

1 To Journal of Clinical Microbiology, Category: **Bacteriology**

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3 **Multicenter Clinical Evaluation of the Automated**

4 **ARIES<sup>®</sup> *Bordetella* Assay**

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18 **Running title:** *Bordetella* PCR assay clinical validation

19 **Keywords:** *Bordetella pertussis*, *Bordetella parapertussis*, pertussis, whooping cough, PCR

20

21 **ABSTRACT**

22 Molecular methods offer superior sensitivity and specificity, and reduce testing turnaround time  
23 from days to hours for detection of *Bordetella pertussis* and *Bordetella parapertussis*. In this  
24 study, we evaluated the performance of the automated PCR-based ARIES *Bordetella* Assay,  
25 which detects both *B. pertussis* and *B. parapertussis* directly from nasopharyngeal swab  
26 specimens. Limits of detection were 1,800 colony-forming units/mL (CFU·mL<sup>-1</sup>) for *B. pertussis*  
27 and 213 CFU·mL<sup>-1</sup> for *B. parapertussis*. The assay detected 16/18 unique *B. pertussis* / *B.*  
28 *parapertussis* strains. Of 71 potential cross-reacting organisms, 5 generated false-positives in 1/6  
29 replicates; none of 6 additional *Bordetella* spp. were erroneously detected. Specimens were  
30 stable at 20–25°C for at least 10 h, 4–8°C for 10 days, and at ≤ -70°C for 6 months. Of 1,052  
31 nasopharyngeal specimens from patients with suspected pertussis, 3.0% (n=32) were *B.*  
32 *pertussis*-positive, and 0.2% (n=2) were *B. parapertussis*-positive. After combining these data  
33 with ARIES *Bordetella* Assay data from 57 nasopharyngeal samples with previously confirmed  
34 *B. pertussis* or *B. parapertussis*, and from 50 contrived *B. parapertussis* samples, ARIES assay  
35 respective positive and negative percent agreements with the reference assays were 97.1% and  
36 99.0% for *B. pertussis*, and 100% and 99.7% for *B. parapertussis*. The ARIES *Bordetella* Assay  
37 provides accurate detection and distinction of *B. pertussis* and *B. parapertussis* infection within 2  
38 hours.

## 39 Introduction

40 Pertussis, or whooping cough, is a highly contagious respiratory disease caused by the Gram-  
41 negative bacteria *Bordetella pertussis* and *Bordetella parapertussis*. In some patients, especially  
42 infants and young children, infection with these organisms can lead to potentially life-threatening  
43 complications such as pneumonia, weight loss, dehydration, and seizures. Although there is a  
44 vaccine to prevent disease caused by *B. pertussis*, an estimated 24.1 million cases and over  
45 160,000 deaths, mainly in infants and children less than 5 years old, are recorded annually  
46 worldwide (1). In the US, pertussis prevalence has risen since the 1980s, with cyclical peaks  
47 occurring every 3–5 years (2). In 2012, the most recent peak year, the Centers for Disease  
48 Control and Prevention (CDC) identified 48,277 cases nationwide, though more cases were  
49 likely undiagnosed. In 2016, the most recent year with complete statistics, the CDC confirmed  
50 nearly 18,000 cases in the US (3).

51 Disease signs and symptoms caused by these two pathogens are similar; however, those  
52 associated with *B. parapertussis* infection are often milder and disease duration is generally  
53 shorter (4–7). Both pathogens cause biphasic symptoms that appear following a 5–10-day  
54 incubation period. A 7–10-day prodromal stage, the catarrhal stage resembling coryza,  
55 progresses to classic pertussis symptoms, which include paroxysmal coughing followed by high-  
56 pitched “whooping” during inspiration of air against narrowed airways, and sometimes post-  
57 tussive emesis. In adults, signs and symptoms can persist for several weeks. Early and accurate  
58 diagnosis of pertussis is essential for the optimization of therapy and to curb the transmission of  
59 these pathogens (5–7).

60 Distinguishing between these two pathogens is important from a public health  
61 perspective, because *B. parapertussis* may cause up to 20% of pertussis-like disease in young

62 children, and co-infection with *B. pertussis* is common (4–7). Contemporary options for the  
63 detection of *B. pertussis* and *B. parapertussis* infections include immunoserological tests to  
64 detect either bacterial antigens in respiratory specimens or pathogen-specific antibodies in serum,  
65 culture of nasopharyngeal specimens using specialized media, and nucleic acid amplification  
66 tests to detect *B. pertussis* and *B. parapertussis* DNA in nasopharyngeal swab and aspirate  
67 specimens (5–7). Although culture affords excellent specificity (nearly 100%), it requires up to 7  
68 days to obtain results, is often labor-intensive, and can have poor sensitivity, especially if  
69 specimens were obtained after the initiation of antimicrobial therapy. Also, culture is most  
70 successful during the first 1–2 weeks following cough onset in unvaccinated patients that have  
71 not received antibiotics (7). Serologic tests for pertussis infection can be helpful, but have limited  
72 utility early after infection, and no commercial kits have been approved by the U.S. Food and  
73 Drug Administration (FDA) for diagnostic use (8). Molecular assays for detection of *B. pertussis*  
74 and *B. parapertussis* detection are much faster than culture, offer higher sensitivity, are  
75 continuously improving in test turnaround time and protocol simplification, and have received  
76 FDA clearance. The CDC Best Practices guidelines indicate that PCR-based assays are  
77 recommended as first-line approaches for diagnosing pertussis in symptomatic patients (9).

