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SHORT COMMUNICATION

Patterns of Possible False-Positive Results for Three Commercial Real-Time PCR Kits for SARS-CoV-2

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SUMMARY

Background: **We retrospectively examined all initial positive SARS-CoV-2 test results using three real-time PCR tests from patients without a history of COVID-19 collected from September to October 2021 at a university-affiliated hospital.**
Methods: **We defined a possible false-positive (PFP) case as a positive case that showed negative results upon performing a confirmatory test on the same specimen. Positivity% and PFP% were defined as the number of first positive and the number of PFP cases divided by the total test numbers, respectively.**
Results: **The positivity%/PFP% values were 0.76%/0.10%, 0.29%/0.02%, and 0.21%/0.03% for the Xpert, Allplex, and cobas tests, respectively. Six (75%) cobas PFP cases were *RdRp*-only positive. All PFP cases analyzed by Xpert except one had cycle threshold values ≥ 40. Contamination during extraction was suspected in five of the 10 PFP cases analyzed by Allplex, which requires a separate extraction step.**
Conclusions: **Care must be taken when analyzing first-positive cases as these may be false-positive signals.**
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KEYWORDS

SARS-CoV-2, COVID-19, false positive, real-time PCR, rapid PCR

INTRODUCTION

According to the World Health Organization, approximately 250 million people worldwide have had COVID-19, which has resulted in 5 million deaths [1]. Since the onset of the COVID-19 pandemic, numerous nucleic acid amplification test (NAAT) panels have been developed to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of COVID-19 [2,3]. NAATs are currently considered to be the gold standard for COVID-19 diagnosis, as they have the highest levels of sensitivity and specificity. Antigen tests [4,5] and serological tests [6,7] have relatively higher false-positive rates due to their intrinsic cross-reactivity. The false-positive rates associated with NAAT panels are low but not zero, ranging from 0.04% to 0.1% [8]. This is important as false-positive NAAT test results can lead to unnecessary epidemiologic investiga-

tions and surveillance testing [9]. Furthermore, false-positive results may lead to delays in the management of critically ill patients [10]. False positives can arise from contamination [11], cross-reactions, or software problems [12].

False-positive cases are usually associated with high cycle threshold (Ct) values [9], making it difficult to differentiate false-positive cases from COVID-19 cases in the early and convalescent phases of infection, which also lead to high Ct values in SARS-CoV-2 NAATs. Therefore, care is needed to distinguish true-positive cases from false-positive cases. This study aimed to evaluate the patterns and rates of possible false-positive cases associated with three commonly used commercial SARS-CoV-2 real-time RT-PCR assays [cobas SARS-CoV-2 and Influenza A/B test (cobas; Roche Molecular, NJ, USA), Allplex SARS-CoV-2 assay (Allplex; Seegene, Seoul, Korea), and Xpert Xpress SARS-CoV-2 test (Xpert; Cepheid, CA, USA)] at a university-affiliated hospital in Korea.

MATERIALS AND METHODS

Routine SARS-CoV-2 NAAT testing is performed using the cobas test with a cobas 6800 instrument (Roche, CA, USA), the Allplex test with a Bio-Rad CFX96 instrument (Bio-Rad, CA, USA), or the STARlet system (Seegene, Seoul, Korea). Additionally, rapid PCR tests, with turn-around times of < 1 hour, are performed using the Xpert Xpress SARS-CoV-2 test with a GeneXpert system (Cepheid, CA, USA). The limits of detection (LoDs), which was calculated by probit analysis with 10 repeats of four dilutions (10^4, 10^3, 10^2, and 10 copies/mL) of AccuPlex SARS-CoV-2 Reference Material (SeraCare, USA), were determined to be 92.5/46.4 copies/mL (*RdRp*/*E*) for the cobas test and 2,306.4/214.5/1,664.6 copies/mL (*E*/*RdRp*/*N*) for the Allplex test (unpublished data). According to the manufacturer, the LoD of the Xpert system is 250 copies/mL. Nasopharyngeal swabs (NPS), obtained with SG Medical swabs (Seegene, Seoul, Korea) and Copan's Universal Transport Medium (COPAN, Brescia, Italy), are used for routine and rapid testing. Sputum and throat swabs are used only for routine testing. Samples from patients with confirmed COVID-19 are submitted for Allplex testing, and these samples are tested as a separate batch. All respiratory specimens are stored at 4℃ if needed, and separate nucleic acid extractions are performed for the Allplex tests using the STARlet system (Seegene, Seoul, Korea). To reduce false positives, it is generally recommended to take the patient's history of related symptoms and exposure and to retest new samples [12-14]. Immediate follow-up testing may be inadequate due to issues regarding the incubation period and the LoD; however, follow-up tests are considered to be effective for ruling out early phase infection. Samples from patients without any exposure are submitted for confirmatory tests, and follow-up specimens are obtained from the patients if possible. However, if the patient is an outpatient, they are retested by the public healthcare body and positive results are reported to us.

