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# External Quality Assessment for Molecular Detection of *Bordetella pertussis* in European Laboratories

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**Although the PCR for the detection of *Bordetella pertussis* is routinely performed in diagnostic laboratories, no quality assessment program has so far been described. We report on the results obtained with two external quality assessment proficiency panels sent to European laboratories. The first proficiency panel contained a series of dilutions of three previously characterized *B. pertussis* clinical isolates and two negative controls. No false-positive results were reported by six laboratories providing seven data sets. The reported limits of detection of the three *B. pertussis* strains varied between 4 and 4,000, 9 and 9,000, and 3 and 30,000 CFU/ml, respectively. The second proficiency panel, composed of a series of dilutions of reference strains of *B. pertussis*, *B. holmesii*, *B. hinzii*, and *B. bronchiseptica*, as well as negative controls, was sent to nine laboratories. One laboratory reported a negative result for a sample and reported a *B. parapertussis*-positive sample to be positive for *B. pertussis*. By using the *B. pertussis*-specific target gene pertactin, one laboratory detected *B. pertussis* with 100% specificity. All other laboratories, which used IS*481*-based assays, reported positive results for the samples containing *B. holmesii* and *B. bronchiseptica*, species that have occasionally been recovered from human respiratory samples. These data show that the choice of the target gene is particularly critical for the species specificity of *B. pertussis* PCR assays.**

Despite the introduction of large-scale vaccination for pertussis in many countries, whooping cough is still an endemic disease (5, 17). Several major and minor outbreaks have been described in countries with large-scale vaccination programs, such as Australia (7), The Netherlands (11, 30), the United States (3), Norway and Sweden (24), and Israel (20, 38).

Evidence suggests that pertussis cases might be grossly underreported (8, 9, 40). Moreover, since pertussis is thought to be an uncommon disease in industrialized countries and the clinical presentation resembles that of other illnesses associated with prolonged cough, health care providers often do not consider pertussis in the differential diagnosis (10).

Confirmation of the diagnosis of pertussis in the laboratory is challenging (16). Although culture of *B. pertussis* is still considered the "gold standard," it is not the ideal diagnostic tool. It has 100% specificity; but its sensitivity can vary greatly and is dependent on the stage of illness at the time of specimen collection, the technique used for specimen collection, specimen adequacy and transport, and culture conditions. Moreover, culture provides no rapid diagnosis: 7 to 10 days may be required to isolate and confirm or exclude the presence of *B. pertussis*.

During the last decade, the application of nucleic acid detection techniques has had a major impact on the diagnosis of pertussis. Numerous applications of extraction, amplification, and detection methods have been investigated (13, 14, 18, 25, 28, 37, 43, 45, 46). An improved sensitivity over that of culture was observed in all instances. Until now, no commercial assay has been available, and this has resulted in the development and introduction of a variety of methods developed in-house. With the improved and automated means of nucleic acid isolation, as well as the availability of real-time detection methods, a new generation of assays is being developed (1, 4, 6, 12, 21, 22, 39, 42). These technological developments have resulted in assays with very short turnaround times.

Although PCR assays for the detection of *B. pertussis* are routinely performed in diagnostic laboratories, no external quality assessment (EQA) program has so far been described. In order to define standards for tests that are clinically relevant, the Belgian Centres for Molecular Diagnostics (CMD) organized an EQA program. The Belgian public health service Rijksinstitut voor Ziekte- en Invalid:teitsverzekering, l'Institut National d'Assurance Maladie Invalidité requires that all members of CMD that perform molecular biology-based tests participate in this program. Laboratories from other European countries joined on a voluntary basis.

Here we describe the design, the panel assembly, and the analysis of the results obtained with two independent proficiency panels used for two blinded quality control studies distributed to six and nine participating European laboratories, respectively.

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TABLE 1. Genetic characteristics of *B. pertussis* strains used for the EQA panel 1

| Strain | Pertactin type | Pertussis type | PFGE type[a] |
|---|---|---|---|
| Bord191 | *prn2* | S1A | D1 |
| Bord200 | *prn2* | S1A | E1 |
| Bord201 | *prn3* | S1A | B1 |

[a] As determined by XbaI and SpeI enzyme restriction of chromosomal DNA and PFGE analysis (19).

One panel was used for determination of the lower limit of detection of *B. pertussis* isolates, while the other panel was used for determination of both analytical sensitivity and analytical specificity.

