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Assessment on residual buffer sample mixture of rapid antigen assay performance in SARS-CoV-2 genomic surveillance: A cross-sectional study in a Malaysian teaching hospital

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ABSTRACT

Aims: Initially, COVID-19 PCR samples were frequently collected for genetic sequencing, thereby prolonging the processing time for diagnosing, screening and surveillance of SARS-CoV-2 virus. The direct utilisation of residual buffer-sample mixtures (RBSM) from COVID-19 antigen rapid test kits for whole genome sequencing (WGS) offers a potential solution to expedite processing time. Thus, this study aimed to perform genomic surveillance of SARS-CoV-2 variants utilising samples obtained from RBSM of rapid antigen test kits.
Methodology and results: For sample collection, nasopharyngeal swabs were collected from inpatients and outpatients of a teaching hospital in Pahang, Malaysia, between October 2022 and April 2023. Antigen fluorescent immunoassays (Ag-FIA) were conducted on all samples. RNA extraction and SARS-CoV-2 real-time PCR (RT-PCR) were then performed on positive Ag-FIA RBSM. Subsequently, variant analysis was carried out using GISAID (https://gisaid.org/). A total of 12,059 swabs were collected, with an Ag-FIA positivity rate of 2.7% (321/12059). Upon RT-PCR, 224/321 (69.8%) samples met the sequencing criteria with a Ct value of less than 30. To date, 66 samples have been completely sequenced, with less than half of the total samples (45.5%) revealing the presence of Omicron subvariants (BA.1.1.529, BA.2.75, XBB and XBB.1.5).
Conclusion, significance and impact of study: All in all, this study demonstrates that the RBSM from Ag-FIA generates valid sequencing results and, hence, can be optimised for rapid detection of viral variants.

Keywords: Diagnostic testing, genome sequencing, reagent kit, RT-PCR, SARS-CoV-2

INTRODUCTION

The sudden emergence of Coronavirus Disease-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has tremendously affected the global economy and mortality rate. Prophylactic vaccines, accurate and timely diagnostic tools, and the endorsement of other preventive measures have been shown to be effective in reducing the number of cases. To date, various types of diagnostic tools are available for detecting SARS-CoV-2. Jiang *et al.* (2022) categorized the SARS-CoV-2 detection methods into nucleic acid detection-based approaches (i.e., Loop-mediated isothermal amplification (LAMP), clustered regularly interspaced short palindromic repeats (CRISPR) and polymerase chain reaction), protein-based detection including antibody-based detection (i.e., enzyme-linked immunosorbent assay (ELISA) and lateral flow assay (LFA), as well as antigen-based methods (i.e., rapid diagnostic tests (RDT) and fluorescent immunoassays (FIA) and lastly, genome sequencing (i.e., Sanger sequencing, illumina sequencing and nanopore sequencing). Despite the comparable sensitivity and specificity of the mentioned methods to RT-PCR - the gold standard of SARS-CoV-2 diagnostic detection - these methods have yet to be employed in large-scale detection (Li *et al.*, 2022). Consequently, there is still a notable demand for a simpler and more rapid method.

In early 2023, the World Health Organization (2023) declared an end to COVID-19 as a public health emergency. However, global health leaders maintained a vigilant focus on monitoring genomic surveillance

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activities for any unprecedented new variants, as the virus could evolve rapidly. Diligent monitoring of viral variants could also enhance regional management and response to COVID-19 spread. Whole-genome sequencing (WGS) of SARS-CoV-2 is a powerful tool for studying its variants. While PCR and Ag-FIA detect the presence of viral antigens, WGS analysis enables deeper insight into the virus's spread through phylogenetic analysis (Forster *et al.*, 2020), revealing the dynamics of subtype evolution (Liu *et al.*, 2021). However, both PCR and WGS require sophisticated equipment and are unsuitable for rapid screening. To address this gap, real-time surveillance can be achieved by incorporating several diagnostic tools, such as Ag-FIA for rapid screening, followed by PCR and WGS. Hence, the main objective of the study is to assess the performance of the residual buffer-sample mixture (RBSM) of the COVID-19 Ag-FIA as the primary source for WGS. WGS is the primary laboratory technique for identifying the origin or variant of the pathogen. To complement the sequencing machine principle, production of a library, which is a set of fragmented DNA/RNA/exome were required. The outcome of WGS is a list of nucleotide sequences that will be used to find the organisms with matching genetic sequences from established databases.

High reliance on the residual buffer-sample mixture (RBSM) of Ag-FIA may facilitate hospital management, including point-of-care testing and transmission preventive measures, while also reducing the number of sample sources needed for WGS. Additionally, several similar studies have been conducted on direct post-Ag-RDT sample sequencing, primarily utilizing the extracted strips from the diagnostic cassette (Martin *et al*., 2022; Nazario-Toole *et al.*, 2022). Nevertheless, extraction from diagnostic cassette strips is laborious and requires additional time and resources. Therefore, it is proposed that the normally discarded RBSM may be a more convenient sample for sequencing, ultimately yielding reliable results.