78         The ARIES *Bordetella* Assay (Luminex Corp., Austin, TX) is an automated nucleic acid  
79 amplification assay that is designed to rapidly, simultaneously, and differentially identify *B.*  
80 *pertussis* and *B. parapertussis* in nasopharyngeal swab specimens. The assay detects the  
81 pertussis toxin (*ptxA*) promoter and IS1001 of *B. pertussis* and *B. parapertussis*, respectively.  
82 The current study evaluated the analytical performance of the ARIES *Bordetella* Assay system  
83 by conducting a large-scale multi-site evaluation of the assay using clinical specimens obtained  
84 from subjects with suspected and/or known pertussis infection.

85 **MATERIALS AND METHODS**

86 **Clinical study design.**

87 *Inclusion Criteria and Study Oversight.* Inclusion criteria were subjects: i) with signs and  
88 symptoms consistent with *B. pertussis* and/or *B. parapertussis* infection; ii) for whom a  
89 *Bordetella* test had been requested; and iii) who provided a nasopharyngeal swab specimen  
90 collected in Universal Transport Medium (UTM) with volume  $\geq 1,000$   $\mu\text{L}$ . Specimens were  
91 excluded if subjects had antibiotic use within 24 h of specimen collection.

92 Clinical specimens were exempted from informed consent requirements by the  
93 Institutional Review Board at all participating sites per FDA advisory *Guidance on Informed*  
94 *Consent for In Vitro Diagnostic Device Studies using Leftover Human Specimens that are not*  
95 *Individually Identifiable, January 2006* (10); results were not used for patient management.  
96 Chart review data were anonymized before compilation and analysis. This study was registered  
97 on ClinicalTrials.gov (NCT02862262), and conformed to the Declaration of Helsinki and the  
98 Health Insurance Portability and Accountability Act.

99

100 *Specimens.* Diagnostic accuracy of the ARIES *Bordetella* Assay was evaluated in 1,053  
101 prospectively collected, de-identified nasopharyngeal swab specimens from subjects presenting  
102 with suspected *B. pertussis* or *B. parapertussis* infection at five geographically diverse sites in  
103 the United States (New Mexico, Wisconsin, Ohio, Michigan, and Indiana) between July and  
104 November, 2015. The Ohio samples were assayed at the New Mexico site. Investigators,  
105 technicians performing *Bordetella* testing, and the study sponsor were masked with respect to  
106 specimen identification. Swab specimens were collected in UTM, refrigerated at 2–8°C within 4

107 h of collection, and specimen aliquots ( $\geq 250$   $\mu\text{L}$ ) were stored at 2–8°C (and tested within 72 h)  
108 or frozen at -70°C for testing at a later time.

109

110 *Study Groups.* This clinical study comprised three study arms. Arm 1 was the blinded  
111 prospective evaluation of nasopharyngeal swabs collected from subjects with symptoms of *B.*  
112 *pertussis* or *B. parapertussis* infection, but without accompanying diagnostic information. We  
113 estimated *B. pertussis* to be present in 5% of samples in Arm 1. Because this frequency was not  
114 guaranteed, per protocol we generated a second arm, Arm 2, containing additional banked pre-  
115 selected known *Bordetella*-positive swab specimens (37 *B. pertussis* and 20 *B. parapertussis*)  
116 and an equal number (n=57) of unique *B. pertussis*-negative/*B. parapertussis*-negative clinical  
117 specimens from study sites. All Arm 2 samples had been stored frozen and were evaluated in  
118 random order. Because *B. parapertussis* is particularly rare, and we wished to accurately  
119 estimate positive and negative percent agreement (PPA and NPA, respectively) between the  
120 ARIES and reference assays, Arm 3 contained 50 contrived specimens that were spiked with *B.*  
121 *parapertussis* strains at clinically-relevant titers, and tested in random order among 50 negative-  
122 control specimens.

123

124 **ARIES *Bordetella* Assay operation.** All study sites used the ARIES system with *Bordetella*  
125 Assay Cassettes (Luminex) and ARIES *Bordetella* Assay Protocol Source File-IUO v.5.  
126 Analysis software was SyncT ARIES UDP Desktop Software, Version 1.1 Build 165 (Luminex).  
127 Four specimen aliquots ( $\geq 250$   $\mu\text{L}$ ) were stored at 2–8°C, and tested within 72 h of collection or  
128 frozen (-65°C to -95°C), per manufacturer instructions (11). External positive-controls included  
129 pooled *B. pertussis* A639 and *B. parapertussis* A747 cultures diluted in Natural Negative

130 Nasopharyngeal Matrix (NPM); negative-controls were vehicle-only. The assay cassettes contain  
131 extractable Sample Processing Controls to assess PCR extraction and amplification fidelity.  
132 Specimens testing negative for *Bordetella* were required to have a positive result for these  
133 internal controls (cycles, Ct; and melting temperature, T<sub>m</sub>) for the negative result to be validated.

