American Journal of Molecular Biology, 2024, 14, 166-185
https://www.scirp.org/journal/ajmb
ISSN Online: 2161-6663
ISSN Print: 2161-6620

PCR-HRM for Genomic Surveillance of SARS-CoV-2: A Variant Detection Tool in Côte d'Ivoire, West Africa

Aboubacar Sylla[1,2*], Solange Kakou-Ngazoa[1], Tata Gniré Safiatou Coulibaly[3], Yakoura Karidja Ouattara[4], Mireille Sylvie Kouamé-Sina[1], Zeinab Ouattara[1], David Ngolo Coulibaly[1], Brice Kouakou Bla[2], Mireille Dosso[1]

[1]Molecular Biology Platform, Pasteur Institute Côte d'Ivoire, Abidjan, Côte d'Ivoire
[2]Laboratory of Biology and Health, UFR Biosciences, University Félix Houphouët Boigny, Abidjan, Côte d'Ivoire
[3]Genetic and Cancer Laboratory, Molecular Genetics Platform, Pasteur Institute Côte d'Ivoire, Abidjan, Côte d'Ivoire
[4]Epidemic Virus Department, Pasteur Institute Côte d'Ivoire, Abidjan, Côte d'Ivoire
Email: *syabnet@yahoo.fr

How to cite this paper: Sylla, A., Kakou-Ngazoa, S., Coulibaly, T.G.S., Ouattara, Y.K., Kouamé-Sina, M.S., Ouattara, Z., Coulibaly, D.N., Bla, B.K. and Dosso, M. (2024) PCR-HRM for Genomic Surveillance of SARS-CoV-2: A Variant Detection Tool in Côte d'Ivoire, West Africa. *American Journal of Molecular Biology*, **14**, 166-185.
https://doi.org/10.4236/ajmb.2024.143013

Received: May 16, 2024
Accepted: July 20, 2024
Published: July 23, 2024

Abstract

The rise of new viruses, like SARS-CoV-2 causing the COVID-19 outbreak, along with the return of antibiotic resistance in harmful bacteria, demands a swift and efficient reaction to safeguard the health and welfare of the global population. It is crucial to have effective measures for prevention, intervention, and monitoring in place to address these evolving and recurring risks, ensuring public health and international security. In countries with limited resources, utilizing recombinant mutation plasmid technology in conjunction with PCR-HRM could help differentiate the existence of novel variants. cDNA synthesis was carried out on 8 nasopharyngeal samples following viral RNA extraction. The P1 segment of the SARS-CoV-2 Spike S protein was amplified via conventional PCR. Subsequently, PCR products were ligated with the pGEM-T Easy vector to generate eight recombinant SARS-CoV-2 plasmids. Clones containing mutations were sequenced using Sanger sequencing and analyzed through PCR-HRM. The P1 segment of the S gene from SARS-CoV-2 was successfully amplified, resulting in 8 recombinant plasmids generated from the 231 bp fragment. PCR-HRM analysis of these recombinant plasmids differentiated three variations within the SARS-CoV-2 plasmid population, each displaying distinct melting temperatures. Sanger sequencing identified mutations A112C, G113T, A114G, G214T, and G216C on the P1 segment, validating the PCR-HRM findings of the variations. These mutations led to the detection of L452R or L452M and F486V protein mutations within the protein sequence of the Omicron variant of SARS-CoV-2. In summary, PCR-HRM is a vital and affordable tool for distinguishing SARS-CoV-2

variants utilizing recombinant plasmids as controls.

Keywords

Genomic Surveillance, SARS-CoV-2, PCR-HRM, Variants, Côte d'Ivoire

1. Introduction

The emergence or re-emergence of viruses with epidemic or pandemic potential remains a persistent problem for human health [1] [2]. Infectious viral diseases pose a never-ending challenge that can emerge or re-emerge in unpredictable regions and at unpredictable times [3] [4]. In addition, the resurgence of resistance to several antimicrobial agents among pathogenic bacteria has become a significant threat to public health [5] [6], notably the epidemic of methicillin-resistant staphylococci at the end of the 90s, then enterococci resistant to glycopeptides, finally enterobacteria producing extended-spectrum beta-lactamases (EBLSE) [7] [8]). The emergence of its new deadly viral [9] and bacterial [10] diseases is sparking intense efforts to improve the understanding of molecular and cellular biology. However, despite concerted efforts to elucidate the complexity of these emerging pathogens, there is little new information on the evolving threats [9].

Indeed, The World Health Organization (WHO) keeps track of diseases that have the potential to become epidemics or pandemics and keeps track of their global spread. Chikungunya, cholera, Lassa fever, Marburg virus disease, Ebola, Hendra, meningitis Neisseria, MERS-CoV, monkeypox, Nipah virus infection, novel coronavirus (COVID-19), plague, Rift Valley fever, SARS, smallpox, tularemia, yellow fever, and Zika virus disease are among the diseases that are currently listed [11]-[13].

