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Evaluation of 3 analyte-specific reagents for detection of *Bordetella pertussis* and *Bordetella parapertussis* in clinical specimens

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ABSTRACT

The performance of 3 analyte-specific reagents (ASRs), Elitech Biosciences, EraGen Biosciences, and Focus Diagnostic, was evaluated for detection of *Bordetella pertussis* (BP) and *Bordetella parapertussis* (BPP) in nasopharyngeal swab specimens. A total of 104 frozen, leftover clinical specimens obtained from pediatric patients during 2011–2012 were included in this study. Performance was compared to the *Bordetella* real-time polymerase chain reaction (PCR) laboratory-developed test (LDT). The positive percent agreement for detection of BP by Elitech was 96% (95% confidence interval [CI]: 85.14–99.30); EraGen and Focus was 98% (95% CI: 87.99–99.89) in comparison to LDT PCR assay. The negative percent agreement of Elitech, EraGen, and Focus in comparison to LDT was 96% (95% CI: 85.14–99.30), 92% (95% CI: 79.89–97.41), and 96% (95% CI: 85.14–99.30), respectively. Limit of detection (LOD) for BP was 0.1 CFU/reaction by both Focus and EraGen and 1.0 CFU/reaction by Elitech. However, LOD for BPP was lower by EraGen (0.1 CFU/reaction) compared to Focus (1.0 CFU/reaction) and Elitech (1.0 CFU/reaction). These results demonstrate that all 3 ASRs tested are comparable and reliable for routine clinical diagnosis of pertussis and parapertussis.

1. Introduction

Whooping cough is a severe contagious disease caused mainly by *Bordetella pertussis* (BP) and, less frequently, by *Bordetella parapertussis* (BPP) and affects primarily infants and young children and, to a lesser extent, can also affect adults (CDC 2002, CDC, 2005, Heininger, 2010). Clinically, it presents with a prolonged cough and vomiting. If untreated, these symptoms can lead to hypoxia, permanent brain damage, cardiopulmonary complications, and death, especially in infants. It is critical to accurately identify pertussis infection in early stage for better patient care management and proper antibiotic treatment.

There are several diagnostic methods for pertussis detection. Among them, culture remains the gold standard for laboratory diagnosis due to high specificity, but culture possesses 2 main disadvantages: i) long turnaround time (usually 3–7 days) and ii) low sensitivity, especially during paroxysmal phase (2 weeks after cough onset). In recent years, nucleic acid amplification test by polymerase chain reaction (PCR) has become a common diagnostic procedure for BP and BPP. Real-time PCR assays are rapid (2–6 h), specific (86–100%), and sensitive (70–99%) in detecting *Bordetella* spp. (He et al., 1996, Loeffelholz et al., 1999, Loeffelholz, 2012). Most of the PCR assays are based on the detection of insertion sequence IS*481* for BP and IS*1001* for BPP (Riffelmann et al., 2005). To date, there are only 2 Food and Drug Administration (FDA)–cleared molecular assays available for detection of BP. Film array respiratory panel from Biofire (formerly known as Idaho Technology, Salt Lake City, Idaho, USA) is a multiplex assay that detects BP along with 20 other respiratory pathogens, and more recently, illumigene® pertussis assay, an isothermal LAMP assay (Meridian Bioscience Inc., Cincinnati, Ohio, USA), was cleared for detection of BP. None of these FDA-cleared assays detect BPP. However, there are several analyte-specific reagents (ASRs) available commercially that can detect both BP and BPP in single reaction by real-time PCR. To our knowledge, no comparative studies have been published on performance of the ASRs available in the US market. In this study, we evaluated the performance of 3 ASRs, namely, Elitech Biosciences, (Princeton, New Jersey, USA) EraGen Biosciences (Madison, Wisconsin, USA), and Focus Diagnostic (Cypress, California, USA) all of which detect and identify BP and BPP in the same reaction by multiplex real-time PCR. Results from these tests were analyzed for accuracy, precision, limit of detection (LOD), and other clinical test parameters according to the CLSI guidelines (CLSI, 2006, 2nd edition).

