Multisite Clinical Validation of Isothermal Amplification-Based SARS-CoV-2 Detection Assays Using Different Sampling Strategies

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ABSTRACT Isothermal amplification-based tests have been introduced as rapid, low-cost, and simple alternatives to real-time reverse transcriptase PCR (RT-PCR) tests for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection. The clinical performance of two isothermal amplification-based tests (Atila Biosystems iAMP coronavirus disease of 2019 [COVID-19] detection test and OptiGene COVID-19 direct plus RT-loop-mediated isothermal amplification [LAMP] test) was compared with that of clinical RT-PCR assays using different sampling strategies. A total of 1,378 participants were tested across 4 study sites. Compared with standard of care RT-PCR testing, the overall sensitivity and specificity of the Atila iAMP test for detection of SARS-CoV-2 were 76.2% and 94.9%, respectively, and increased to 88.8% and 89.5%, respectively, after exclusion of an outlier study site. Sensitivity varied based on the anatomic site from which the sample was collected. Sensitivity for nasopharyngeal sampling was 65.4% (range across study sites, 52.8% to 79.8%), for midturbinate was 88.2%, for saliva was 55.1% (range across study sites, 42.9% to 77.8%), and for anterior nares was 66.7% (range across study sites, 63.6% to 76.5%). The specificity for these anatomic collection sites ranged from 96.7% to 100%. Sensitivity improved in symptomatic patients (overall, 82.7%) and those with a higher viral load (overall, 92.4% for cycle threshold [C_T] of ≤25). Sensitivity and specificity of the OptiGene direct plus RT-LAMP test, which was conducted at a single study site, were 25.5% and 100%, respectively. The Atila iAMP COVID test with midturbinate sampling is a rapid, low-cost assay for detecting SARS-CoV-2, especially in symptomatic patients and those with a high viral load, and could be used to reduce the risk of SARS-CoV-2 transmission in clinical settings. Variation of performance between study sites highlights the need for site-specific clinical validation of these assays before clinical adoption.

IMPORTANCE Numerous SARS-CoV-2 detection assays have been developed and introduced into the market under emergency use authorizations (EUAs). EUAs are granted primarily based on small studies of analytic sensitivity and specificity with limited clinical validations. A thorough clinical performance evaluation of SARS-CoV-2 assays is important to understand the strengths, limitations, and specific applications of these

assays. In this first large-scale multicentric study, we evaluated the clinical performance and operational characteristics of two isothermal amplification-based SARS-CoV-2 tests, namely, (i) iAMP COVID-19 detection test (Atila BioSystems, USA) and (ii) COVID-19 direct plus RT-LAMP test (OptiGene Ltd., UK), compared with those of clinical RT-PCR tests using different sampling strategies (i.e., nasopharyngeal, self-sampled anterior nares, self-sampled midturbinate, and saliva). An important specific use for these isothermal amplification-based, rapid, low-cost, and easy-to-perform SARS-CoV-2 assays is to allow for a safer return to preventive clinical encounters, such as cancer screening, particularly in low- and middle-income countries that have low SARS-CoV-2 vaccination rates.

KEYWORDS COVID-19, SARS-CoV-2, isothermal amplification, clinical validation, cancer screening

The coronavirus disease of 2019 (COVID-19) pandemic has led to major disruptions in health services worldwide. In many developed nations, widespread severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing and mass vaccination have allowed for a return to most elective health services. However, many low- and middle-income countries (LMICs) have limited access to testing and vaccination and continue to struggle to contain COVID-19 (1, 2). As the COVID-19 crisis continues, considerable reductions in cancer screening, cancer control, and elective clinical services remain (3). The safe return to cancer screening and elective testing and procedures during the pandemic, especially in low vaccination regions, requires reliable SARS-CoV-2 testing for both providers and patients.

Numerous SARS-CoV-2 detection assays have been developed and introduced into the market under emergency use authorizations (EUAs) (4). EUAs are granted primarily based on analytic sensitivity (i.e., limit of detection [LOD]) and analytic specificity (i.e., cross-reactivity) with limited clinical validations. Yet, a thorough clinical performance evaluation of SARS-CoV-2 assays is important for understanding the strengths, limitations, and specific applications of these assays (5). Current Centers for Disease Control and Prevention (CDC) guidelines recommend the use of the laboratory-based nucleic acid amplification test (NAAT) (e.g., reverse transcriptase PCR [RT-PCR]) for confirmatory testing. Specimens that are considered optimal for detection include nasopharyngeal (NP), nasal midturbinate, and anterior nasal swabs. Currently, the CDC does not recommend NAATs that use oral specimens (e.g., saliva) for confirmatory testing (6–8).

In addition to clinical performance, several other factors are important to consider when assessing the feasibility of an assay for use in different environments and clinical settings. These factors include time to run the assay, hands-on time, throughput, ease of implementation, and cost. Furthermore, the possibility to use different sampling approaches, including self-collection, can be an important distinguishing feature since many LMICs have limited personal protective equipment (PPE). While RT-PCR assays fulfill the desired clinical performance criteria, they are not ideal for primary care clinics in resource-limited settings as point-of-care SARS-CoV-2 screening tests due to high costs as well as longer turnaround times and the need for technical expertise (9). While rapid antigen-based tests address these limitations, they lack sensitivity to rule out an active infection (10). Isothermal amplification-based reverse transcription assays may fill this gap as they are more rapid (take ∼1 to 2 hours), less expensive (∼5 to 15 USD per test), and simpler (do not need RNA extraction) than RT-PCR based tests (11), but they require clinical validation.