## MATERIALS AND METHODS

**Participating laboratories.** Four laboratories participated in both EQA panels, two participated in the first panel only, and five participated in the second panel only. Each laboratory followed its standard procedures for *Bordetella* PCR; thus, the procedures varied from laboratory to laboratory. The participating laboratories are as follows, in alphabetical order: Academisch Ziekenhuis Vrije Universitieit Brussel, Brussels, Belgium; C.H. de Versailles, Le Chesnay, France; C.H. Régional de la Citadelle, Liège, Belgium; GG&GD, Municipal Health Laboratory, Amsterdam, The Netherlands; Health Protection Agency, London, United Kingdom; Institut de Bactériologie, Strasbourg, France; Institut de Pathologie et de Génétique, Gerpinnes, Belgium; Klinikum Krefeld, Krefeld, Germany; St. Elisabeth Hospital, Tilburg, The Netherlands; ULB-Erasme, Brussels, Belgium; and University Hospital, Örebro, Sweden.

**Preparation of proficiency panels.** Proficiency panel 1 contained a series of dilutions of three *B. pertussis* clinical isolates collected in 2000 in the organizing laboratory. The isolates were typed previously (19) by analysis of the DNA sequence polymorphism of the pertussis toxin gene (*ptxS1*) and the pertactin gene (*prn*), as well as by analysis of chromosomal polymorphism by pulsed-field gel electrophoresis (PFGE) (Table 1). Five *Bordetella* species were selected for proficiency panel 2: *B. pertussis* (ATCC 9340), *B. parapertussis* (ATCC 15237), *B. hinzii* (LMG 1872), *B. holmesii* (LMG 15946), and *B. bronchiseptica* (ATCC 4617).

Physiologic saline solution was inoculated with cells from a single *B. pertussis* colony grown on Regan-Lowe charcoal agar (Charcoal Agar; Oxoid Ltd., Basingstoke, England) containing 10% horse blood and 40 mg of cephalexin (*Bordetella* Selective Supplement; Oxoid Ltd.) per liter. One to 10 serial dilutions were prepared in physiologic saline solution, the dilutions were aliquoted, and the number of CFU in each fraction was determined by plating 100 μl on the charcoal agar.

Similarly, the cells from a single colony each of *B. hinzii*, *B. holmesii*, and *B. bronchiseptica* grown on 3.7% tryptone soy agar (LabM, Lancashire, United Kingdom) containing 5% horse blood were used to spike 10 ml of physiologic saline solution. After the spiked samples were aliquoted, the number of CFU per milliliter was determined.

Panel 1 consisted of 20 frozen samples (Table 2): 6 samples of each of the 1/10 series of dilutions of strain Bord191 (range, 4 to $4 \times 10^5$ CFU/ml), strain Bord200 (range, 9 to $9 \times 10^5$ CFU/ml), and strain Bord201 (range, 3 to $3 \times 10^5$ CFU/ml) and two samples of physiologic saline solution without *B. pertussis* (as negative controls).

Panel 2 consisted of 15 frozen samples (Table 3): 6 samples of the dilution series of *B. pertussis* (range, 30 to $3 \times 10^6$ CFU/ml), 2 samples of the dilution series of *B. parapertussis* ($2 \times 10^6$ and $2 \times 10^4$ CFU/ml), 1 sample of *B. holmesii* ($>1 \times 10^6$ CFU/ml), 1 sample of *B. hinzii* ($>1 \times 10^6$ CFU/ml), 1 sample of *B. bronchiseptica* ($>1 \times 10^6$ CFU/ml), and 4 samples of physiologic saline solution (as negative controls).

In order to assess sample quality and homogeneity, three and four different technologists in the organizing laboratory tested the samples from panel 1 and panel 2, respectively, before distribution.

**Distribution of EQA samples.** The panels were distributed on dry ice by courier service. The participants were asked to report whether the samples were positive or negative for *B. pertussis* and whether another *Bordetella* species was detected. In order to obtain additional information on the procedures used, a questionnaire was sent to each participating laboratory.