134

135 **Comparator assays.** One frozen aliquot was shipped on dry ice to Luminex Molecular  
136 Diagnostics (Toronto, ON) for comparator testing, with two frozen aliquots remaining on site as  
137 reserves. All ARIES assay results, whether positive or negative, were confirmed by two real-time  
138 PCR assays per organism (total = 4 PCR assays), with results validated by conventional  
139 (endpoint) PCR amplification followed by bidirectional sequencing of the amplicons, in  
140 accordance with FDA guidance. The reference real-time PCR assays for *B. pertussis* and *B.*  
141 *parapertussis* targeted unique regions of the *ptxA* gene promoter and *IS1001* insertion sequence,  
142 respectively, which differed from the regions targeted by the ARIES assay. Specimens were  
143 considered positive for *B. pertussis* or *B. parapertussis* if one or both comparator PCR assays  
144 was positive (Ct values  $\leq 40$ ) and confirmed by bidirectional sequencing. All samples from all  
145 three arms were evaluated using the ARIES system, as well as by the comparator assays.

146 Arm 1 samples had previously been tested by each site using their in-house standard-of-  
147 care (SOC) assay. Each site used their own laboratory-developed real-time PCR assay targeting  
148 *IS481* and *IS1001* for *B. pertussis* and *B. parapertussis*, respectively, with initial findings  
149 remaining undisclosed until study terminus. Two study sites performed a laboratory-developed  
150 test (LDT) using Luminex MultiCode Analyte Specific Reagents, one site used a TaqMan-based  
151 (Thermo-Fisher; Foster City, CA) LDT, and one site used DiaSorin reagents (DiaSorin; Cypress,  
152 CA) for their LDT.

153

154 **Analytical performance overview.** The ARIES *Bordetella* Assay was evaluated for limit-of-  
155 detection (LoD), intra-lab/inter-operator and site-to-site reproducibility, detectability of multiple  
156 *B. pertussis* and *B. parapertussis* strains, potential interfering substances and microorganisms,  
157 run-to-run carryover, swab and media interference effects, and sample stability during storage,  
158 with outcomes detailed in the Luminex ARIES *Bordetella* Assay 510(k) FDA Decision Summary  
159 (12). Reference bacterial strains *B. pertussis* A639 and *B. parapertussis* A747 from  
160 (ZeptoMetrix, Buffalo, NY) were used in all assays except where noted. Dilution medium and  
161 vehicle-only negative-controls were NPM, derived from nasopharyngeal swab extracts pooled  
162 from 242 asymptomatic individuals (BioIVT, Westbury, NY) or UTM, unless otherwise  
163 indicated. Data points are the average of triplicate determinations for a given condition, unless  
164 otherwise noted.

165

166 **Limit of detection.** Six 20-fold serial dilutions of two *B. pertussis* strains (A639 and ATCC  
167 BAA-589) and two *B. parapertussis* strains (A747 and ATCC BAA-587 [ATCC, Manassas,  
168 VA]) in NPM were assayed to estimate the LoD; this concentration was subsequently tested in  
169 20 replicates, and the analytical LoD was defined as the concentration in CFUs that had  $\geq 95\%$   
170 positive detection rate.

171

172 **Cross reactivity.** Cross-reactivity of the ARIES *Bordetella* Assay was evaluated in 18 strains of  
173 *B. pertussis* and *B. parapertussis* in samples created with 3 $\times$ LoD, 10 $\times$ LoD, and 100 $\times$ LoD of  
174 these organisms (Supplementary Table S1). We also evaluated erroneous detection of high-  
175 concentration suspensions (in NPM) of 71 non-*B. pertussis*/*B. parapertussis* potential cross-

176 reacting organisms (CROs), many of which cause infections with symptoms resembling those of  
177 *Bordetella* infection (12). This included six additional *Bordetella* species, including 4 strains  
178 each of *B. bronchiseptica* and *B. holmesii*, at concentrations ranging between  $1.0 \times 10^6$  CFU·mL<sup>-1</sup>  
179 to  $1.9 \times 10^6$  CFU·mL<sup>-1</sup>.

180 Microbial interference was evaluated by adding the same potential CROs at the same  
181 concentrations as in cross-reactivity testing to *B. pertussis* and *B. parapertussis* suspensions  
182 containing 3×LoD of either target *Bordetella* pathogen. Cross-target interference in simulated co-  
183 infection scenarios was assessed by assaying suspensions containing 100×LoD of both target  
184 organisms (high/high) or 3×LoD of both (low/low). For high/low co-infection testing, high  
185 concentrations ( $\geq 10^6$  CFU·mL<sup>-1</sup>) of *B. pertussis* or *B. parapertussis* were spiked into 3×LoD  
186 suspensions of the other *Bordetella* species.

187  
188 **Specimen stability.** We evaluated positive detection of 3×LoD *B. pertussis* and *B. parapertussis*  
189 samples suspended in NPM that were stored at 20 - 25°C for 0, 2, 4, 8 and 10 h, or at 4 - 8°C for  
190 0, 1, 3, 7, and 10 days, or after storage at -70°C for up to 6 months (n=3 samples per timepoint  
191 under each condition). In a separate experiment, suspensions of *B. pertussis* or *B. parapertussis*  
192 (3×, 10×, and 100×LoD) were assayed immediately or after freezing at -70°C for 2 days (15–30  
193 replicates per condition/time). In total, 214 specimens were evaluated by the clinical testing sites  
194 for stability under freezing conditions (114 from the Arm 2 prospectively collected samples and  
195 100 from the Arm 3 pre-selected retrospective samples).

