagNA Pure	144	94.4 (87.4–98.2)	90.9 (80.0–97.0)
	Crude boil, heat lysis	72	100 (91.6–100)	100 (88.4–100)
	Spin column, Qiagen	92	100 (93.7–100)	100 (90.0–100)
	Not specified	3	100 (29.2–100)	

^a *n*, number of tests reported for each organism, including tests of positive and negative samples.

tamination. If so, each of the four laboratories had a single instance of contamination.

Although there is no assurance or expectation that the C_T s across laboratories would be consistent, there was in fact a high inverse correlation between C_T values and specimen concentration, as one might ideally expect (Fig. 1 and Table 2). This is a reassuring finding in regard to the common use of a C_T cutoff, e.g., <35 for target IS481 in the CDC assay, but warrants further study in regard to harmonization.

The expansion of the U.S. performance exercise to include two other *Bordetella* species demonstrated high sensitivity and specificity of *B. parapertussis* and *B. holmesii* detection by laboratories with species-level detection capacity. A future target for improvement is a higher percentage of USPHLs that correctly detect all three *Bordetella* species, such as that seen in the 2013 external quality assurance exercise for *Bordetella* identification reported by the European CDC (10).

As anticipated, *B. pertussis* detection sensitivity was lower in samples with the lowest *B. pertussis* concentration (1×10^3 CFU/ml), suggesting that the samples were close to the limit of detection of the PCR assays (Table 3). Sensitivity was higher in low-concentration samples of *B. parapertussis* and *B. holmesii* than in low-concentration *B. pertussis* samples. This may be due to the need to confirm *B. pertussis* diagnoses by a second PCR when using the IS481 target, most commonly targeting a single-copy gene, such as *ptxS1*, which is a slightly less sensitive assay (5). All six USPHLs that submitted a false-negative result for a *B. pertussis* 1×10^3 -CFU/ml sample included *ptxS1* as one of their targets, and five out of six of the laboratories employed the MagNA Pure sys-

tem for DNA extraction (data not shown). The most common targets to identify *B. parapertussis* and *B. holmesii* (*pIS1001* and *hIS1001*, respectively) occur in multiple copies per genome and do not require additional confirmation. The CDC assay, designed for outbreak investigations, includes an upper-limit C_T of <35 for IS481 to avoid false-positive results (5). In contrast, the CDC has set the upper limit for *pIS1001* and *hIS1001* at a C_T of <40 (5). It is important to have accurate species-level diagnostics of pertussis and pertussis-like infections for public health response, vaccine efficacy, and epidemiological studies (11). Inclusion of additional targets such as *ptxS1* in pertussis PCR diagnostics is important to avoid misidentification of *B. holmesii* as *B. pertussis*, to prevent other false-positive results, and to detect the rare coinfection with *B. pertussis* and a second *Bordetella* species (12–14).

The lower *B. pertussis* sensitivities achieved for some samples by laboratories that extract DNA with MagNA Pure or manual Qiagen spin columns suggest that these two methods may be less efficient at DNA extraction than the EasyMAG platform or simple heat lysis. A disadvantage of using heat lysis to release DNA for PCR is that it does not remove potential PCR inhibitors. Although human DNA was added to the bacterial saline suspensions to simulate clinical specimens, actual specimens may contain inhibiting components. An additional concern with manual extraction or heat lysis is the increased possibility for DNA cross-contamination (15). An efficacy comparison of DNA extraction kits to detect *Bordetella* species in nasopharyngeal swabs or aspirates without the added complication of multiple laboratories and PCR protocols will be required to definitively resolve this question.

Limitations of the 2012 performance exercise included confu-

sion for using the BpBh category, which led to a few results that were reported in two categories. Bias may have been introduced into the analysis by choosing one result per sample to avoid double counting. Another possible source of bias is the choice to include indeterminate and equivocal results in the positive category. The current performance exercise did not include a detailed survey of laboratory protocols or validation of assays and did not request that USPHLs provide positive cutoff C_T values for their assays. The analysis was based solely on the result interpretations made by each USPHL. The previous performance exercise found that most participating laboratories took appropriate precautions to avoid contamination, such as having separate areas for working with the PCR master mix, template, and positive controls (8), indicating that additional surveys of laboratory protocols were not necessary at this time.

In conclusion, the 2012 pertussis rt-PCR performance exercise demonstrated that USPHLs detect *Bordetella* species with high sensitivity and specificity, despite the continued variety of DNA extraction methods, PCR platforms, targets, and PCR protocols adopted by the laboratories. The majority of participating laboratories differentiate between *B. pertussis*, *B. parapertussis*, and *B. holmesii*; and 47% have adopted the CDC rt-PCR assay. Intensive training and communication with USPHLs increased harmonization of the rt-PCR assay protocol and improved sensitivity for pertussis diagnostics. Suggestions for additional improvement include encouraging all USPHLs to expand their rt-PCR assays to detect *B. pertussis*, *B. parapertussis*, and *B. holmesii*. All assays should include an internal control, and, where feasible, DNA extraction should be automated to avoid potential cross-contamination of specimens.

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