TABLE 2. Results for EQA panel 1

| Sample code | Strain | No. of CFU/ml | Result for laboratory[a]: | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4a | 4b | 5 | 6 |
| B-A10 | Bord191 | 400,000 | + | + | + | + | + | + | + |
| B-A18 | Bord191 | 40,000 | + | + | + | + | + | + | + |
| B-A5 | Bord191 | 4,000 | + | + | + | + | + | + | Inh − |
| B-A3 | Bord191 | 400 | + | + | + | + | + | + | − |
| B-A9 | Bord191 | 40 | + | + | + | + | + | − | − |
| B-A1 | Bord191 | 4 | + | + | − | + | + | − | − |
| B-A21 | Negative control | | − | − | − | − | − | − | − |
| B-A15 | Bord200 | 900,000 | + | + | + | + | + | + | + |
| B-A8 | Bord200 | 90,000 | + | + | + | + | + | − | + |
| B-A17 | Bord200 | 9,000 | + | + | + | + | + | + | + |
| B-A4 | Bord200 | 900 | + | + | − | + | + | − | Inh − |
| B-A7 | Bord200 | 90 | + | + | − | + | + | − | − |
| B-A20 | Bord200 | 9 | − | − | − | − | + | − | Inh − |
| B-A12 | Negative control | | − | − | − | − | − | − | − |
| B-A2 | Bord201 | 300,000 | + | + | + | + | + | + | + |
| B-A16 | Bord201 | 30,000 | + | + | + | + | + | + | + |
| B-A13 | Bord201 | 3,000 | + | + | + | + | + | − | − |
| B-A19 | Bord201 | 300 | + | + | − | + | + | − | − |
| B-A6 | Bord201 | 30 | + | + | + | + | + | − | − |
| B-A14 | Bord201 | 3 | − | − | − | − | + | − | − |

[a] Laboratory 4a, results obtained without extraction; laboratory 4b, results obtained with MagnaPure extraction; Inh −; negative result for *B. pertussis* PCR, but inhibition was shown.

## RESULTS

**Proficiency panel 1.** The results obtained with the seven data sets from six laboratories are shown in Tables 2 and 4. All results were obtained by in-house methods. One laboratory (laboratory 4) reported two data sets, one with and one without DNA extraction, leading to different sensitivities. The results obtained by the first technologist in the organizing laboratory (laboratory 1 in Table 2) were also included in the data sets.

No false-positive results were reported (as determined with negative control samples B-A12 and B-A21). The detection limit for the three strains varied between 4 and 4,000, 9 and 9,000, and 3 and 30,000 CFU/ml, respectively. Laboratory 4, which reported two data sets (one with data obtained by use of MagnaPure DNA extraction and one obtained by use of no DNA extraction step), reported a higher sensitivity rate after extraction of the samples. However, one laboratory (laboratory 5) that used the QIAamp extraction procedure with a concentrated sample and another laboratory (laboratory 6) that used a boiling step with sample treatment with a reducing agent (*N*-acetylcysteine) reported results with very low analytical sensitivities.

There were considerable variations in the procedures used. Only one laboratory (laboratory 3) performed a nested PCR. The nested procedure was less sensitive than two procedures that used a single round of PCR (laboratories 1 and 4) but was more sensitive than two other single-round PCR assays (laboratories 5 and 6).

Of the six laboratories that reported the procedures that they used, five targeted the IS*481* insertion element. The other laboratory used the pTx promoter as the target gene.

**Proficiency panel 2.** The results obtained with the nine data sets for proficiency panel 2 are shown in Table 3. All results

TABLE 3. Results for EQA panel 2

| Sample code | Species | No. of CFU/ml | Result for laboratory[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 7 | 8 | 9 | 10 | 11 |
| B-B1 | None | | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| B-B5 | None | | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| B-B7 | None | | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| B-B11 | None | | Neg | BP | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| B-B2 | *B. pertussis* | $3 \times 10^6$ | BP | BP | BP | BP | BP | BP | BP | BP | BP |
| B-B6 | *B. pertussis* | $3 \times 10^5$ | BP | BP | BP | BP | BP | BP | BP | BP | BP |
| B-B13 | *B. pertussis* | $3 \times 10^4$ | BP | BP | BP | BP | BP | BP | BP | BP | BP |
| B-B12 | *B. pertussis* | $3 \times 10^3$ | BP | BP | BP | BP | BP | BP | BP | BP | BP |
| B-B8 | *B. pertussis* | $3 \times 10^2$ | BP | BP | BP | BP | BP | BP | BP or BH | BP | BP |
| B-B3 | *B. pertussis* | $3 \times 10^1$ | Neg | BP | Neg | Neg | BP | BP | Neg | BP | Neg |
| B-B9 | *B. parapertussis* | $2 \times 10^6$ | BPP | BP | BPP | BPP | BPP | Neg | Neg | Neg | Neg |
| B-B14 | *B. parapertussis* | $2 \times 10^4$ | BPP | ± | BPP | BPP | BPP | Neg | Neg | Neg | Neg |
| B-B10 | *B. holmesii* | $>1 \times 10^6$ | BP | BP | BP | BP | BP | BP | BP or BH | BP | Neg |
| B-B4 | *B. hinzii* | $>1 \times 10^6$ | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| B-B15 | *B. bronchiseptica* | $>1 \times 10^6$ | Neg/BP[b] | BP | BP | BP | BP | BP | BP or BH | BP | Neg |