196  
197 **Statistical analysis.** To ensure sufficient statistical power, we calculated the required number of  
198 prospectively collected specimens to achieve  $\geq 95\%$  sensitivity with a lower bound of the

199 two-sided 95% confidence interval (CI) >85%. Based on an estimated *B. pertussis* prevalence of  
200 approximately 5% in the intended population, we calculated that 1,000 specimens were needed  
201 to obtain  $\geq 50$  positive specimens. Data are presented as n (%) or value  $\pm$  95% CIs, as indicated.  
202 Positive and negative percent agreement (PPA and NPA, respectively) of the ARIES assay with  
203 comparator assays were derived from 2 $\times$ 2 contingency tables, with minimum desired  $\geq 95\%$  PPA  
204 with lower bound of two-sided 95% CI >85%. Statistical software included SyncT UDP desktop  
205 software v.1.1, build 165 (Luminex), SAS v.9.2 (SAS Corp, Cary, NC), and Prism v.5.0  
206 (GraphPad Software, La Jolla, CA).

207

## 208 RESULTS

209 **Clinical trial subject and specimen accounting.** A total of 1,053 unique nasopharyngeal swab  
210 specimens from 1,053 subjects presenting with pertussis-like disease were collected at five study  
211 sites during July – November 2015. Individual sites provided between 25–427 (2–41%) of total  
212 prospective Arm 1 samples. One specimen was excluded from performance calculations due to  
213 refrigerator storage >72 h, leaving 1,052 evaluable specimens. Most subjects (74%) were  
214 <18-years-old, 56% were female, and nearly 74% visited outpatient clinics (Table 1). ARIES  
215 testing was performed on 63.4% of samples (n=667) within 72 h of storage at 4–8°C; the  
216 remaining 36.6% (n=385) were stored frozen at -70°C for up to 12 days prior to assay.

217

218 **Comparison to SOC results.** After completing the clinical performance assessment of the study,  
219 we compared the results of the ARIES assay to the result initially obtained by each site using  
220 their SOC test method. Of the 1,052 evaluated specimens in Arm 1, 23 samples were negative  
221 for *B. pertussis* by ARIES and by the ARIES-positive comparator sequencing assay, but had

222 initially tested *B. pertussis*-positive by the study sites' in-house SOC assay for *IS481*. These  
223 samples were further tested by Luminex using a validated high-sensitivity endpoint nested-PCR  
224 assay, followed with bidirectional sequencing for *B. pertussis* using primers targeting regions  
225 distinct from those used in the ARIES assay and in the clinical trial comparator sequencing  
226 assay, to reassess *B. pertussis*. In addition, validated single-plex real-time PCR assays and  
227 bidirectional sequencing were performed for *B. holmesii* and *B. bronchiseptica*. Of the 23  
228 discordant specimens, this analysis identified 5 *B. pertussis*-positive and 3 *B. holmesii*-positive  
229 specimens (Table 3).

230

231 **Clinical performance.** Of 1,052 unique specimens in prospective Arm 1, 1,043 (99.1%) yielded  
232 valid results on the first run. The remaining 9/1,052 (0.9%) initially produced invalid results due  
233 to run failure or instrument error, but generated valid results (positive or negative) upon re-  
234 testing. The ARIES assay produced 30 positive *B. pertussis* results and 1,011 negative results  
235 (Table 2). Compared to the PCR/sequencing comparator controls, the PPA for *B. pertussis*  
236 detection was 93.8% and the NPA was 98.9%. For *B. parapertussis* detection, the ARIES assay  
237 identified two positives in this population, yielding a PPA of 100% and an NPA of 98.9%.  
238 Because the observed prevalences of *B. pertussis* and *B. parapertussis* in the prospective study  
239 were lower than expected (3.0%, 32/1,052; and 0.2%, 2/1,052, respectively), the initial study  
240 design (Arm 1, prospective) did not achieve the desired minimum criteria of  $\geq 95\%$  PPA with a  
241 lower bound of the two-sided 95% CI  $> 85\%$ . Per protocol, we supplemented this sample set with  
242 pre-selected specimens that tested positive for *B. pertussis* and *B. parapertussis* by comparator  
243 assays (Arm 2; pre-selected). In addition, we tested *B. parapertussis* suspensions at clinically  
244 relevant titers (Arm 3, contrived).

245           Percent agreement to a reference method in part depends on the frequency of the studied  
246 pathogen. To increase *Bordetella* frequency, a set of 57 pre-selected nasopharyngeal swabs (37  
247 for *B. pertussis* and 20 for *B. parapertussis*) were obtained from study sites that had been  
248 previously characterized as positive for these pathogens. These Arm 2 samples underwent  
249 ARIES *Bordetella* testing at 3 study sites, with comparator real-time PCR and bidirectional  
250 sequencing performed at Luminex. An equal number of clinical samples negative for *B. pertussis*  
251 and *B. parapertussis* (n=57) were included as controls. All specimens were anonymized and their  
252 order of analysis was randomized. The PPA for *B. pertussis* in the 37 pre-selected samples was  
253 100%, with a lower bound of the 95% CI: 90.5%; the NPA was also 100%, with lower 95% CI  
254 bound: 95.3% (Table 2). In the 20 pre-selected *B. parapertussis* samples, the PPA was 100%,  
255 although the lower 95% CI bound was 83.2%; the NPA was 99% with the lower 95% CI bound:  
256 98.9%. In 50 contrived *B. parapertussis* samples in Arm 3, both PPA and NPA were 100%, with  
257 the lower CI bound satisfying the desired criterion (Table 2). After pooling results from all study  
258 arms, the PPA and NPA for both *B. pertussis* and *B. parapertussis* satisfied all pre-defined  
259 acceptability criteria.