Monitoring multi-antibiotic-resistant bacteria and emerging viruses has become a major public health issue. Actually, medical microbiology laboratories have access to new high-throughput DNA sequencing (NGS) techniques [14]-[16]. Sequencing has been increasingly used in recent years for outbreak research in emerging diseases, as seen in the recent Ebola virus outbreak in Africa [17] and arbovirus outbreaks in South America [18]-[21]. In contrast, the scale of genomic surveillance undertaken during the current pandemic that of COVID-19 is unprecedented [22].

The most recent example concerns the new 2019 coronavirus called Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2). This virus gave rise to an outbreak of viral pneumonia in December 2019. According to the World Health Organization (WHO), more than 600 million cases of coronavirus have been confirmed and more than six million deaths worldwide [23]. SARS-CoV-2, a member of the Betacoronavirus, is an enveloped positive-sense single-stranded RNA virus, infectious to humans and mammals [24]. The rapid spread of the virus across the world has highlighted the "gap" existing in the global surveillance system and particularly with regard to the notable differences in technical

capacity between the different regions of the world.

The rapid development of the COVID-19 pandemic has highlighted the shortcomings of the existing laboratory testing strategy for SARS-CoV-2 diagnosis [25] [26]. Rapid, efficient and reliable diagnostic methods have been of paramount importance in combating the COVID-19 pandemic [27].

Next-generation sequencing (NGS) has enabled the most technologically advanced countries to rapidly study the genomic diversity of SARS-CoV-2 [28]-[30]. However, these approaches require highly specialized equipment and personnel that are not readily available in many laboratories within developing countries [31] [32].

A highly sensitive molecular biology technique based on the melting temperature (Tm) of amplified double-stranded DNA is post-PCR high-resolution melting (HRM) analysis [33]. HRM analysis has been used to identify single-nucleotide polymorphisms in DNA sequences [34] and in bacterial strains [35]. It has also been utilized in our Institution for point mutation detection in tumor cell genomes [36]. Previous research confirms that HRM analysis can successfully identify human immunodeficiency virus (HIV) variants [37], which, like SARS-CoV-2, are RNA viruses. These findings suggest that HRM analysis could potentially be a general and versatile method for determining SARS-CoV-2 transmission [38].

Several recent studies have highlighted the utility of PCR-HRM in distinguishing mutations in SARS-CoV-2 variants. For instance, [39] employed the PCR-HRM technique to identify new variants of the SARS-CoV-2 virus, such as the Alpha variant in Iran, as well as the Delta and Omicron variants [22]-[33] showcased the efficacy of PCR-HRM in detecting the Omicron BA.1 and BA.2 variants in Japan. Additionally, [36] utilized the HRM method to identify KRAS mutations in colorectal cancer patients for the first time in Côte d'Ivoire. Furthermore, [40] employed recombinant plasmid-based controls for routine detection of known mutations linked to drug resistance using the PCR-HRM method in Brazil.

The spike protein (S) of the virus has been the subject of countless investigations because of its crucial role in host invasion. Many of these studies have been related to potential therapeutic targets, such as preventing binding with the receptor [41], DNA vaccines, or RNA based on the S protein sequence [42], among other methods. However, as the disease progressed, transmission rates increased and the appearance of variants with mutations in their S protein sequences was discovered, seriously affecting the effectiveness of diagnosis and treatment [42] [43]. For the tracing of emergent viruses, the needs of rapid, cost-effective and reliable detection are urgent and crucial.

In this research, we have implemented a strategy to divide the SARS-CoV-2 Spike S protein gene into multiple mutated segments. These mutations are characteristic of the majority of SARS-CoV-2 variants. Following traditional PCR gene detection, we inserted them into a pGEM plasmid to create recombinant plasmids containing various segments of the Spike S protein. These recombinant

plasmids, referred to as clones, served as positive controls for the PCR-HRM technique, utilizing the Tm of the primers. This approach enables the identification of a new variant within the viral population. The amplicons were subsequently sequenced to pinpoint known mutations in SARS-CoV-2.