2. Materials and methods

2.1. Bacterial strains

The following organisms were obtained from ATCC (Manassas, VA, USA): BP, BPP, *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. *Bordetella holmesii*

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was kindly provided by Dr Marcon, Nationwide Children's Hospital, Columbus, OH. BP and BPP were used as positive controls in our study.

2.2. Clinical specimens and extraction of DNA

A total of 104 frozen nasopharyngeal (NP) swabs collected from pediatric patients seen at the Children's Mercy Hospitals and Clinics from January 2011 to June 2012 as a part of routine clinical procedures were included. This study was reviewed and approved by institutional review board at Children's Mercy Hospitals and Clinics. The NP swabs were collected in normal saline and transported immediately to the laboratory for testing by a laboratory-developed real-time PCR. Leftover specimens were immediately stored at −80 °C until used in this study. 200 μL of these frozen specimens was used for DNA extraction. Total DNA was extracted by NucliSens easyMAG automated extraction system according to manufacturer's instruction (bioMerieux Inc, Durham, North Carolina, USA), aliquoted in 3 separate Eppendorf tubes (15 μL each), and stored at −20 °C until further used. One aliquot was used for 1 ASR, thus limiting repeated freeze–thaw cycle. All discrepant specimens that differed in results between any of the 3 ASRs were sequenced for resolution of the discrepant result.

2.3. Performance specification of ASRs

The performance specifications of ASRs were analyzed as per the MM03-A2 CLSI (2006) guidelines. The analytical sensitivity assay was evaluated with known colony-forming unit (CFU) of BP and BPP that were obtained from ATCC. ASRs were analyzed starting with BP and BPP at 10 CFU/reaction followed by 10-fold dilution up to 0.001 CFU/reaction.

The cross-reactivity of the ASR reagents was tested against the following organisms: *B. holmesii*, *B. bronchiseptica*, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*.

The characteristics of ASRs were further analyzed by precision studies by spiking the ATCC cultures of BP and BPP into 5 CFU/reaction. PCR was performed on 3 different days in triplicates with fresh nucleic acid extracted for each run. Accuracy in detection of BP and BPP DNA was estimated based on the number of true-positive specimens detected accurately by each ASR assay.

2.4. Nucleic acid amplification

Laboratory developed *Bordetella* real-time PCR: samples were boiled at 95 °C for 10 min, centrifuged at 13,000 RPM for 5 min, and 2.0 μL of supernatant was used for PCR testing using the primers and probes for IS*481* (BP) (TIB Molbiol LLC, New Jersey, USA).

PCR was performed in Light Cycler II (Roche Inc, Indianapolis, Indiana, USA) at total of 20-μL reaction volume. Total reaction volume included 2.0 μL of FastStart DNA master Hybridization probe mix (TIB Molbiol LLC), 5 mmol/L $MgCl_2$, 0.5 μmol/L each primer, and 0.2 μmol/L each probe. The instrument was configured with the following thermal cycling protocol: 95 °C for 10 min (denature), 45 cycles of 95 °C for 10 s, 50 °C for 10 s, 72 °C for 20 s (PCR amplification) followed by 1 cycle of 95 °C for 10 s, 40 °C for 10 s, 95° for 0 s, and 40 °C for 2 s (melting). Samples with <37.0 threshold cycle (Ct) value were considered positive; 37.1–39.9, considered intermediate; and >40.0, considered negative. Retesting of intermediate specimens was performed to confirm positive specimens.

Elitech molecular diagnostics *Bordetella*-specific ASR real-time PCR analysis was carried out using primers specific for BP (IS*481*) and BPP (IS*1001*) and internal control DNA. PCR was performed on Light Cycler II (Roche Inc) at 20-μL total reaction volume. Total reaction volume included 10 μL 2× MGB Alert Hot Start Master Mix, 1.2 μL 25 mmol/L $MgCL_2$, 1.8 μL PCR Enhancer, 1.0 μL 20× BP/BPP/internal control primer and probe mix, 1.0 μL internal control reference DNA, and 5.0 μL extracted DNA template. The instrument was configured with the following thermal cycling protocol: 50 °C for 120 s (uracil N-glycosylase activation), 93 °C for 120 s (enzyme activation), 50 cycles of 93 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s (PCR amplification), followed by 1 cycle of 95 °C for 15 s, 40 °C for 15 s, and 80 °C for 15 s (melting). At the completion of the run, amplification products resulting in a Ct of <42 for BP and <38 for BPP were confirmed by melt curve analysis with a melting peak at the appropriate temperature (68 ± 1.0 °C for BP and 60 ± 1.0 °C BPP).