The primary objective of this study was to evaluate the clinical performance and operational characteristics of two isothermal amplification-based SARS-CoV-2 tests, namely, (i) the iAMP COVID-19 detection test (Atila BioSystems, USA) targeting N and ORF1ab genes of SARS-CoV-2 virus, which is the Food and Drug Administration (FDA; USA) EUA-approved test, and used in the United States and (ii) COVID-19 direct plus RT-loop-mediated isothermal amplification (LAMP) test (OptiGene Ltd., UK) targeting
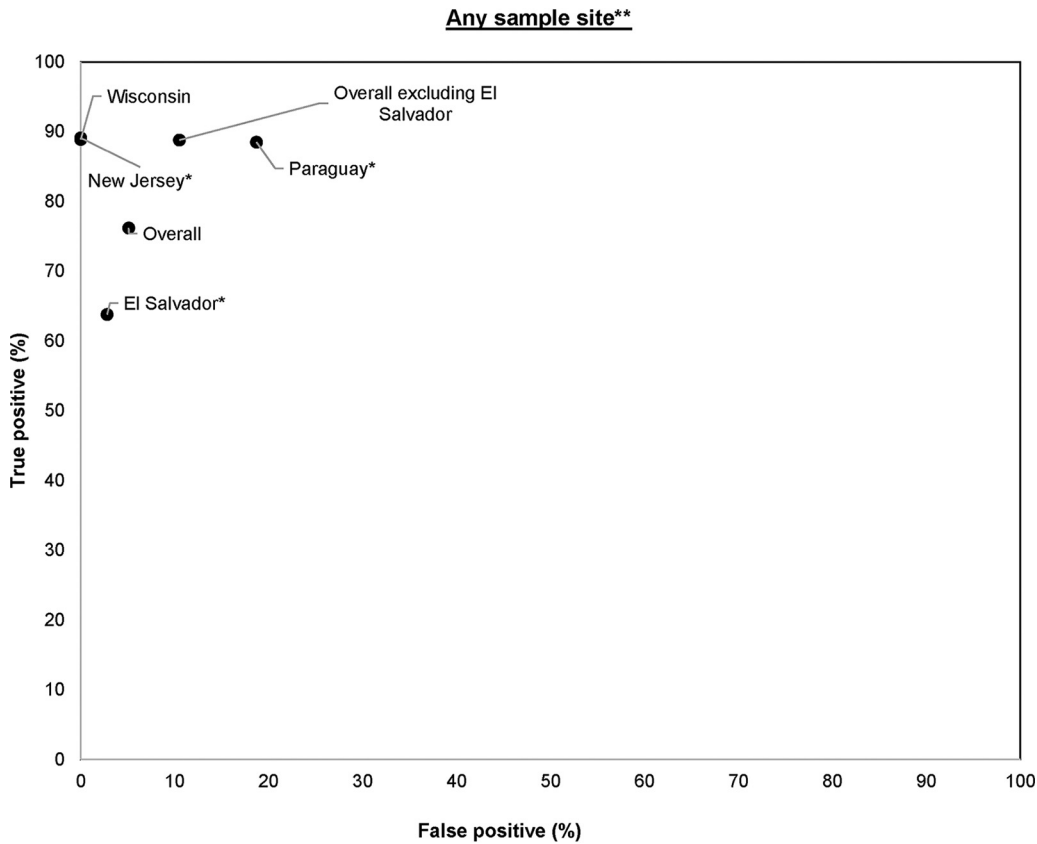
FIG 1 Study site-specific analysis of the validity of the Atila iAMP assay against PCR (reference) test (not stratified by sample collection site). *, P value of < 0.05 for McNemar's test (continuity corrected); **, any sample collection site positive out of the total samples collected is considered positive.

the ORF1ab gene of SARS-CoV-2 virus, which is European Conformity for *in vitro* diagnostic devices (CE-IVD) marked and used in the United Kingdom, compared with those of clinical RT-PCR tests. The secondary objective was to evaluate the influence of different sampling strategies on the detection of SARS-CoV-2. One specific use for such assays is rapid SARS-CoV-2 testing to allow for a safer return to preventive clinical encounters, such as cancer screening in low- and middle-income countries.

RESULTS

Atila iAMP test. In the overall analysis (Fig. 1), the sensitivity of the Atila iAMP test was 76.2% (95% confidence interval [CI], 71.1 to 80.7) and the specificity was 94.9% (95% CI, 93.3 to 96.1) for detection of SARS-CoV-2. Stratified by study site, the sensitivity was 63.8% (95% CI, 55.9 to 71.2) in El Salvador, 88.5% (95% CI, 79.9 to 94.3) in Paraguay, 88.9% (95% CI, 65.3 to 98.6) in Wisconsin, and 89.1% (95% CI, 77.8 to 95.9) in New Jersey. The specificity was 97.2% (95% CI, 95.7 to 98.2), 81.3% (95% CI, 74.7 to 86.7), 100% (95% CI, 96.6 to 100), and 100% (95% CI, 88.4 to 100), respectively. Since the sensitivity of El Salvador site was significantly lower than that of all the other sites, and considered an outlier, we conducted an overall pooled analysis excluding El Salvador, which demonstrated an overall sensitivity of 88.8% (95% CI, 82.8 to 93.2) and an overall specificity of 89.5% (95% CI, 85.6 to 92.7).

We evaluated the clinical performance of individual sampling strategies (Fig. 2). The sensitivity and specificity of the provider-collected NP sample was 65.4% (95% CI, 59.9 to 70.6) and 97.6% (95% CI 96.5 to 98.4). Since sensitivity at the El Salvador site was significantly different from that of all the other sites, we recalculated the overall sensitivity
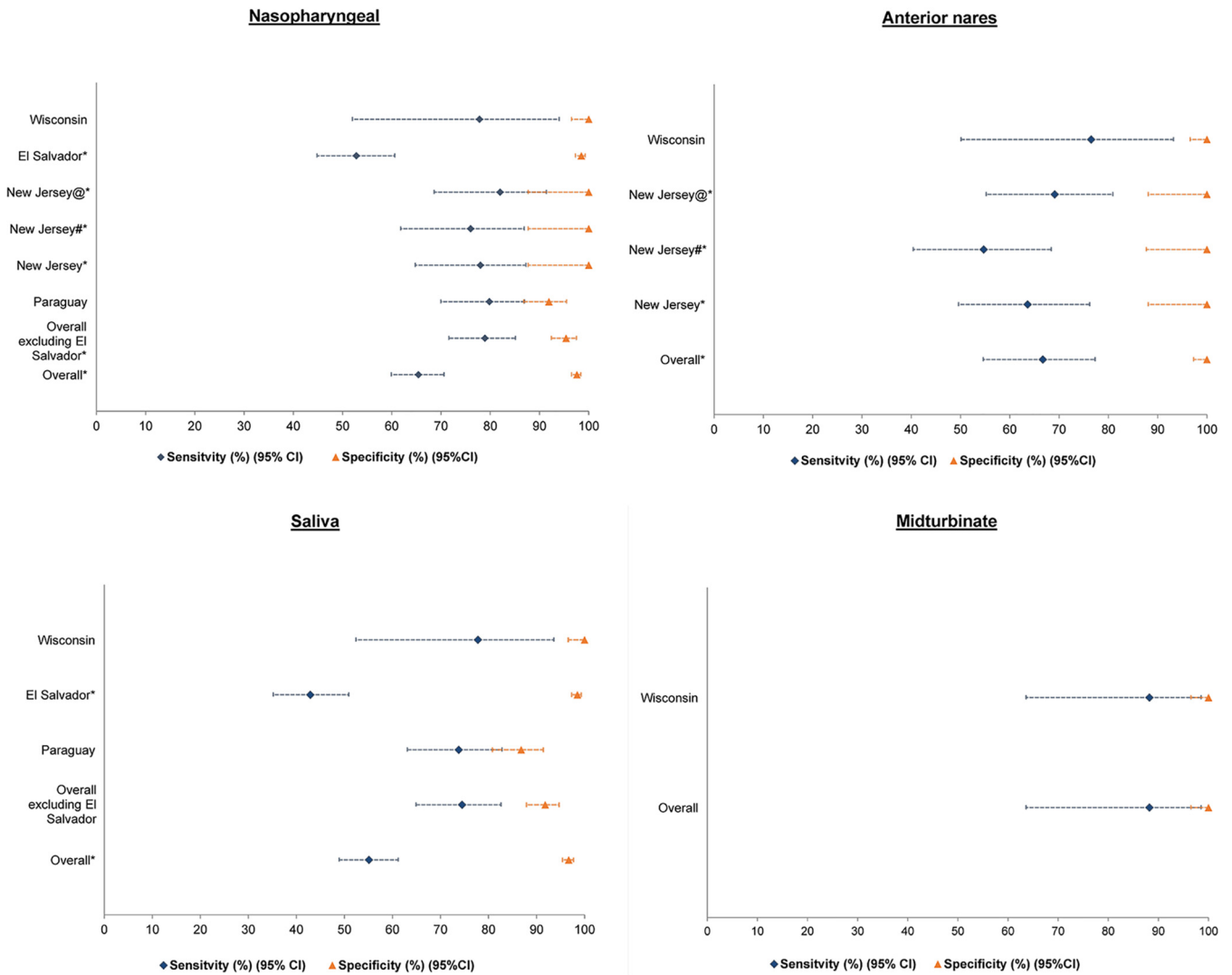
Nasopharyngeal