260

261 **LoD.** The LoD was defined as the lowest concentration of *Bordetella* that was detectable in  $\geq$   
262 95% of 20 replicates. In *B. pertussis* strains A639 and BAA-589, LoDs were 1,640 CFU·mL<sup>-1</sup>  
263 and 1,800 CFU·mL<sup>-1</sup>; in *B. parapertussis* strains A747 and BAA-587, LoDs were 172 CFU·mL<sup>-1</sup>  
264 and 213 CFU·mL<sup>-1</sup>, respectively. Thus, 1,800 CFU·mL<sup>-1</sup> and 213 CFU·mL<sup>-1</sup> were considered to  
265 be the LoDs for *B. pertussis* and *B. parapertussis*, respectively.

266

267 **Cross-reactivity.** Of the 71 unique potential CROs tested alone, 66 were non-reactive with the  
268 ARIES *Bordetella* Assay at high titers, including 4 strains each of *B. bronchiseptica* and *B.*  
269 *holmesii* (12). All of the 5 CROs (*Fusobacterium necrophorum* and *Proteus vulgaris* for *B.*  
270 *pertussis*, and human coronavirus OC43, influenza B virus, and *Moraxella catarrhalis* for *B.*  
271 *parapertussis*) generated a false-positive in 1/3 replicates each, all with late Cts (38–40 cycles);  
272 no cross-reactivity was observed when these CROs were assayed in 3 additional replicates.

273 Cross-reactivity of the ARIES *Bordetella* Assay was also evaluated in 18 strains of *B.*  
274 *pertussis* and *B. parapertussis* (Supplementary Table S1). Nine of 11 (81.8%) *B. pertussis* strains  
275 were detected at 100% positivity at 3×LoD while two *B. pertussis* strains, ATCC 8478 and  
276 ATCC 9797, were not detected at up to 100×LoD. All seven *B. parapertussis* strains were  
277 detected at 100% positivity when tested at 3×LoD.

278 In interference assays with *Bordetella* suspensions spiked with CROs, *B. pertussis* was  
279 correctly detected in 3/3 (100%) replicates in the presence of 66 CROs. Five CROs in total, *B.*  
280 *bronchiseptica* (strains 1 and 2), *Bordetella petrii*, *Klebsiella aerogenes*, and *Klebsiella*  
281 *pneumoniae* required testing of 3 additional replicates, per protocol. For these 5 CROs, *B.*  
282 *pertussis* was detected in 5/6 (83.3%) replicates. *B. parapertussis* was correctly detected in 3/3  
283 (100%) replicates when tested in presence of all 71 CROs.

284 Microbial interference was also evaluated in simulated *B. pertussis*/*B. parapertussis* co-  
285 infection settings with variable ratios of *B. pertussis* to *B. parapertussis* (e.g., high [100×LoD]  
286 -low [3×LoD], low-high, high-high, and low-low). All replicates in all combinations yielded  
287 expected positivity for *B. pertussis* and *B. parapertussis* (12). The overall invalid rate was 0.9%  
288 (7/744) during the microbial interference and cross-reactivity study; all invalids were re-run and  
289 gave the expected result.

290

291 **Specimen stability.** One-hundred percent of both *B. pertussis*- and *B. parapertussis*-positive  
292 (3×LoD) and NPM-only negative controls were accurately distinguished after storage at 20–  
293 25°C for at least 10 hours, 4–8°C for at least 10 days, and -70°C for up to 6 months (n=3 samples  
294 per timepoint under each condition; not shown). No differences were seen in detection of  
295 identical suspensions of *B. pertussis* or *B. parapertussis* (3×, 10×, and 100×LoD) that were  
296 assayed immediately after production or after freezing at -70°C for 2 days (12).

297

## 298 **DISCUSSION**

299 This study characterized the performance of the ARIES *Bordetella* Assay for identifying  
300 and differentiating *B. pertussis* and *B. parapertussis* in nasopharyngeal swabs from patients with  
301 suspected or known pertussis. Whereas microbiological culture of nasopharyngeal swab or  
302 aspirate samples is the most specific means of detecting and delineating *B. pertussis* and *B.*  
303 *parapertussis*, these pathogens can be fastidious to grow *in vitro* and frequently require 5–7 days  
304 to acquire results (5–7, 13). This time lag delays implementation of appropriate antimicrobial  
305 therapy and infection control measures, often when the infected individual is most contagious (2,  
306 3). While the World Health Organization (WHO) surveillance guidelines still include paired  
307 serology as a *B. pertussis* diagnostic measure, this requires that sequential sampling and testing  
308 occur at least 1 week apart (14), conferring the same delay in obtaining results that occurs with  
309 culture. The CDC does not recognize a role for serological testing in its pertussis surveillance  
310 guidelines (7) and essentially all current *Bordetella* surveillance guidelines concur on the evident  
311 utility of nucleic acid amplification testing as a first-line approach for diagnosing *Bordetella*

312 infection (9, 14, 15). The ARIES *Bordetella* Assay accurately identifies two primary *Bordetella*  
313 pathogens in less than 2 hours.