We reviewed all new positive SARS-CoV-2 tests performed from September to October 2021. We defined a possible false-positive (PFP) result as a positive test result that was negative upon confirmatory testing. A probable false-positive (PrFP) was defined as a PFP that was negative upon repeat sampling of the patient. Cases without resampling were considered indeterminate. All tests performed using each method and all positive cases in the laboratory information system were reviewed. Positivity% and PFP% were calculated as the number of first-positive and PFP cases divided by the total number of tests, respectively. The false discovery rate (FDR) was calculated as the number of PFP cases divided by the sum of the PFP cases and positive cases. Specimen types and the epidemiological characteristics of patients with PFP results were retrieved from electronic medical records. Amplification curves and the results of testing batches were reviewed for PFP results. This study was approved by the Institutional Review Board (IRB) of Asan Medical Center (#2021-2774).

RESULTS

A total of 84,012 samples were submitted for SARS-CoV-2 testing. The overall positive rates and PFP rates were 0.30% and 0.03%, respectively. For routine testing, 154 (0.29%) positives and 10 (0.02%) PFPs were reported from 53,210 Allplex tests, and 53 (0.21%) positives and eight (0.03%) PFPs were reported from 24,756 cobas tests. For rapid PCR testing, 46 (0.76%) positives and six (0.10%) PFPs were reported from 6,046 Xpert tests. The FDRs for the Allplex, cobas, and Xpert tests were 6.1%, 13.1%, and 11.5%, respectively. The details of the 24 PFPs are summarized in Table 1. Of these, 19 were confirmed with resampling tests 1 day later and subsequently classified as PrFPs. The remaining five were classified as indeterminate.

All PFP samples were NPSs. Six PFP results from the cobas test were *RdRp*-only positive with Ct values > 35, and the rest were *E*-only positive with Ct values > 35. All PFP results from the Xpert test were positive for *E* and/or *N2* with Ct values > 40 except one that had a Ct value of 37.4 for the *N2* target. *N2*-only positives dominated the PFP results at this institution. Contamination during nucleic acid extraction was suspected in 5 of 10 PFP cases tested using the Allplex test (Figure 1). Three PFP samples evaluated using the cobas test and two PFP samples evaluated using the Allplex test were not tested the day after acquiring the fresh sample because these were from individuals who were outpatients. Over the 2 months, one N2-only positive patient, with a Ct value of 41.50 on rapid PCR testing, and negative when tested a day later with Allplex was diagnosed with COVID-19 a few days later. Therefore, this sample was not counted as a PFP.

Table 1. Patient characteristics and test results of probable false-positive cases.