◆ Sensitvity (%) (95% CI)   ▲ Specificity (%) (95%CI)

Anterior nares

◆ Sensitvity (%) (95% CI)   ▲ Specificity (%) (95%CI)

Saliva

◆ Sensitvity (%) (95% CI)   ▲ Specificity (%) (95%CI)

Midturbinate

◆ Sensitvity (%) (95% CI)   ▲ Specificity (%) (95%CI)

FIG 2 Study site-specific analysis of the validity of the Atila iAMP assay against PCR (reference) test (stratified by sample collection site). *, P value of <0.05 for McNemar's test (continuity corrected); #, samples were tested in duplicates and the test was considered positive only if both were positive; @, samples were tested in duplicates and the test was considered positive if either was positive.

excluding El Salvador, which led to the sensitivity of 78.9% (95% CI, 71.6 to 85.1) and specificity of 95.4% (95% CI, 92.4 to 97.5).

By comparing the other sampling strategies to the reference standard NP sample, we found that the self-collected dry midturbinate sample (only collected at Medical College of Wisconsin [MCW]) was most sensitive (88.2% [95% CI, 63.6 to 98.5]) and specific (100% [95% CI, 96.5 to 100]). Self-collected saliva samples, excluding El Salvador (due to a significantly different estimate than that of other sites), had an overall sensitivity of 74.5% (95% CI, 64.9 to 82.6) and overall specificity of 91.8% (95% CI, 87.9 to 94.7). The self-collected dry anterior nares sample was the least sensitive strategy, with an overall sensitivity of 66.7% (95% CI, 54.6 to 77.3) and overall specificity of 100% (95% CI, 97.3 to 100). Since the anterior nares sample was not collected at the El Salvador study site and none of the study sites had a significantly different estimate than other sites for the anterior nares sample, no exclusion was made.