314 Molecular diagnostic tests for identifying *Bordetella* species in nasopharyngeal swabs  
315 and aspirates are more sensitive than serological testing, provide faster results than culture, and  
316 are applicable to specimens collected up to 3 weeks after the onset of cough. Nucleic acid  
317 amplification assays can potentially match culture's high specificity and ability to  
318 simultaneously identify the presence of multiple *Bordetella* species (9, 16–18). The *B.*  
319 *parapertussis* infection rate is increasing, thought in part to be due to a competitive advantage  
320 conferred by vaccination against *BP* (4, 6, 19). The overlapping symptomatology caused by *B.*  
321 *pertussis* and *B. parapertussis* increases the importance of being able to confidently identify and  
322 differentiate these two most common human-pathogenic *Bordetella* species.

323 The ARIES *Bordetella* Assay consistently detected the presence of low titers of 9 *B.*  
324 *pertussis* and 7 *B. parapertussis* strains. Two *B. pertussis* strains that went undetected contained  
325 similar nucleotide mismatches in the ARIES primer binding regions (19), which presumably  
326 hindered assay detection of these strains. A search of the National Center for Biotechnology  
327 Information database revealed a very low (2.9%) prevalence of two similarly mismatched *B.*  
328 *pertussis* strains, both collected over a decade ago, and only one of which is known to have a  
329 human origin. This suggests a current paucity of similar mismatched *B. pertussis* strains  
330 circulating in the human population. Coinfection with *B. pertussis* and *B. parapertussis* occurs  
331 and might affect both treatment and outcome (20, 21). The ARIES assay was able to detect both  
332 of these organisms at low titers in the presence of extremely high concentrations of the sister  
333 pathogen, thereby demonstrating utility for accurately characterizing the most likely *Bordetella*  
334 species coinfection scenarios.

335 The ARIES *Bordetella* Assay has a low likelihood of falsely detecting a potential CRO as  
336 *B. pertussis* or *B. parapertussis*, or of a CRO interfering with intended *B. pertussis*/*B.*  
337 *parapertussis* detection, even near the *Bordetella* species' LoDs. The single-copy *ptxA* gene is  
338 present only in *B. pertussis*, which confers increased specificity to the ARIES assay in accurately  
339 diagnosing true pertussis compared to nucleic acid assays that target *IS481*, a multi-copy gene  
340 that is also present in the confounding pathogens *B. holmesii* and some strains of *B.*  
341 *bronchiseptica* (22–25). Of other currently FDA-cleared *B. pertussis* diagnostic assays, one  
342 system (FilmArray RP and RP2; BioFire Diagnostics) targets the *ptxA* gene, while another  
343 (Illumigene; Meridian Bioscience) targets the *IS481* gene and is known to additionally detect *B.*  
344 *holmesii* and *B. bronchiseptica* (26, 27).

345 The ARIES assay identifies *B. parapertussis* by detecting *IS1001*, present at  
346 approximately 20 copies per genome (17). Although *IS1001* has been reported in occasional  
347 strains of *B. bronchiseptica* (4/73 human isolates), this low expression level coupled with the  
348 very rare involvement of this organism in suspected pertussis outbreaks makes it unlikely to be a  
349 meaningful impediment to ARIES diagnostic utility (23, 28). The BioFire RP2 also evaluates  
350 *IS1001*; the Illumigene does not assess *B. parapertussis*. *B. bronchiseptica* was not detected in  
351 any sample in the current study.

352 Of 71 potential CROs assayed, 66 (93%) were not detected by ARIES. Two organisms  
353 produced a single false-positive for *B. pertussis* that was not replicated in triplicate re-testing;  
354 similarly, three CROs generated a single false-positive for *B. parapertussis* that was not  
355 repeatable. Human specimens and contrived samples remained stable during common storage  
356 temperatures and durations. Together these observations demonstrate the ARIES assay's

357 reliability in selectively identifying low titers of the two most common *Bordetella* pathogens in  
358 diverse testing scenarios.