Sample number	Patient age (years)	Gender	Specimen	Panel	*E*	*RdRp*	*N*	*N2*	Follow-up test	Conclusion
1	33	F	NP swab	cobas	ND	36.6			negative	PrFP
2	35	M	NP swab	cobas	ND	37.15			negative	PrFP
3	20	F	NP swab	cobas	36.2	ND			NT	indeterminate
4	55	M	NP swab	cobas	ND	36.6			negative	PrFP
5	28	F	NP swab	cobas	35.93	ND			negative	PrFP
6	73	F	NP swab	cobas	ND	37.01			negative	PrFP
7	2	M	NP swab	cobas	ND	37.1			NT	indeterminate
8	19	M	NP swab	cobas	ND	37.18			NT	indeterminate
9	47	F	NP swab	Allplex	ND	ND	36.56		negative	PrFP
10	66	M	NP swab	Allplex	ND	ND	37.6		NT	indeterminate
11	68	F	NP swab	Allplex	34.82	ND	35.24		negative	PrFP
12	56	F	NP swab	Allplex	37.66	ND	ND		negative	PrFP
13	60	F	NP swab	Allplex	ND	ND	38.44		NT	indeterminate
14	63	F	NP swab	Allplex	35.34	36.85	35.94		negative	PrFP
15	57	M	NP swab	Allplex	36.32	37.71	34.82		negative	PrFP
16	36	F	NP swab	Allplex	35.34	36.18	35.82		negative	PrFP
17	68	M	NP swab	Allplex	ND	ND	37.54		negative	PrFP
18	81	F	NP swab	Allplex	ND	ND	37.96		negative	PrFP
19	0	M	NP swab	Xpert	ND			42.3	negative	PrFP
20	76	F	NP swab	Xpert	42.3			ND	negative	PrFP
21	47	F	NP swab	Xpert	42.4			41.5	negative	PrFP
22	60	F	NP swab	Xpert	42.3			42.6	negative	PrFP
23	58	M	NP swab	Xpert	ND			37.4	negative	PrFP
24	71	F	NP swab	Xpert	ND			41.3	negative	PrFP

Abbreviations: NA - not applicable, ND - not detected, NT - not tested, NP - nasopharyngeal, PrFP - probable false-positive, cobas - cobas SARS-CoV-2 and Influenza A/B test (Roche Molecular Systems, Inc., NJ, USA), Allplex - Allplex SARS-CoV-2 assay (Seegene, Seoul, Korea), Xpert - Xpert Xpress SARS-CoV-2 (Cepheid, CA, USA).

DISCUSSION

This retrospective study identified an overall PFP rate of 0.02% between September and October 2021. This rate is slightly lower than the false-positive rates in previous studies (e.g., 0.04%) [8]. This low false-positive rate may have been possibly achieved due to the low rates of cross-contamination rate due to separate batch testing of true-positive samples despite the low prevalence. Nevertheless, a false-positive rate above zero is alarming, and all new positive cases should be confirmed with a second test.

The PFP rate was the highest in the Allplex panel. However, all confirmed COVID-19 cases were tested with the Allplex panel. We reasoned that due to the higher positivity rates in this panel, there is a greater possibility of contamination, which may have resulted in higher PFP rates. Furthermore, contamination in open systems, such as the Allplex system, was not limited to adjacent wells, as shown for sample 11 (Figure 1) [15]. Therefore, rigorous decontamination must be performed throughout the testing process, and the occurrence of contamination must be closely monitored.

The Ct values of PFP samples tested using Xpert and cobas were high: > 40 for Xpert and > 35 for cobas. This finding is in line with a previous report that samples with high Ct values are suspected to be false positives [9]. Surprisingly, the FDR rates were higher for the cobas and Xpert tests, which are closed systems, than for the Allplex test, which is an open system. These higher FDR rates are probably related to spurious amplification, leading to false-positive readings by the automated interpretation system [16]. This suggests that the cobas and Xpert tests may be too sensitive and routinely detect non-infectious levels of SARS-CoV-2 nucleic acid, which does not develop into a SARS-CoV-2