Assuming that viral load would influence accuracy, we analyzed the sensitivity at different cycle threshold (C_T) values by conducing RT-PCR among the positive subjects (Fig. 3). Restricting the analysis to samples with a C_T of ≤35, ≤30, ≤25, and ≤20 increased the sensitivity to 82.6%, 97%, 100%, and 100% for NP samples and 68%, 86.1%, 88.9%, and 100% for anterior nares samples in New Jersey (Rutgers New Jersey
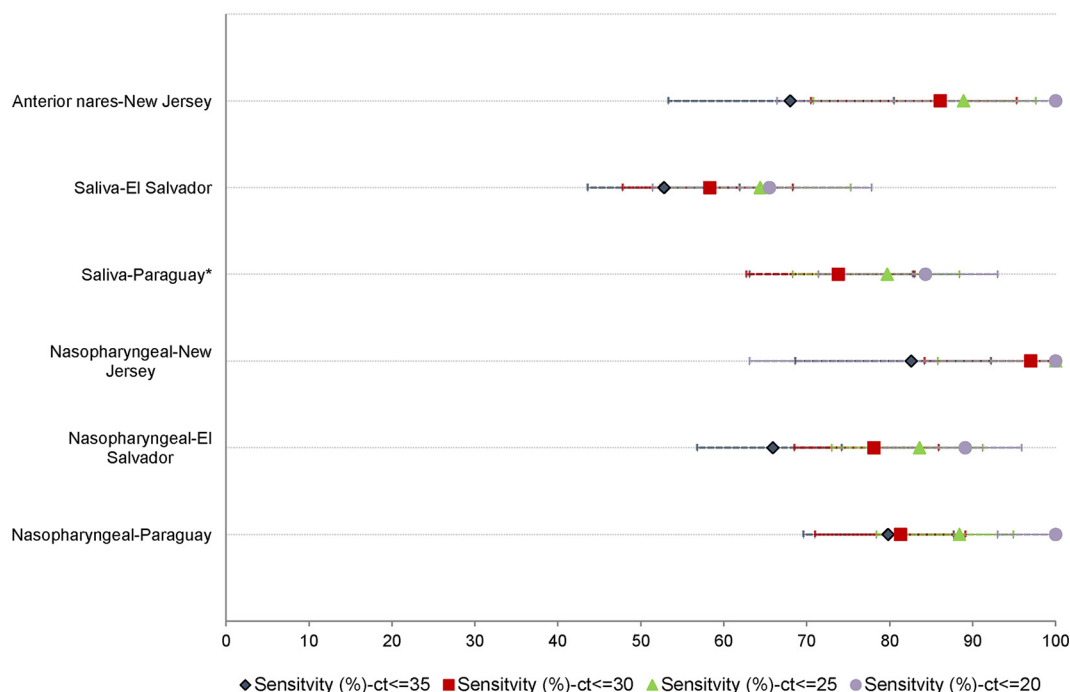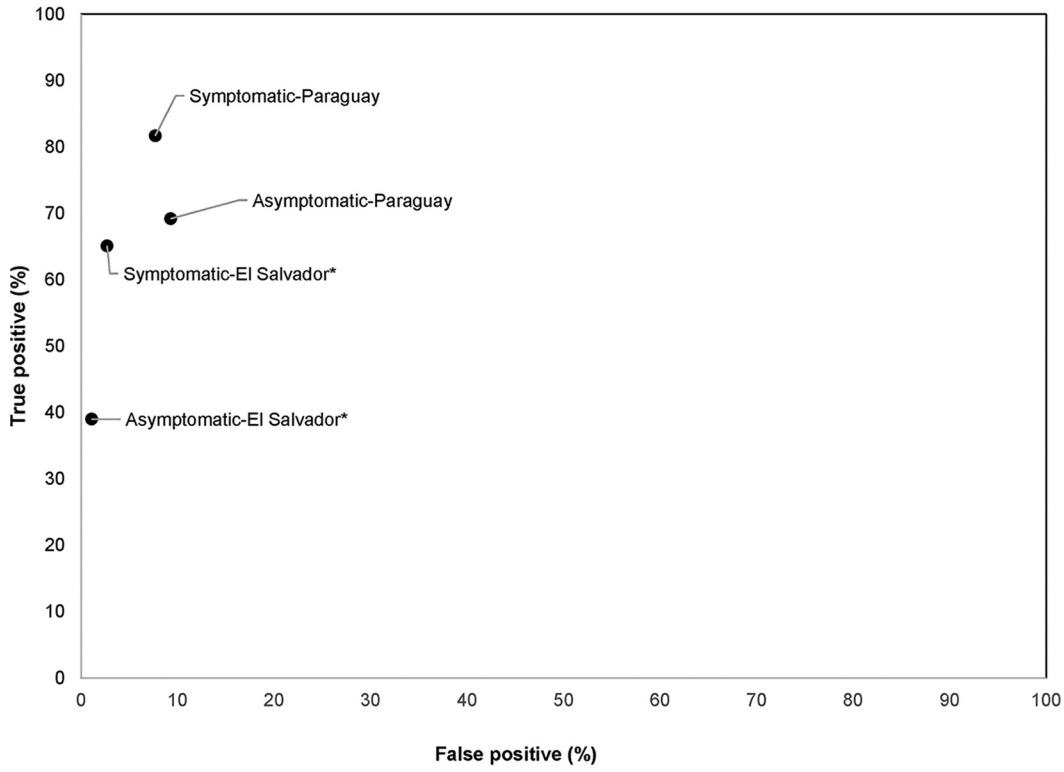
Sensitivity by viral load

FIG 3 Study site- and sample site-specific analysis of the sensitivity of the Atila iAMP assay against PCR (reference) test stratified by the C_T values. *, sensitivity for C_T of <35 and C_T of <30 was equal.

Medical School [NJMS]); and 79.8%, 81.3%, 88.4%, and 100% for NP samples and 73.8%, 73.8%, 79.7%, and 84.3% for saliva samples in Paraguay. The respective corresponding percentages for El Salvador were 65.9%, 78.1%, 83.6%, and 89.1% for NP samples and 52.8%, 58.3%, 64.4%, and 65.5% for saliva samples. Although the sensitivity increased in El Salvador with an increase in viral load (i.e., at lower C_T values), within each C_T value strata, the sensitivity in El Salvador was still lower than that in Paraguay and New Jersey for each anatomic collection sites.

The mean C_T value determined by RT-PCR testing in El Salvador among the RT-PCR-positive asymptomatic subjects was 30.0 (95% CI, 25.6 to 30.4) and among symptomatic subjects was 22.5 (95% CI, 20.2 to 24.8). The respective corresponding values in Paraguay were 18 (95% CI, 13.7 to 22.4) and 17.5 (95% CI, 15.8 to 19.2). The mean C_T value in New Jersey among the RT-PCR-positive subjects was 25.7 (95% CI, 23.9 to 27.5); all RT-PCR-positive subjects were hospitalized for observation and management of COVID-19 and so were likely symptomatic. The sensitivity of the NP sample in El Salvador among symptomatic subjects was significantly higher (65.1% [95% CI, 54.1 to 75.1]) than that among asymptomatic subjects (39.0% [95% CI, 28.0 to 50.8]) (Fig. 4). The difference was not significant between symptomatic and asymptomatic subjects for NP samples in Paraguay, saliva in El Salvador, and saliva in Paraguay. Among the symptomatic subjects, the sensitivity was significantly higher for saliva samples in Paraguay (74.3% [95% CI, 62.4 to 84.0]) than that in El Salvador (51.2% [95% CI, 40.1 to 62.1]); the difference was not significant for the NP samples in Paraguay (81.7% [95% CI, 70.7 to 89.9]), New Jersey (78% [95% CI, 64 to 88.5]), and El Salvador (65.1% [95% CI, 54.1 to 75.1]). Among the asymptomatic subjects, the difference for either NP (69.2% [95% CI, 38.6 to 90.9] in Paraguay and 39% [95% CI, 28.0 to 50.8] in El Salvador) or saliva samples (71.4% [95% CI, 41.9 to 91.6] in Paraguay and 33.8% [95% CI, 23.4 to 45.4] in El Salvador) was not significantly different between Paraguay and El Salvador.