[a] BP, positive reaction for *B. pertussis*; BPP, positive reaction for *B. parapertussis*; BH, positive reaction for *B. holmesii*; ±, unresolved result for *B. pertussis*; Neg, negative reaction for *B. pertussis* and *B. parapertussis* (when tests for *B. parapertussis* were conducted).
[b] A positive result for *B. pertussis* was reported by two of four technologists.

were obtained by in-house methods. The results obtained by the technologist performing the first predistribution test in the organizing laboratory (laboratory 1) were also included in the data sets.

One laboratory (laboratory 2) reported that one of the four negative controls (sample B-B11) was positive for *B. pertussis*. This laboratory uses real-time PCR, three physically separate areas for the different steps of the procedure, and dUTP and uracil glycosylase as a measure for the prevention of contamination (Table 5).

All nine laboratories detected the samples spiked with *B. pertussis* (samples B-B2, B-B6, B-B13, B-B12, and B-B8) to a dilution of 300 CFU/ml. Four of the nine laboratories (laboratories 2, 7, 8, and 10) reported a positive result for the sample containing 30 CFU/ml (sample B-B3). These four laboratories did not perform the PCR with sample volumes higher than those used by the five laboratories reporting a negative result for this sample (for the first set of four laboratories, mean volume, 10 μl; median volume, 8.75 μl; and volume range, 5 to 20 μl; for the second set of five laboratories, mean volume, 10 μl; median volume, 12.5 μl; volume range, 1 to 20 μl), nor did the extraction protocol influence the detection limit. Nested PCR assays demonstrated no improved analytical sensitivities compared to those of the single-round PCR assays.

The four laboratories that used the IS*1001* insertion element from *B. parapertussis* as the target detected an amplicon in both samples spiked with *B. parapertussis* (samples B-B9 and B-B14). Laboratory 2 reported the results for these samples as positive and weakly positive for *B. pertussis*, respectively.

None of the nine laboratories detected *Bordetella* spp. in the sample spiked with *B. hinzii* (sample B-B4). One laboratory (laboratory 11) that used the pertactin gene from *B. pertussis* as the target correctly reported the sample spiked with *B. holmesii* (sample B-B10) to be negative for *B. pertussis*. Use of a combination of the IS*481* and *ptxA* promoter region as targets for the detection of *B. pertussis* allowed another laboratory (laboratory 9) to answer "*B. pertussis* or *B. holmesii*." The remaining seven laboratories reported this sample to be *B. pertussis* positive.

Laboratory 11 correctly reported the sample spiked with *B. bronchiseptica* (sample B-B15) to be negative for *B. pertussis*. Two of four technologists performing the predistribution test in the organizing laboratory (laboratory 1) as well as the remaining seven laboratories reported this sample to be *B. pertussis* positive. These unexpected false-positive reactions for the majority of the participating laboratories suggest the presence of a homologous sequence in the *B. bronchiseptica* strain used.

TABLE 4. Methods used by participating laboratories with EQA panel 1

| Characteristic | Lab 1 | Lab 2 | Lab 3 | Lab 4a | Lab 4b | Lab 5 | Lab 6 |
|---|---|---|---|---|---|---|---|
| Extraction method | Boil | MagnaPure[a] | Boil | Boil | MagnaPure[a] | QIAamp[b] | Boil |
| Target gene | IS*481* | IS*481* | IS*481* | IS*481* | IS*481* | IS*481* | *ptxA* Pr[c] |
| Type of PCR | Single | Real time | Nested | Single | Single | Single | Single |
| No. of cycles | 40 | 50 | 30 + 30 | 33 | 33 | 30 | 35 |
| Eq. sample used for PCR[d] | 1 | 5 | 4 | 5 | 20 | 30 | 10 |

[a] MagnaPure (Roche Diagnostics).
[b] QIAamp DNA mini kit (Qiagen).
[c] *ptxA* Pr, pertussis toxin promoter.
[d] Equivalent (Eq.) sample used for PCR (in microliters), calculated as the starting volume used for concentration and extraction × fraction of extracted volume added to the amplification mixture.