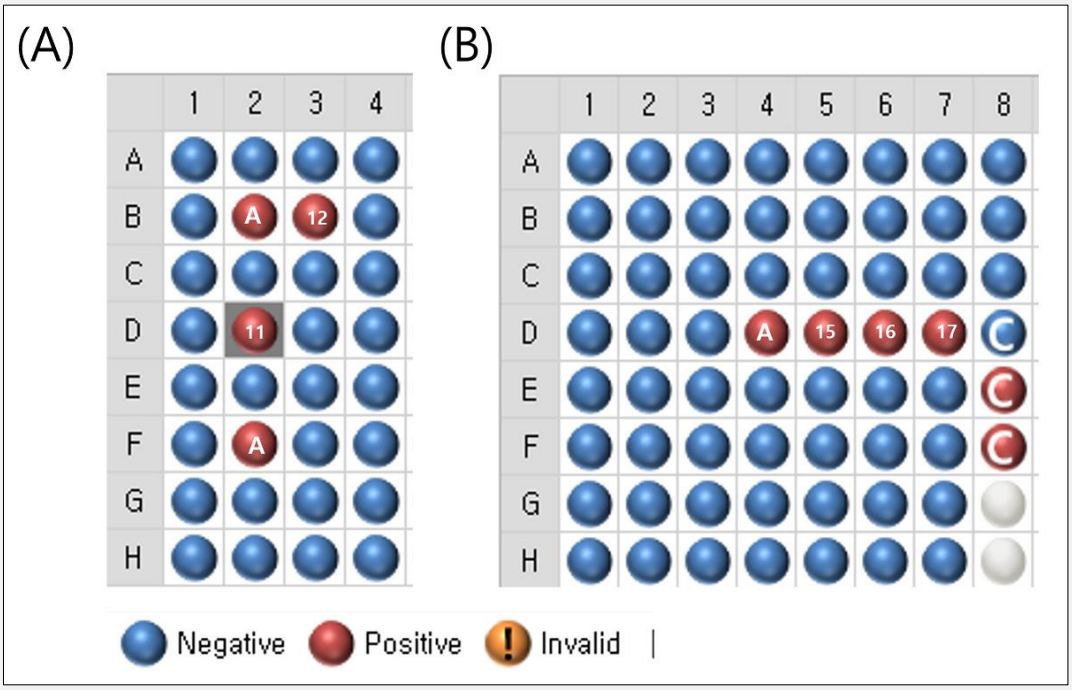

Figure 1. Probable cases of contamination during nucleic acid extraction.

Red circles containing "A" are true positives, and red circles with sample numbers are possible false positives. (A) Sample 12 is a typical case of contamination, which is next to a true positive, whereas sample 11 was two wells away from a positive well, but it was also considered to be a case of contamination. (B) Three false-positive cases of contamination. The Ct values associated with these cases were delayed with increased distance from the positive wells.

infection.

A borderline-positive case that was negative in both confirmatory tests and repeat sampling was diagnosed as positive a few days later. Due to the variation in the LoD of different tests, a borderline-positive result can be negative, inconclusive (if only part of the target gene is positive), or positive [17]. Thus, to be truly confident that a result is not a false positive, fresh sample, confirmatory testing, and follow-up tests 2 weeks later are required, considering a mean incubation period of 7 days [18].

This study had intrinsic limitations of retrospective nature. Additionally, repeating tests for all initial positives may be demanding in terms of reagent supplies, labor, time, and laboratory capacity and may lead to delays in epidemiologic measures, such as contact tracing and isolation.

Overall, the PFP rates for all three panels were very low, but they were not zero. False-positive signals with high Ct values in the cobas and Xpert tests and contamination in the Allplex tests may result in false positivity. The findings of this study suggest that all new positive cases should be epidemiologically investigated, and samples should be requested for resampling in cases without an epidemiologic linkage.

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Declaration of Interest:

The authors report no potential conflicts of interest relevant to this article.

References:

1. World Health Organization. Weekly epidemiological update on COVID-19 - 9 November 2021 2021 [Available from: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19--9-november-2021]

2. Penarrubia L, Ruiz M, Porco R, et al. Multiple assays in a real-time RT-PCR SARS-CoV-2 panel can mitigate the risk of loss of sensitivity by new genomic variants during the COVID-19 outbreak. Int J Infect Dis 2020;97:225-9. (PMID: 32535302)

3. Ravi N, Cortade DL, Ng E, Wang SX. Diagnostics for SARS-CoV-2 detection: A comprehensive review of the FDA-EUA COVID-19 testing landscape. Biosens Bioelectron 2020;165:112454. (PMID: 32729549**)**