359 We reconciled ARIES findings by comparison to results from two unique real-time PCR  
360 reference assays for each pathogen (four assays total), supplemented with bidirectional  
361 sequencing of PCR amplicons when either one or both of the two PCR assays (per organism)  
362 was positive. In the prospective arm, 99.1% of specimens (1,043/1,052) yielded valid results on  
363 the first attempt, and remaining nine samples provided a definitive conclusion when retested.  
364 Because our population contained lower than the estimated *B. pertussis* incidence of 5%, we  
365 supplemented our sample set with known *B. pertussis*-positive (n=37) or *B. parapertussis*-  
366 positive (n=20) clinical samples to increase pathogen frequency; all were accurately assessed by  
367 the ARIES *Bordetella* Assay. Because *B. parapertussis*-positive samples were expectedly lower  
368 in number than *B. pertussis*-positive samples, we also included 50 contrived *B. parapertussis*  
369 specimens to increase the accuracy of our percent agreement calculations, along with appropriate  
370 vehicle-only controls, all of which were also accurately identified. The ARIES' cumulative PPA  
371 and NPA exceeded the minimum acceptable performance criteria by a substantial margin. The  
372 respective PPAs for *B. pertussis* and *B. parapertussis* were 97.1% and 100%, with lower 95% CI  
373 bounds above the minimum 85% specification for both organisms. The respective NPAs for *B.*  
374 *pertussis* and *B. parapertussis* were 99.0% and 99.7%, respectively, again with lower 95% CI  
375 bounds above 85% for both species. Thus, the diagnostic accuracy of the ARIES *Bordetella*  
376 Assay is acceptable for effectively detecting *BP* and *BPP* in nasopharyngeal swabs from patients  
377 suspected of having respiratory tract infection attributable to either pathogen.

378 Initial SOC testing performed by each study site produced 23 cases of putative *B.*  
379 *pertussis* infection in Arm 1 (23/1,502=1.5%) that were not detected as *B. pertussis*-positive by

380 ARIES or by the bidirectional sequencing comparator. All study sites used PCR assays that  
381 targeted IS481, an insertion sequence present in high copy number in *B. pertussis* ( $\geq 50$   
382 copies/genome) but that is also present in *B. holmesii* at a lower copy number (e.g., 8–10  
383 copies/genome) (22, 25). Highly sensitive follow-up testing of the 23 discordant samples  
384 identified 5 additional *B. pertussis*-positive specimens, 3 *B. holmesii*-positive specimens, and 15  
385 confirmed *Bordetella*-negative samples. This reflects the utility of IS481 assays as a sensitive  
386 screening tool for *B. pertussis*, but also highlights its limited diagnostic specificity that can  
387 potentially misidentify other less insidious *Bordetella* spp. as true pertussis. For example, *B.*  
388 *holmesii* was present in up to 20% of suspected pertussis cases during a 2009–2011 outbreak in  
389 France (29). Whereas only approximately 5% of *B. bronchiseptica* strains reportedly express  
390 IS481, a commonly used primer set used for PCR detection of IS481 erroneously produced  
391 diagnostic amplicons of the predicted size for this gene in 78% of 149 tested *B. bronchiseptica*  
392 isolates, including in 22/24 that were of human origin (24). The IS481 assay may also produce  
393 false-positive *B. pertussis* findings by detecting high-copy IS481 DNA on lightly contaminated  
394 laboratory surfaces (30), or even from aerosolized pertussis vaccines in the clinic setting in the  
395 absence of viable organisms being present in samples (31). Interestingly, 12/15 (80%) of the  
396 study site SOC IS481-positive samples that ultimately tested negative for *B. pertussis* had initial  
397 IS481 Ct values  $>35.0$  (Table 3), suggesting that it detected a low total starting target sequence  
398 copy number. Together, these limitations of IS481-based assays may be responsible for  
399 identifying pertussis pseudo-outbreaks that do not truly constitute public health emergencies  
400 (32), and may constitute more of a public health concern than the low false-negative rate using  
401 the *ptxA*-based ARIES assay.

402 Study strengths include the multicenter prospective design, a large initial clinical sample  
403 size and the availability of supplemental known-positive *BP* and *BPP* clinical samples necessary  
404 to increase the accuracy of PPA and NPA determinations in low-frequency illnesses, particularly  
405 with *BPP*, and the availability of three robust reference techniques for comparison with ARIES  
406 findings. Study limitations include the inability of the qualitative ARIES assay to discern  
407 symptomatic, subclinical, and resolving *Bordetella* infection, although the likelihood of  
408 misidentifying an active infection is negated by judicious evaluation of clinical symptoms (5–7).  
409 The low natural prevalence of *B. pertussis* and *B. parapertussis* in the prospectively collected  
410 samples required inclusion of additional specimens with known *Bordetella* PCR outcomes or the  
411 use of contrived samples. Because the ARIES assay targets genetic sequences specific to *B.*  
412 *pertussis* and *B. parapertussis*, it is unable to identify the two other *Bordetella* species that can  
413 cause pertussis-like symptoms in humans, *i.e.*, *B. bronchiseptica* and *B. holmesii*, although  
414 infection with these organisms is comparatively rare (4–6).

415 The ARIES system can simultaneously evaluate up to 12 samples with minimal hands-on  
416 time required during its 2-hour runtime. The self-contained assay cartridges contain all reaction  
417 components and an internal sample processing control to monitor PCR fidelity. This study  
418 demonstrated that the ARIES *Bordetella* diagnostic assay reproducibly and accurately detects  
419 and discerns the two most clinically relevant *Bordetella* species that cause pertussis-like disease.

420

#### 421 **ACKNOWLEDGEMENTS**

422 Materials and financial support were provided by Luminex Corporation. Matthew Silverman  
423 PhD (Biomedical Publishing Solutions, Delray Beach, FL) provided expert writing assistance.