MATERIALS AND METHODS

Study area and population

The study was carried out between October 2022 and April 2023 at a teaching hospital in Pahang, Malaysia. This hospital is one of the leading COVID-19 treatment centres in the East Coast region of Malaysia. The study population consists of both outpatients and inpatients who were accepted for COVID-19 testing.

Ag-FIA procedure

The Ag-FIA test procedure was conducted in accordance with the manufacturer's manual (SD BIOSENSOR, Korea). Briefly, a swab taken from the nasopharynx of a suspected COVID-19 patient was placed into an extraction buffer tube and mixed with the buffer by squeezing it five times. Four drops of the extracted specimen were then placed onto the test device. Positive results were indicated by the appearance of two bands on the test device. The RBSM in the tubes of positive results were stored at 4 °C for RT-PCR screening.

RNA extraction and screening RT-PCR

RNA extraction from the RBSM was conducted following the manufacturer's guidelines (Maxwell® RSC simplyRNA Cells kit, Promega, Wisconsin). For RT-PCR, the Allplex™ SARS-CoV-2 Master Assay Kit (Seegene Inc., Seoul) was used, according to the manufacturer's instructions, to screen for RdRP/S, E and N target genes (i.e., genes that encode spike (S), envelope (E) and matrix (M) structural proteins). The results yielded three different Ct values, each representing one of the genes. In this study, a mean cycle threshold (Ct) value of less than 30 was used as the criteria to determine the eligibility of the RBSM for WGS. This criteria was chosen based on an agreement (dated November 2021) by the COVID-19 National Genomic Surveillance Project consortium members, including all teaching hospitals under the Ministry of Health Malaysia and the Institute of Medical Research, Malaysia. No other parameters were collected during the study due to limited access.

Whole genome sequencing (WGS)

The genomic sequencing and sequencing analysis for this study was conducted by the Medical Genetic Laboratory of the teaching hospital. The laboratory is accredited and recognised for its proficiency in genomic research, ensuring the obtained data's reliability and accuracy and providing various molecular diagnostic services. Oxford Nanopore Technologies' (ONT) Rapid Barcoding Kit 96 (SQK-RBK110.96) was used for library preparation. Samples were barcoded and combined to produce a pooled library. After a priming mixture was added, the 1000 ng eluted and pooled library was then mixed with sequencing reagents before being loaded into the MinION SpotON flow cell. The flow cell was placed on the sequencing device (MinION Mk1B) beforehand. The results of the viral gene sequences were available in the EPI2ME software, which was then uploaded to the GSAID website for variant detection.

Data analysis

Data, including Ag-FIA results, RT-PCR Ct values of E, N and RdRp genes, and viral gene sequencing results, were tabulated and analysed using IBM Statistical Package for Social Sciences (SPSS) version 28.0. The rate of Ag-FIA positivity was calculated as the number of positive results from specimen extraction over the total number of swabs taken. The rate of RT-PCR results qualified for WGS (i.e., Ct values of less than 30) was calculated as the number of qualified RT-PCR samples over the total number of Ag-FIA positive samples.

The viral gene sequences were uploaded to the Global Initiative on Sharing All Influenza Data (GISAID; https://gisaid.org/) database to identify the COVID-19

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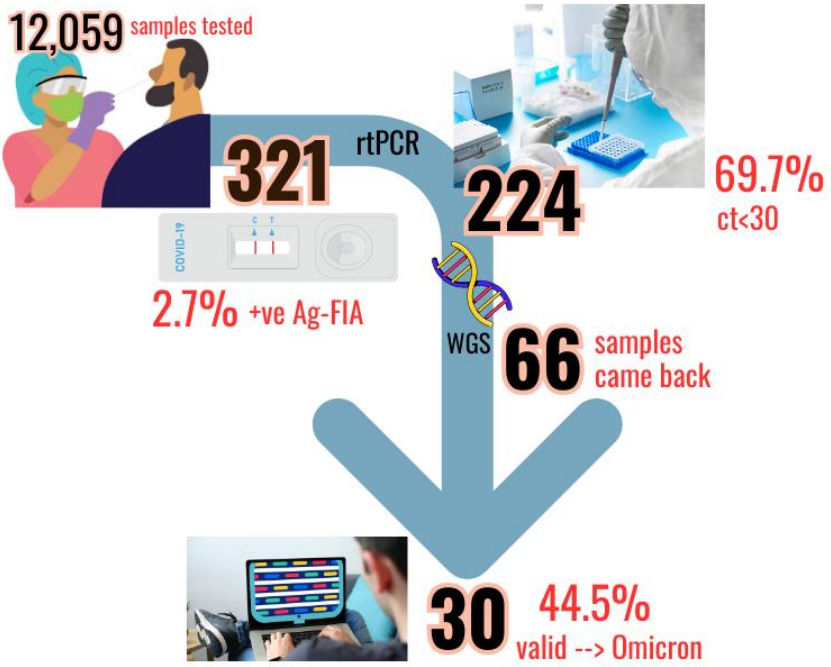

Figure 1: Summary of results showing the number of samples tested and success rates of antigen rapid test kit (Ag-FIA), real-time PCR (RT-PCR) and finally whole genome sequencing (WGS).

virus variants. The identification numbers ranged between EPI_ISL_17637514 and EPI_ISL_18009410. The success rate of WGS in determining the SARS-CoV-2 variants was calculated by dividing the successful sequencing results (i.e., valid data) by the total number of samples sent for sequencing.