4. Itoh K, Kawamitsu T, Osaka Y, et al. False positive results in severe acute respiratory coronavirus 2 (SARS-CoV-2) rapid antigen tests for inpatients. J Infect Chemother 2021;27(7):1089-91. (PMID: 33810971)

5. Otake S, Miyamoto S, Mori A, Iwamoto T, Kasai M. False-positive results in SARS-CoV-2 antigen test with rhinovirus-A infection. Pediatr Int 2021;63(9):1135-7. (PMID: 33963633)

6. Henry BM, Benoit SW, Lippi G, et al. False-Positive Rates in Pediatric SARS-CoV-2 Serology Testing [published online ahead of print, 2021 Sep 4]. Am J Clin Pathol 2021;aqab118. (PMID: 34480168)

7. To KK, Chua GT, Kwok KL, et al. False-positive SARS-CoV-2 serology in 3 children with Kawasaki disease. Diagn Microbiol Infect Dis 2020;98(3):115141. (PMID: 32795776)

8. Chandler CM, Bourassa L, Mathias PC, Greninger AL. Estimating the False-Positive Rate of Highly Automated SARS-CoV-2 Nucleic Acid Amplification Testing. J Clin Microbiol 2021; 59(8):e0108021. (PMID: 33972455)

9. Basile K, Maddocks S, Kok J, Dwyer DE. Accuracy amidst ambiguity: false positive SARS-CoV-2 nucleic acid tests when COVID-19 prevalence is low. Pathology 2020;52(7):809-11. (PMID: 33087255)

10. Schizas N, Michailidis T, Samiotis I, et al. Delayed Diagnosis and Treatment of a Critically Ill Patient with Infective Endocarditis Due to a False-Positive Molecular Diagnostic Test for SARS-CoV-2. Am J Case Rep 2020;21:e925931. (PMID: 32980852)

11. Montgomery TL, Paavola M, Bruce EA, Botten JW, Crothers JW, Krementsov DN. Laboratory Worker Self-Contamination with Noninfectious SARS-CoV-2 DNA Can Result in False-Positive Reverse Transcriptase PCR-Based Surveillance Testing. J Clin Microbiol 2021;59(7):e0072321. (PMID: 33952597)

12. Braunstein GD, Schwartz L, Hymel P, Fielding J. False Positive Results With SARS-CoV-2 RT-PCR Tests and How to Evaluate a RT-PCR-Positive Test for the Possibility of a False Positive Result. J Occup Environ Med 2021;63(3):e159-62. (PMID: 33405498)

13. Layfield LJ, Camp S, Bowers K, Miller DC. SARS-CoV-2 detection by reverse transcriptase polymerase chain reaction testing: Analysis of false positive results and recommendations for quality control measures. Pathol Res Pract 2021;225:153579. (PMID: 34385110)

14. Wilson MJ, Sparkes D, Myers C, et al. Streamlining SARS-CoV-2 confirmatory testing to reduce false positive results. J Clin Virol 2021;136:104762. (PMID: 33607351)

15. Martinez RJ, Pankratz N, Schomaker M, et al. Prediction of False-Positive Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Molecular Results in a High-Throughput Open-Platform System. J Mol Diagn 2021;23(9):1085-96. (PMID: 34116245)

16. Navarathna DH, Sharp S, Lukey J, et al. Understanding false positives and the detection of SARS-CoV-2 using the Cepheid Xpert Xpress SARS-CoV-2 and BD MAX SARS-CoV-2 assays. Diagn Microbiol Infect Dis 2021;100(1):115334. (PMID: 33571862)

17. Hur KH, Park K, Lim Y, Jeong YS, Sung H, Kim MN. Evaluation of Four Commercial Kits for SARS-CoV-2 Real-Time Reverse-Transcription Polymerase Chain Reaction Approved by Emergency-Use-Authorization in Korea. Front Med (Lausanne) 2020;7:521. (PMID: 32903503)

18. Cheng C, Zhang D, Dang D, et al. The incubation period of COVID-19: a global meta-analysis of 53 studies and a Chinese observation study of 11 545 patients. Infect Dis Poverty 2021;10(1): 119. (PMID: 34535192)