TABLE 5. Methods used by participating laboratories with EQA panel 2

| Characteristic | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 7 | Lab 8 | Lab 9 | Lab 10 | Lab 11 |
|---|---|---|---|---|---|---|---|---|---|
| In-house method | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Concentration of sample | No | No | No | No | No | No | No | No | Centrifugation |
| Extraction method | Boil | Silica[a] | GuSCN[b] | MagnaPure[c] | Qiagen[d] | Qiagen | Qiagen | Amplicor[e] | Qiagen |
| Eq. sample used for PCR[f] | 1 | 5 | 4 | 20 | 8 or 20 | 10 | 12.5 | 5 | 12.5 |
| Amplification method | Single PCR | Real time | Nested PCR | Real time | Real time | Nested PCR | Real time | Real time | Real time |
| Target gene | IS*481*–IS*1001* | ?[g] | IS*481*–IS*1001* | IS*481*–IS*1001* | IS*481*–IS*1001* | ? | IS*481* + *ptxA* Pr[h] | IS*481* | Pertactin |
| Detection method | Gel | Taqman[i] | Gel | Taqman | Taqman | Gel | FRET probe | Taqman | Taqman |
| Analytical sensitivity (per ml) | 1,000 CFU | Unknown | Unknown | Unknown | 125 CFU | 10 cells | 200 CFU | Unknown | Unknown |
| No. of separate areas | 3 | 3 | 4 | 1 | 3 | 4 | 3 | 3 | 2 |
| Carryover prevention | No | dUTP[j] | No | dUTP | No | No | dUTP | dUTP | dUTP |

[a] Silica extraction method.
[b] GuSCN, guanidine thiocyanate lysis and isopropanol precipitation.
[c] MagnaPure (Roche Diagnostics).
[d] QIAamp DNA mini kit (Qiagen).
[e] Amplicor respiratory specimen preparation kit.
[f] Equivalent (Eq.) sample used for PCR (in microliters), calculated as the starting volume used for concentration and extraction × fraction of extracted volume added to the amplification mixture.
[g] ?, participant did not answer.
[h] *ptxA* Pr, pertussis toxin promoter.
[i] Taqman double dye probe.
[j] Addition of dUTP and uracil glycosylase.

## DISCUSSION

Numerous laboratories demonstrated that nucleic acid amplification techniques improved the sensitivity and turnaround time for the detection of *B. pertussis* and/or *B. parapertussis* and have demonstrated their clinical value for the diagnosis of pertussis (13, 14, 18, 25, 28, 37, 43, 45, 46). More recently, some laboratories introduced real-time PCR and have demonstrated the reliability of this technique as well as the shorter turnaround time (1, 4, 6, 12, 21, 22, 39, 42).

As molecular analysis is increasingly used for the diagnosis of whooping cough, standardization and EQA programs are recommended (23). The EQA program described here, in which representative laboratories from several European countries participated, is the first study on this scale to evaluate the molecular biology-based methods for the diagnosis of *Bordetella* spp.

The 11 laboratories participating in either one or two EQA schemes used a PCR method developed in-house, as no commercial assay is available. Evaluation of the various PCR protocols for which information was available showed no apparent association between their performance and the particular variables of the PCR method used. However, the results of such a comparison must be interpreted with caution due to the relatively small number of samples, the small number of participating laboratories, and the diversity of the methods used.

The variable analytical sensitivity observed suggests that false-negative PCR results with clinical specimens may be a problem.

The results with EQA panel 2 demonstrated a good analytical sensitivity for most participating laboratories, but the two laboratories with lower detection limits with samples from EQA panel 1 did not participate in this program. One of the two laboratories modified its procedures and uses real-time-based methods targeting the IS*481* and IS*1001* genes of *B. pertussis* and *B. parapertussis*.