424

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**Table 1** Demographics of the clinical study population in prospective Arm 1.

| <b>Gender</b>           | <b>Site 1</b> | <b>Site 2</b> | <b>Site 3</b> | <b>Site 4</b> | <b>Site 5</b> | <b>All Sites</b> |
|-------------------------|---------------|---------------|---------------|---------------|---------------|------------------|
| Male                    | 56 (40.9%)    | 180 (42.3%)   | 56 (45.2%)    | 11 (44.0%)    | 164 (48.2%)   | 467 (44.4%)      |
| Female                  | 81 (59.1%)    | 246 (57.7%)   | 68 (54.8%)    | 14 (56.0%)    | 176 (51.8%)   | 585 (55.6%)      |
| <b>Age, yrs</b>         |               |               |               |               |               |                  |
| <18                     | 76 (55.5%)    | 263 (61.7%)   | 92 (74.2%)    | 22 (88.0%)    | 326 (95.9%)   | 779 (74.0%)      |
| ≥18                     | 61 (44.5%)    | 163 (38.3%)   | 32 (25.8%)    | 3 (12.0%)     | 14 (4.1%)     | 273 (26.0%)      |
| <b>Subjects by Site</b> | 137           | 424           | 124           | 25            | 339           | 1052             |

**Table 2** ARIES *Bordetella* Assay PPA and NPA for *B. pertussis* and *B. parapertussis* detection versus comparator real-time PCR assays and bidirectional sequencing.

| <i>Bp</i>            | PPA                |       | 95% CI      | NPA                |       | 95% CI      |
|----------------------|--------------------|-------|-------------|--------------------|-------|-------------|
| Prospective (Arm 1)  | 30/32 <sup>a</sup> | 93.8% | 79.2%–99.2% | 1,009/1020         | 98.9% | 98.1%–99.5% |
| Pre-selected (Arm 2) | 37/37              | 100%  | 90.5%–100%  | 77/77              | 100%  | 95.3%–100%  |
| Total                | 67/69              | 97.1% | 89.9%–99.6% | 1,086/1097         | 99.0% | 98.2%–99.5% |
| <i>Bpp</i>           | PPA                |       | 95% CI      | NPA                |       | 95% CI      |
| Prospective (Arm 1)  | 2/2                | 100%  | 15.8%–100%  | 1,048/1050         | 99.8% | 99.3%–100%  |
| Pre-selected (Arm 2) | 20/20              | 100%  | 83.2%–100%  | 93/94 <sup>b</sup> | 98.9% | 94.2%–100%  |
| Contrived (Arm 3)    | 50/50              | 100%  | 92.9%–100%  | 50/50              | 100%  | 92.9%–100%  |
| Total                | 72/72              | 100%  | 95.0%–100%  | 1,191/1194         | 99.7% | 99.3%–99.9% |

<sup>a</sup>Two prospective specimens generated false-negative results by ARIES *Bordetella* assay when compared to the composite comparator method. One of these two specimens gave a positive result in only one of two comparator real-time PCR assays. The other specimen generated Ct values between 37.1 and 38.8 by comparator real-time PCR assays; in this specimen, the ARIES *Bordetella* assay detected low levels of DNA (Ct=40.1), which was close to the assay cut-off.

<sup>b</sup>One pre-selected specimen generated a false-positive *BPP* result by ARIES *Bordetella* assay that was not confirmed by comparator real-time PCR and bi-directional sequencing.

*Bp*, *B. pertussis*; *Bpp*, *B. parapertussis*; NPA, PPA, positive percent agreement; NPA, negative percent agreement; CI, confidence interval.

**Table 3** Reconciliation results of 23 discordant samples that were negative by ARIES for *B. pertussis*, but positive by each study site's standard-of-care IS481 PCR assays.

| Study Site | Sample | Sequencing Result                           | Site IS481 Assay Ct |
|------------|--------|---|---------------------|
| Site A     | 02-003 | POS <i>Bordetella pertussis</i> *           | 37.0                |
|            | 02-006 | NEG   |                     |
|            | 02-007 | POS <i>Bordetella holmesii</i> <sup>†</sup> |                     |
|            | 02-037 | POS <i>Bordetella pertussis</i> *           | 37.3                |
|            | 02-059 | NEG   |                     |
|            | 02-076 | POS <i>Bordetella pertussis</i> *           |                     |
|            | 02-103 | POS <i>Bordetella pertussis</i> *           |                     |
|            | 02-119 | NEG   | 36.9                |
|            | 02-165 | NEG   | 36.5                |
|            | 02-407 | NEG   | 38.1                |
| 02-501     | NEG    | 38.6  |                     |
| Site B     | 05-005 | NEG   | 40.1                |
|            | 05-013 | NEG   | 38.5                |
|            | 05-024 | NEG   | 39.2                |
| Site C     | 06-031 | NEG   | 36.7                |
|            | 06-076 | NEG   | 36.0                |
|            | 06-125 | POS <i>Bordetella holmesii</i>              | 32.8                |
|            | 06-156 | NEG   |                     |
|            | 06-172 | NEG   | 31.2                |
|            | 06-174 | POS <i>Bordetella pertussis</i>             | 33.5                |
|            | 06-179 | POS <i>Bordetella holmesii</i> <sup>†</sup> |                     |
|            | 06-314 | NEG   | 33.5                |
|            | 06-332 | NEG   | 36.9                |

\* Detected in the forward read only

<sup>†</sup> Detected in the reverse read only