OptiGene direct plus RT-LAMP test. The overall sensitivity and specificity of the OptiGene direct plus RT-LAMP test were 25.5% (95% CI, 14.7 to 39) and 100% (95% CI,

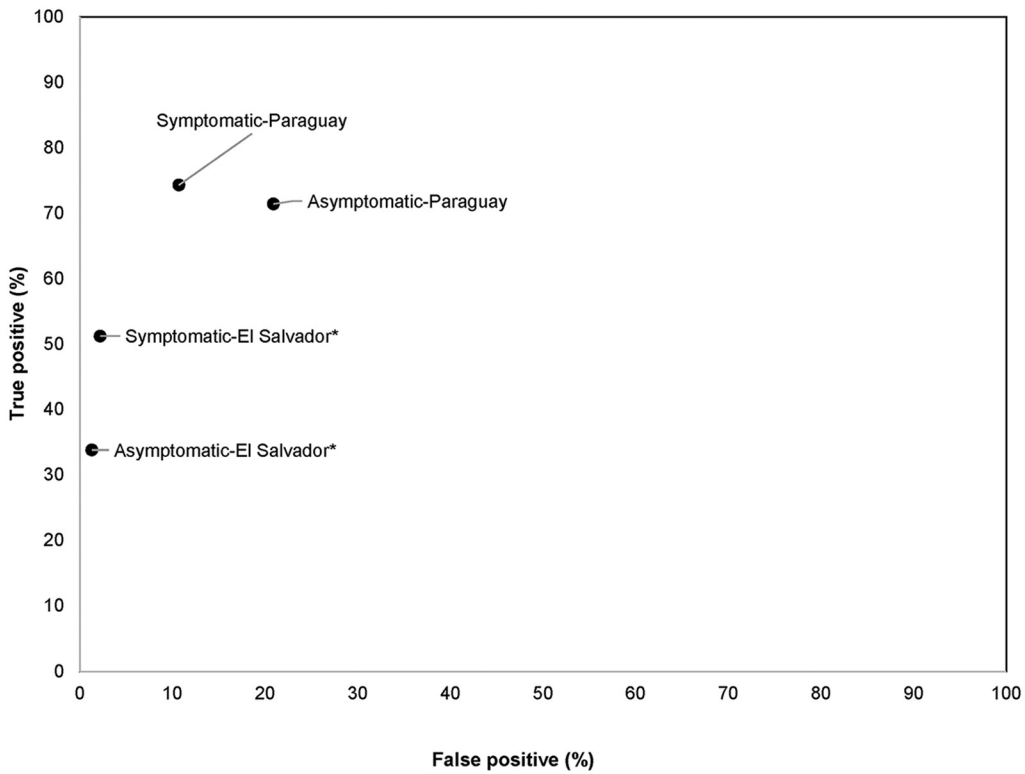## Nasopharyngeal

Saliva

FIG 4 Study site- and sample site-specific analysis of the validity of the Atila iAMP assay against PCR (reference) test stratified by the symptoms. *, P value of <0.05 for McNemar's test (continuity corrected).
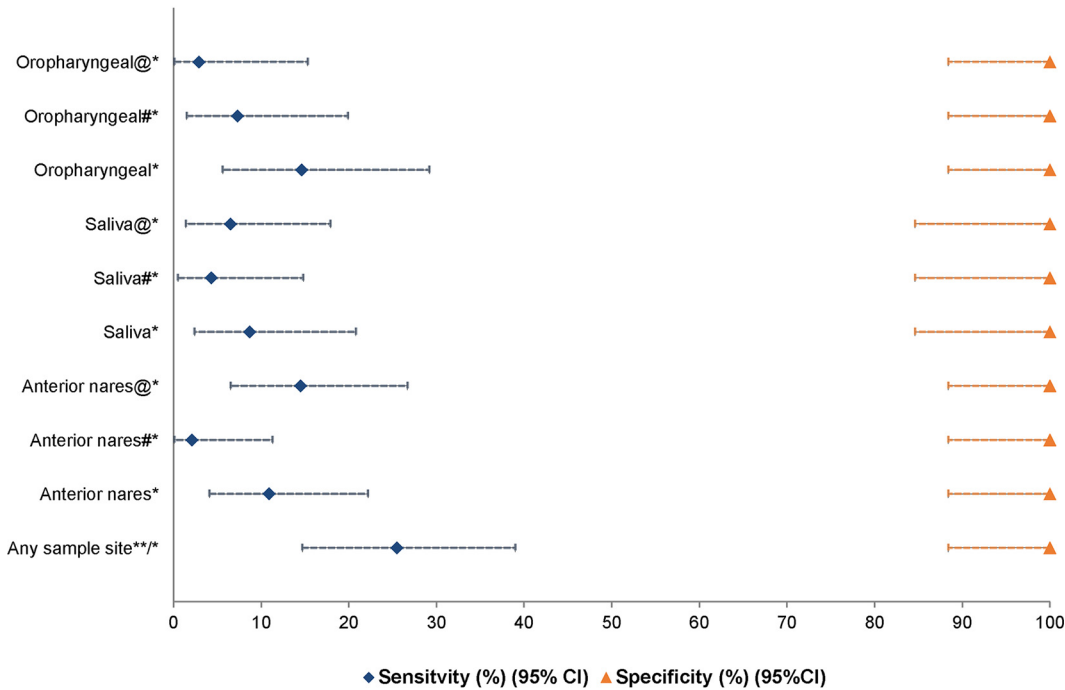
Optigene

FIG 5 Study site-specific analysis of the validity of the OptiGene direct plus RT-LAMP assay against PCR (reference) test (overall and stratified by sample collection site). *, P value of <0.05 for McNemar's test (continuity corrected); **, any sample collection site positive out of the total samples collected is considered positive; #, samples were tested in duplicates and the test was considered positive only if both were positive; @, samples were tested in duplicates and the test was considered positive if either was positive.

88.4 to 100), respectively (Fig. 5). The estimates did not differ significantly by sampling strategies or duplicate testing. Furthermore, when we limited the analysis to test samples collected within 24 h of RT-PCR sample collection, the overall sensitivity was still only 33.3%.

DISCUSSION

The current study evaluated the clinical performance of two isothermal amplification tests for the detection of SARS-CoV-2. The overall sensitivity and specificity of the Atila iAMP test for detection of SARS-CoV-2, excluding the outlier study site, were 88.8% and 89.5%, respectively. The sensitivity, excluding the outlier study site, was 78.9% for nasopharyngeal, 88.2% for self-sampled midturbinate, 74.5% for direct saliva, and 66.7% for anterior nares samples. The specificity for these sites ranged from 91.8% to 100%. The sensitivity increased with higher viral load (i.e., at lower C_T values) and among symptomatic compared with asymptomatic participants. The sensitivity and specificity of the OptiGene direct plus RT-LAMP test, conducted at a single site, were 25.5% and 100%, respectively.