Only one laboratory (laboratory 2) reported a false-positive result for a sample containing no *Bordetella* spp. The information provided on the questionnaire did not point to a particular risk for false-positive results (Table 5). Similar problems of specificity have been reported for the molecular biology-based assay detection of hepatitis B virus (44), *Mycobacterium tuberculosis* (31), *Toxoplasma gondii* (33), and hepatitis C virus (36) as well. The same laboratory reported the result for a sample spiked with *B. parapertussis* as *B. pertussis* positive, respectively.

By use of the *B. pertussis*-specific target gene pertactin, only one laboratory (laboratory 11) performed the tests with 100% specificity for *B. pertussis*. The eight other laboratories participating in EQA panel 2 reported positive results for *B. pertussis* for the samples spiked with *B. holmesii* and *B. bronchiseptica*. These unexpected false-positive reactions for *B. pertussis* by the majority of the participating laboratories suggest the presence of a homologous sequence in the strains of these two species used. The cross-reactivity with *B. holmesii* was expected, since the presence of IS*481* in *B. holmesii* was described previously (34, 35) and these laboratories used this insertion element as the target gene. Laboratory 9 therefore performed a supplementary PCR specific for the toxin promoter region, which allowed that laboratory to report *B. pertussis* in the case of a positive result and *B. pertussis* or *B. holmesii* in the case of a

negative result. A negative result by a PCR specific for the toxin promoter might be indicative of the presence of *B. holmesii* (sample B-B10) in the sample as well as a small amount of *B. pertussis* (30 CFU/ml in sample B-B8) due to the lower analytical sensitivity of this PCR compared to that of the IS*481*-based PCR.

Sequencing of the complete genome of representative strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* demonstrated the presence of the IS*481* target gene only in *B. pertussis* (32). However, Gladbach et al. (15) demonstrated that 2 of 12 *B. bronchiseptica* isolates tested were PCR positive for IS*481*. Furthermore, the sequence of a 200-bp amplicon from these isolates showed 97% homology with the *B. pertussis* IS*481* sequence. Despite the absence of the IS*481* sequence from *B. bronchiseptica* strain RB50, the complete sequence of which is available, a positive PCR result for the *B. bronchiseptica*-positive sample in our panel was observed by eight of the nine participating laboratories. This is very surprising, since it was previously demonstrated that the IS*481*-based assay performed by some of these laboratories showed no cross-reactivity with *B. bronchiseptica* (2, 22). It is therefore tempting to suggest that *B. bronchiseptica* might be rather genetically diverse, so that some isolates contain an IS*481*-like sequence, which makes these isolates more similar to *B. pertussis* than *B. bronchiseptica* strain RB50, whose genome was recently sequenced (32).

The presence of the IS*481* sequence in *B. holmesii* (34, 35) and the isolation of *B. holmesii* from patients with pertussis-like symptoms (29, 49) or respiratory failure (41) were described previously. Likewise, *B. bronchiseptica* has been isolated from AIDS patients (27, 47) and cystic fibrosis patients (48). Although both *B. holmesii* and *B. bronchiseptica* have been implicated as infrequent causes of a pertussis-like syndrome and other respiratory illnesses (26), the clinical relevance of the presence of these rare microorganisms in human respiratory samples needs further investigation. The false-positive results for *B. pertussis* for samples containing *B. holmesii* and *B. bronchiseptica* strains in our EQA program suggest that the specificity and the positive predictive value of IS*481*-based PCR assays for the diagnosis of pertussis may be compromised. It could therefore be more appropriate to follow the suggestions of Fry et al. (13) and report the results of assays based only on IS*481* sequences as evidence of the presence of *Bordetella* species. Otherwise, the development of an additional PCR that targets other *B. pertussis*-specific genes, such as the pertussis toxin promoter, the porin gene, the pertactin gene, or the adenylate cyclase gene, could be evaluated further. Although IS*481* is present in multiple copies, whereas the other genes are not, assays targeting either may have comparable sensitivities, as shown by laboratory 11 in this study.

Finally, this study underlines the importance of proficiency panels for monitoring the quality of the PCR protocols performed in diagnostic laboratories. This study highlights the lack of homogeneity between PCR protocols and performance and underlines the need for an external quality assurance scheme which could provide reference samples that could be used by any laboratory wanting to establish and maintain an accurate diagnostic test based on PCR.

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