EraGen Biosciences *Bordetella*-specific ASR real-time PCR analysis was carried out using primers specific for BP (IS*481*) and BPP (IS*1001*) and internal control DNA. PCR was performed on Light Cycler II (Roche Inc) at 25-μL total reaction volume. Total reaction volume included 5 μL MultiCode-RTX solutions, 1.0 μL primers for BP and BPP each, 1.0 μL primers for internal control, 1. 0 μL DNA universal reference, 0.5 μL TITANIUM *Taq*, 11.5 μL of nuclease free water, and 5.0 μL DNA template. The instrument was configured with the following thermal cycling protocol: 95 °C for 120 s, 45 cycles of 95 °C for 5 s, 58 °C for 10 s, 72 °C for 20 s followed by 1 cycle of 60 °C for 20 s. At the end of the run, data were imported and analyzed by MultiCode-RTx analysis software (version 4.05). Amplification products with Ct <42 for BP and <36 for BPP were confirmed by analyzing appropriate melting temperature (Tm; 79.5 ± 1.0 °C for BP and Tm 83.0 ± 1.0 °C for BPP).

Focus Diagnostic *Bordetella*-specific ASR real-time PCR analysis was carried out using primers and probes specific for BP (IS*481*) and BPP (IS*1001*) and internal control DNA. PCR was performed on 3 M integrated cycler (Focus Diagnostics, Cypress, California, USA) at 10-μL total reaction volume. Total reaction volume was 10 μL including 4 μL universal master mix, 0.4 μL primers and probe for BP (FAM dye) and BPP (CFR610 dye) each, 0.2 μL internal control (Q670 dye), and 5.0 μL DNA template. The instrument was configured with the following thermal cycling protocol: 97 °C for 120 s, 40 cycles of 97 °C for 10 s, and 60 °C for 30 s (with capture). Specimens with a Ct of <38 for BP and <33 for BPP were considered positive.

Any PCR amplification with Ct values that were either outside the range of manufacturer's recommended Tm (±1.0 °C) or without any Tm were considered non-specific PCR amplification and were not considered as positive identification. Proper amplification of internal control was also documented.

2.5. Discrepant analysis

Any inconsistent results among 3 ASRs were considered as discrepant. All discrepant specimens were de-identified and sent to a third-party vendor for sequencing.

3. Results

All of the ASRs were kept and maintained at appropriate temperature as suggested by the manufacturers. All ASRs detected BP and BPP by targeting IS*481* and IS*1001* for BPP. Total of 104 samples were selected based on historical laboratory-developed test (LDT) results. Among them, 50 samples were positive for BP by laboratory-developed real-time PCR, 4 samples were culture positive for BPP. The rest of the samples were negative by the LDT-PCR. Positive percent agreement (PPA) and negative percent agreement (NPA) for BP were calculated against LDT PCR assay and found to be almost

Table 1
Detection of BP by 3 ASRs.

ASR	Pos[a]	Pos[a]	Pos[a]	Neg[b]	Neg[b]	Neg[b]
	ASRs vs LDT	PPA (%)	95% CI	ASR vs LDT	NPA (%)	95% CI
Elitech	48[c]/50	96%	85.14–99.30	48[d]/50	96	85.14–99.30
EraGen	49[c]/50	98	87.99–99.89	46[e]/50	92	79.89–97.41
Focus	49[c]/50	98	87.99–99.89	48[d]/50	96	85.14–99.30

Due to limited number of BPP samples, PPA and NPA could not be calculated.
[a] Samples were positive by LDT PCR assay.
[b] Samples were negative by LDT PCR assay.
[c] Sample #21 was negative by all 3 ASRs.
[d] Two samples were positive by respective ASR (sample #139 and 143).
[e] Four samples were positive by respective ASR (sample #113, 126, 139, and 143).