There is scant literature on the performance of the Atila iAMP COVID test. We identified only one clinical performance evaluation of the Atila iAMP COVID test on the direct, nonextracted samples, which is the recommended application as per EUA by the manufacturer. This small-scale evaluation ($n = 197$) showed a sensitivity of 44.1% and specificity of 96.6% for the Atila iAMP test on NP swabs with a large number (35.5% [70/197]) of invalid results (12). A small ($n = 50$) analytic and clinical validation study on the Atila iAMP assay showed the analytic LOD for the assay to be 50 to 100 copies/reaction for ORF1ab gene and 1 to 10 for the N gene, which is higher than that of RT-PCR (average range of 1 to 10) (13). This result may explain our finding of lower

clinical sensitivity of the assay at higher C_T values, considering C_T values as a surrogate marker for the viral load, which may not always be precise (14). In the clinical validation by the same group, the assay findings had 100% agreement with the RT-PCR results. However, this validation was on extracted RNA and was based on 46/50 samples that have a C_T of ≤30. Another small ($n = 50$) clinical validation (15), again on extracted RNA, showed the sensitivity and specificity of the assay to be 82.8% and 100%, respectively, with all 5 false-negative samples having C_Ts of ≥35.

The OptiGene direct plus RT-LAMP COVID assay has been clinically validated previously by the National Health Service (NHS) trust (United Kingdom) to have a sensitivity of 70% for swabs and 79% for saliva, with an increase in sensitivity to 100% for swabs at a C_T of ≤25 (16). However, similar to our validation, such a high sensitivity was not confirmed by other groups, which showed the sensitivity in the range of 46.7% (17) to 34%, including false-negative results on symptomatic high viral load subjects (18). Our validation study was based on kits purchased from the manufacturer, using fresh samples (not freeze-thawed samples) collected and placed in the viral transport medium (VTM) recommended by the manufacturer and run as per the instructions provided by the manufacturer. Furthermore, even though the reference RT-PCR used in our assay targeted E or N2 and S gene in addition to the ORF1ab gene, given that an NP swab-based RT-PCR is the accepted reference standard for the SARS-CoV-2 diagnosis (7, 8), we believe that the clinical sensitivity of the assay should not be affected by the differences in gene targets between the assays. While it has been suggested that assays targeting the N gene are not a valid reference standard with which to evaluate the OptiGene direct plus RT-LAMP assay (19), this suggestion is not supported by clinical studies.

It is important to note that the instructions for use (IFUs) for both of the test assays state the need to confirm the negative test result with a more sensitive RT-PCR test and do not claim to provide the final screening answer (20). However, compared with the RT-PCR assays, which sometimes have a >24-h turnaround time (TOT) (variable by the assay but approximately 4 to 5 hours total hands-on and run time, but much longer TOT due to the need for batch testing), the advantage of the isothermal amplification-based assay is its rapid TOT (~1 to 2 hour) (approximately 40 minutes hands-on time, including 20 minutes of incubation period and 60 minutes run time for the Atila iAMP test and approximately 30 minutes hands-on time and 30 minutes run time for the OptiGene Direct Plus RT-LAMP test with no need for batch-testing for either), lower cost, and ease of performance (no nucleic acid extraction needed). We could not compare the isothermal amplification-based test assays to rapid antigen tests due to limitations on the numbers of samples that could be collected for each participant. Compared with performance estimates from the literature, the Atila iAMP assay provided higher sensitivity across NP, midturbinate, and direct saliva sampling than rapid antigen tests (reported to vary from 56% to 73% [9, 21–23]), resulting in more reassurance of a negative test result for iAMP. The higher sensitivity, along with similar advantages concerning the ease of operability and quick TOT as rapid antigen tests (~15 to 30 minutes for rapid antigen tests [24]), as well as compatibility with direct saliva testing (recommended to be nasal or nasopharyngeal only for rapid antigen assays [24]), allows us to identify high viral load subjects who are likely to be most infectious rapidly and at low cost (25, 26). Moreover, there is at least some evidence to suggest that RT-PCR positivity does not necessarily translate into infectivity because it can detect the shedding of postinfectious viral RNA particles, particularly among postsymptomatic patients (27, 28).

Variation in the performance of both the assays across various study sites in our evaluation and notable differences to other studies cannot be ignored. These differences demonstrate the limitations of EUAs, which may not necessarily translate to acceptable clinical performance for all tests in all settings. A thorough clinical validation of diagnostic assays on a standardized panel of samples in clinical settings is advisable before its widespread adoption for clinical use.

We do not fully understand the reason for the variation in test performance across study sites. Importantly, the populations at each site were different with respect to SARS-CoV-2 prevalence, clinical symptoms, and other factors, but stratified analyses

showed a similar performance at all sites except for El Salvador. Given that invalid results were rare and did not differ across the study sites and there was no consistent pattern observed in C_T values for the internal control (mean C_T values for the internal control for NP were 28.4 [95% CI, 28.1 to 28.7; El Salvador], 34.1 [95% CI, 32.8 to 35.1; MCW], 22.7 [21.9 to 23.4; Paraguay], and 25.4 [23.4 to 27.3; NJMS]; those for saliva were 19.4 [95% CI, 19.1 to 19.7], 23.6 [95% CI, 23.1 to 24.2; MCW], and 22.1 [21.3 to 22.8; Paraguay]), we do not attribute the lower sensitivity in our validation in El Salvador to sampling variation. Rather, we hypothesize the lower sensitivity of the Atila iAMP test in El Salvador to be related to multiple factors, as follows: relatively higher proportion of asymptomatic subjects than that in Paraguay (69.8% versus 21.9%) and operator-dependent nature of the assay due to the hands-on time to set up the reaction (29). However, given that on stratified analysis by symptoms and C_T values the sensitivity was still lower in El Salvador than that in other sites within the strata, the second explanation is more likely. Variation in the reference standard RT-PCR method and RNA extraction kits used across the study sites as well as variation in duration of performing the test assay after collection (Table 1) (30–39) is a limitation of our study and may also have influence on the study site-wide variations.

In conclusion, in this first large-scale multisite clinical evaluation of the Atila BioSystems iAMP COVID-19 detection test, the assay showed good sensitivity with high specificity for the detection of SARS-CoV-2, particularly with high viral load (i.e., C_T of ≤25) NP samples. In addition, it also showed moderate sensitivity for NP samples with a C_T of ≤35 and saliva samples with a C_T of ≤25. Overall, the sensitivity was superior for NP and midturbinate samples compared with saliva and anterior nares samples. The rapid TOT, low cost, and lack of a need for nucleic acid extraction make the Atila iAMP test a reasonable alternative screening test for SARS-CoV-2 for patients and providers in outpatient clinics to identify likely infectious subjects. When implemented with other COVID safety measures, such low cost testing can provide an approach for the safe reopening and daily clinical activities of essential medical services for the highest risk population in immediate need of care. However, inconsistency observed in assay performance across the study sites highlights the need for a rigorous site-specific clinical performance evaluation of the isothermal amplification-based assays before their clinical adoption.