Percentage of sequencing success rate (%)
= (Valid result/Total samples sent for sequencing) × 100

The success rate of sequencing results was compared with the Ct values using an independent T-test.

RESULTS AND DISCUSSION

Success rates of Ag-FIA and genomic sequencing

Rapid genomic surveillance assays play a crucial role in controlling and containing new emerging virus variants before they escalate into new pandemics. In this study, RBSM of the COVID-19 Ag-FIA rapid test kit served as the primary source for WGS. A total of 12,059 swab samples were collected from patients in the hospital during the study period (Figure 1). The high number of swab samples were due to the mandatory swabbing practice for all patients upon hospital admission and before undergoing any medical procedures. This practice also extended to caretakers and visitors of the hospital, as well as swab samples outsourced from other private hospitals and clinics, and in cases of special indications such as travel or job requirements, regardless of clinical manifestations. Out of the total samples, only 321 (2.7%) tested positive for COVID-19 Ag-FIA. PCR was conducted for all Ag-FIA positive samples and 224 PCR products (69.7%) met the sequencing criteria with Ct<30. A similar study by Macori *et al.* (2022) reported a sequencing success rate of 100% for 30 samples with Ct values below 34.67. However, no sequencing success rate was mentioned by Nazario-Toole *et al.* (2022) when employing a similar antigen-buffer extraction method for RT-PCR.

When the 224 PCR products were sent for sequencing, only 66 samples returned with WGS results. Thirty out of 66 samples (45.5%) were detected with the presence of the SARS-CoV-2 Omicron subvariants (B.1.1.529, BA.2.75, XBB and XBB.1.5). Generally, the Omicron variant (B.1.1.529) has milder effects on the patient than preceding circulating variants. However, the mutation that led to the emergence of the BA.2.75 subvariant has enhanced its invasion effect and, therefore, becoming more pathogenic. The recombinant product of BA.2.75, XBB and XBB.1.5 subvariants demonstrated increased antibody resistance and faster spread compared to other sublineages (Akash *et al*., 2023).

The high number of invalid outcomes was unexpected in this study. The invalid results were not attributed to Ct values or the viral proteins (p>0.05, Table 1). It was postulated that the invalid results could be due to modifications of the genetic base, i.e., G-C deletion of the ONT genome sequencing kit used. According to Bull *et al.* (2020) and Delahaye and Nicolas (2021), ONT may be susceptible towards insertion and deletion (indel) errors due to repeated nucleotide bases in the low-complexity

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Table 1: Multivariate analysis of variance (MANOVA) of sequencing validity against Ct values.

Sequencing validity	n	Ct values			P-value
		Mean			
		E	*N*	*RdRp*	
Valid result (Omicron variant)	30	28.01	21.34	29.99	0.550
Invalid result	36	28.96	22.92	31.34	

region of the virus. However, despite numerous efforts to repeat the genomic extraction and troubleshoot the errors, the results remained invalid.

Another possible reason for errors could occur in the amplification phase or cDNA synthesis via PCR, where errors may arise from the substitution activity made by DNA polymerases (Potapov and Ong, 2017). Contamination during library preparation may also lead to the production of errors. Contaminants, such as foreign nucleic acids as a result of cross-contamination of cells or primers, may cause false interpretations or invalid results (National Institutes of Health, 2022). Improper handling of RNA samples throughout the pre-analytical and analytical phases could also contribute to invalid WGS results.

CONCLUSION

In conclusion, this study demonstrates that the RBSM from Ag-FIA can generate a substantial number of valid sequencing results with a success rate of at least 45%. The study's limitations include the limited number of positive Ag-FIA samples, the short study duration and the inability to analyse the demographic details of the patients. Despite the limitations, this study has notably demonstrated the feasibility of SARS-CoV-2 variant detection using the Ag-RDT RBSM through the significant results, thus, enhancing the potential to monitor variants of concern in real-time.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The study has been approved by the hospital's research ethics committees and the University's Research Ethics Committees (IREC No. 2023-KAHS/DBMS1).

AUTHORS' CONTRIBUTION STATEMENT

The manuscript was drafted by ASA. Furthermore, ASA performed the literature search and the data analysis. IAS supervised the manuscript writing and contributed to the discussion of results. NK conceptualised and supervised the clinical and laboratory work and contributed to the discussion of results. All authors reviewed and approved the final version of the manuscript.

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