MATERIALS AND METHODS

Study design and population. A cross-sectional study was conducted from December 2020 to April 2021 at four clinical sites, as follows: Hospital Nacional de Santa Ana, El Salvador; Hospital Materno Infantil de San Lorenzo, Ministerio de Salud Pública (MSP-BS), Paraguay; Medical College of Wisconsin (MCW), USA; and Rutgers New Jersey Medical School (NJMS), USA (Table 1). The current evaluation of isothermal SARS-COV-2 amplification assays was conducted to identify low-cost, accurate testing solutions that could be used for women and providers to allow for a safer return to elective medical services, such as cervical cancer screening. The study sites chosen for validation of SARS-COV-2 assays conducted cervical cancer screening studies in collaboration with NCI before the pandemic and were seeking approaches toward cancer screening while reducing the risk of COVID-19 transmission. SARS-COV-2 assays, if validated, could be integrated at these sites to resume the cervical cancer screening programs more safely. Human papillomavirus (HPV) is the primary screening test for cervical cancer screening. Atila BioSystems, the manufacturer of one of the SARS-CoV-2 assays, also has a rapid HPV assay. Both assays could be run on the same platform, simplifying approaches particularly for low- and middle income countries. In addition to their engagement in cervical cancer screening, study sites also needed to do active COVID testing using test and reference assays. The study protocol and sampling strategies varied slightly across the study sites, based on local requirements.

At the El Salvador site, 628 asymptomatic and 272 symptomatic subjects (total 900) presenting for SARS-CoV-2 testing were enrolled. A standard NP swab for RT-PCR testing was collected from all the participants for clinical diagnosis. A second provider-collected dry NP swab and a self-collected direct saliva sample were obtained from study participants in parallel for the Atila iAMP test.

At the Paraguay site, 58 asymptomatic and 207 symptomatic subjects (total 265) presenting for SARS-CoV-2 testing were enrolled in the study. A standard NP swab for RT-PCR was collected from all the participants for clinical diagnosis. In addition, for those consenting to participate in the study, a left-over of the clinical NP swab placed in viral transport medium (VTM) and a second parallel self-collected direct saliva sample were obtained for the Atila iAMP test.

At the Wisconsin (MCW) site, 128 total (symptomatic and asymptomatic) subjects presenting for SARS-CoV-2 testing were enrolled in the study. A standard NP swab for RT-PCR was collected from all the participants for clinical diagnosis. In addition, for those consenting to participate, a second provider-

TABLE 1 Description of the study population

Descriptor	Data by site[a]			New Jersey	
	El Salvador	Paraguay	Wisconsin	Atila	Optigene
Type of PCR (reference) test					
RealTime SARS-CoV-2 assay (Abbott, USA) targeting RdRp and N genes with onboard automated RNA extraction using M2000sp	691/900 (76.8)	0	0	0	0
Allplex 2019-nCoV assay (Seegene, South Korea) targeting E, N, and RdRP-genes with QIAmp viral RNA mini kit (Qiagen) for RNA extraction	209/900 (23.2)				
Cobas SARS-CoV-2 assay (Roche Diagnostics, USA) targeting ORF1ab and E genes with onboard RNA extraction	0	0	72/128 (56.3)	0	
Taqpath COVID-19 combo kit targeting ORF1ab, N, and S genes with MagMAX viral/pathogen nucleic acid kit using Thermo KingFischer extractor	0	0	56/128 (43.8)	0	
STAT-NAT COVID-19 MULTI assay (Sentinel Diagnostics, Italy) targeting RdRP and ORF1b genes with DA0633-RNA/DNA purification kit (magnetic beads) (Da An Gene, China) for RNA extraction	0	265/265 (100.0)	0	0	
Xpert Xpress SARS-CoV-2 assay (Cepheid, USA) targeting N2 and E genes with automated onboard RNA extraction	0	0	0	14/85[b] (16.5)	
Simplexa COVID-19 Direct assay (DiaSorin Molecular, USA) targeting ORF1ab and S genes with no RNA extraction	0	0	0	50/85[b] (58.8)	
Quest Diagnostics lab-developed test	0	0	0	1/85[b] (1.2)	
PCR (reference) result					
Positive	163/900 (18.1)	87/264 (33.0)	18/126[c] (14.1)	55/85 (64.7)	80/85 (94.1)
PCR (reference) result by C_T values					
≤20	57/163 (35.0)	54/87 (62.1)	3/18[d]	10/55[b] (18.2)	
21–25	17/163 (10.4)	20/87 (23.0)	2/18[d]	20/55[b] (36.4)	
26–30	24/163 (14.7)	9/87 (10.3)	0	7/55[b] (12.7)	
31–35	26/163 (16.0)	4/87 (4.6)	1/18[d]	14/55[b] (25.5)	
≥36	39/163 (23.9%)	0	0	2/55[b] (3.6)	
Duration between sample collection for PCR (reference) and test assay					
Parallel (same day)	900/900 (100.0)	265/265 (100.0)	128/128 (100.0)	8/85 (9.4)	
Duration between sample collection for test assay and running the assay					
Same day	900/900 (100.0)	0	N/A[e]	81/85 (95.3)	
Sample collection sites[f]					
Nasopharyngeal	900/900	265/258	128/123	79/78	0/0
Saliva[g]	900/900	265/259[h]	128/126[i]	0/0	84/68[i]
Anterior nares[g]	0/0	0/0	128/126	85/84	85/85
Midturbinate[g]	0/0	0/0	127/122	0/0	0/0
Oropharyngeal	0/0	0/0	0/0	13/11	71/71

(Continued on next page)

TABLE 1 (Continued)

Descriptor	Data by site[a]				New Jersey	
	El Salvador	Paraguay	Wisconsin		Atila	Optigene
Age (yrs)						
18–28	204/900 (22.7)	118/265 (44.5)	N/A		N/A	
29–39	242/900 (26.9)	74/265 (27.9)				
40–50	222/900 (24.7)	41/265 (15.5)				
≥51	232/900 (25.8)	32/265 (12.1)				
Sex						
Male	425/900 (47.2)	106/265 (40.0)	N/A		N/A	
Female	475/900 (52.8)	159/265 (60.0)				
Symptomatic						
Yes	272/900 (30.2)	207/265 (78.1)	N/A		55/85 (64.7)	
Total	900/1,378 (65.3)	265/1,378 (19.2)	128/1,378 (9.3)		85/1,378 (6.2)	

[a]Data are n (%) unless otherwise indicated.
[b]Type of RT-PCR data not available for 20 of 85 samples; C_T values not available for 2 of 55 samples.
[c]RT-PCR results missing for 2 of 128 subjects.
[d]C_T value not available for 12 of 18 samples.
[e]N/A, data not available.
[f]Data are total no. of samples collected/total no. of samples with valid result.
[g]Samples were self-collected (at Wisconsin).
[h]Three samples out of six total invalid runs were due to insufficient sample/mainly phlegm to process.
[i]All invalid runs were due to insufficient sample/mainly phlegm to process.

collected dry NP swab, a self-collected dry midturbinate swab, a self-collected dry anterior nares swab, and a self-collected direct saliva sample were obtained in parallel for the Atila iAMP test.

At the New Jersey (NJMS) site, 55 symptomatic SARS-CoV-2-positive patients, based on a prior RT-PCR assay, who were admitted for observation and management of COVID-19, were enrolled in the study. A total of 28 of 55 (50.9%) of the patients were enrolled within 24 hours, 14 of 55 (25.5%) within 48 hours, and 6 of 55 (10.9%) within 72 hours of the sample collection for the RT-PCR test. In addition, 30 participants expected to be negative for the SARS-CoV-2 infection (i.e., no SARS-CoV-2 symptoms) were enrolled. A negative SARS-CoV-2 RT-PCR test obtained within 5 days of test sample collection was performed on 28 (93.3%) of these 30 participants. Regardless of the RT-PCR status, for everyone enrolled in the study, a provider-collected dry NP swab and a provider-collected dry anterior nares swab were obtained at the time of enrollment for the Atila iAMP test. In addition, a provider-collected oropharyngeal (OP) swab placed in Sigma Virocult medium (MSW, UK) and a self-collected direct saliva sample were also obtained at the time of enrollment for the OptiGene direct plus RT-LAMP test.

The study protocol was approved by the ethical review board of Comite Nacional de Etica de Investigacion en Salud (institutional review board [IRB] no. FWA00010986) in El Salvador; Comité de Ética, Instituto de Investigaciones en Ciencias de la Salud, Universida Nacional de Asución (IRB no. P37/2020) in Paraguay; MCW IRB (IRB no. FWA00000820) in Wisconsin, and the Newark Health Sciences IRB for Rutgers Biomedical Health Sciences (IRB no. Pro2020001801) in New Jersey. Written informed consent was obtained from all study participants.

Test and RT-PCR assays. All the assays were performed as per the manufacturer's instruction for use (IFU).

The Atila iAMP test was performed on the same day of test sample collection for all samples in El Salvador and for 81 of 85 (95.3%) samples in New Jersey (NJMS). The samples not tested on the same day were frozen at −20°C in Paraguay and −80°C in Wisconsin (MCW) and tested in batches. Either of the following volumes and conditions were used for the initial reaction set up: (i) 3 μl of the dry swab was vortexed (2 to 3 sec) and incubated (5 min) with 0.7 ml of 1× COVID-19 elution solution or (ii) 3 μl of the 20 to 100 μl of saliva was heated at 95°C for 10 minutes (of the 500- to 1,000-μl collected volume) without RNA extraction. A validated RT-PCR system (i.e., Bio-Rad CFX96 RT system or Atila PowerGene 9600 plus RT-PCR system) with 6-carboxyfluorescein (FAM)/6-carboxy-2,4,4,5,7,7-hexachlorofluorescein (HEX) fluorescence detection was used for the reaction run and detection. Positive and negative controls were run for each batch, and the batch was considered valid only if both controls were valid. The individual sample test result was determined positive if an exponential amplification curve with a cycle threshold (C_T) of <50 was present in the FAM (ORF1ab or N genes) channel. The test result was determined negative if the FAM channel did not have an amplification curve and the HEX (internal control) channel had an exponential amplification curve with a C_T of <50. The test was determined invalid if no amplification was detected in both FAM and HEX channels, in which case the test was repeated. If the repeat run was also invalid, then that sample was considered invalid. In total, 1.0% of NP, 1.4% of anterior nares, 3.9% of midturbinate, and 0% of saliva samples had invalid results. Less than 1% (0.6%) of saliva samples could not be tested secondary to the samples being predominantly phlegm.

With few exceptions (5 of 85 [6.0%]), the OptiGene direct plus RT-LAMP test was performed on the same day of the test sample collection. Five microliters of either 50 μl of vortexed VTM from the swab container or 50 μl of a neat saliva sample (of the 500 to 1,000 μl of the collected volume) mixed with 50 μl of RapiLyze heated to 98°C for 2 minutes without RNA extraction was used for the initial reaction set up. A Genie III or II platform (OptiGene, UK) was used for the reaction run and detection. Positive and negative controls were run for each batch of samples, and the batch was considered valid only if both controls were valid. The Genie software automatically analyzed the individual sample test results as positive or negative based on the amplification plot and annealing temperature. The test result was reported positive if the fluorescence level of the amplification curve increased above a defined threshold and the peak of the annealing curve was above a defined threshold and lay within a specified temperature range. All of the OP and anterior nares samples were tested. A total of 19% of the saliva samples were not tested because those samples were predominantly phlegm without saliva.

A single run was performed for each sample at all study sites except at NJMS, for which duplicate runs were performed for each sample. To ensure comparability across the sites, for the pooled analysis, the first of the duplicate run at NJMS was used.

Statistical analysis. Pooled and study site-specific analyses were performed overall and stratified by different sampling strategies. For the overall analysis, if any sample anatomic collection site tested positive, that subject was identified as positive for that test assay. If all collection site samples were negative for the subject, the subject was considered negative for that test assay.

The NP sample for the RT-PCR test used for clinical diagnosis was considered the reference method. The sensitivity was defined as the proportion of RT-PCR-positive samples which tested positive by the test assay, and specificity was defined as the proportion of the RT-PCR-negative samples which tested negative by the test assay. Additional stratified analyses by the C_T value for the RT-PCR, as a surrogate marker for the viral load, and a history of symptoms were also conducted wherever the data were available. Since C_T values were available for only 6 of the 18 positives at the MCW site, the C_T value-wise stratified analysis did not include MCW. A history of symptoms was collected from the subjects at the time of sample collection. Also, 95% confidence intervals (CIs) were calculated for the sensitivity and specificity measures. Imbalances in paired sample results were evaluated using McNemar's test, with a P value of <0.05 considered statistically significant. Data analysis was performed using IBM SPSS software.

Data availability. The data sets used in the current study are available from the corresponding author on reasonable request.

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