2. Materials and Methods

2.1. Sample Collection

Following approval from the National Ethics Committee for Life Sciences and Health (N/Ref: 028- 22/MSHPCMU/CNESVS-km), 08 RT-qPCR-positive nasopharyngeal samples have been selected for this study. These samples, identified as the Omicron variant using ONT MinIon sequencing, are part of the Institut Pasteur de Cote d'Ivoire biocollection. Previously employed by [44] for cloning the E, M, and N genes of SARS-CoV-2, these samples with known mutations underwent partial amplification in the S region and subsequent cloning into a plasmid. The resulting clones were instrumental in establishing the PCR-HRM method. For PCR-HRM validation, a wild-type control strain devoid of kit 20219-nCoV mutations (Biosensor, Gyeonggi-do, Republic of Korea) was utilized. This research was conducted at the molecular biology facility of the Institut Pasteur de Cote d'Ivoire. To assess the performance and effectiveness of the qPCR test, we chose 25 nasopharyngeal samples from the Institut Pasteur de Cote d'Ivoire biobank, comprising 19 COVID-19 positive and 6 negative samples for the year 2020. The QIAamp Viral RNA micro kit (Qiagen, Hilden, Germany) was used to extract total RNA in accordance with the manufacturer's instructions.

2.2. cDNA Synthesis

CDNA synthesis was performed using the LunaScript RT Super-Mix kit (New England Biolabs Inc.) following the manufacturer's guidelines. A 3 µL volume of LunaScript was added to 12 µL of RNA. The mixture was lightly centrifuged and then transferred to the Vapoprotect Pro Mastercycler thermal cycler (Eppendorf, Germany) for reverse transcription for 2 min at 25˚C, 55˚C for 10 min, and 95˚C for 1 min.

2.3. RT-PCR Amplification

The Wuhan-Hu-1 reference strain (NC_045512) of SARS-CoV-2 was utilized for designing primers. The nucleotide range nt-22805-23036 within the SARS-CoV-2 Spike protein gene was targeted for primer design, spanning 231 base pairs. This corresponds to amino acid positions 415-490 in the S gene. The specific sequence P1 was amplified using the primers FS-hrm-P1-Fwd: 5'-ACCGGCAAG ATCGCCGA-3' and FS-hrm-P1-Rev: 5'-TCAGAAGTAGCAGTTGAAGCC-3'. Primer's design was conducted using SnapGene software for PCR-HRM and Sanger sequencing. The reaction mixture contained 10 µL of 5X PCR buffer, 4 µL of 25 mM $MgCl_2$, 1.5 µL of 10 mM dNTPs, 0.5 µL of each primer at 20 µM

concentration, 0.4 µL of Go Taq Flexi DNA polymerase (Promega, Madison, USA), 5 µL of cDNA, and the volume was adjusted to 50 µL with H_2O. The amplification program is initiated by a 5 min predenaturation step at 94˚C, followed by 40 cycles of 94˚C for 30 s, 53˚C for 30 s, 72˚C for 1 min, and a final extension at 72˚C for 10 min. The mixture was incubated in the Vapoprotect Pro Mastercycler thermal cycler (Eppendorf, Germany). PCR products were purified using the Wizard® SV PCR kit (Promega, Madison, USA) as per the manufacturer's guidelines.

2.4. SARS-CoV-2 Gene Cloning

The pGEM®-T Easy Vector Systems kit (Promega, Madison, USA) was utilized for the ligation reaction. This reaction took place in a 10 µL volume with 5 µL of T4 DNA ligase 2X ligation buffer, 1 µL of 50 ng pGEM-T Easy Vector, 3 µL of purified PCR products, 1 µL of 3 U/µL T4 DNA ligase enzyme. Competent *E. coli* cells (Promega, Madison, USA) were employed for bacterial transformation. Upon thawing the JM109 competent cells on ice, 3 µL of the ligation reaction was introduced to 50 µL the cells. The mixture was gently mixed by tube squeezing to prevent cell damage, followed by a 20 min incubation on ice. Subsequently, the cells were heat-shocked in a water bath at 42˚C for 45 to 50 s without agitation, then promptly returned to ice for a 2 min incubation. S.O.C medium (Invitrogen Corporation, Carlsbad, USA) was utilized to enhance the transformation efficiency of competent cells. 450 µL SOC medium were added to tubes containing cells that had been transformed with the ligation reaction, and the mixture was incubated at 37˚C for 1 h 30 min with shaking. Following incubation, 100 µL of the transformation culture was spread onto LB (Luria-Bertani) agar plates containing 100 µg ampicillin, 40 µL of 20 mg/mg X-Gal solution (Invitrogen Corporation, Carlsbad, USA), and 40 µL of 100 mM IPTG (Invitrogen Corporation, Carlsbad, USA). The agar plates were then left to incubate overnight at 37˚C.

2.5. qPCR Analytical Efficiency

We established the positive standard curve for plasmid S portion P1 by performing qPCR on one-tenth serial dilutions. Corresponding plasmid standards ranging from 10^{10} to 10^{1} copies (viral gemone/µL) was used. Ct values of reactions wer plotted against $\log_{10}$ values of viral copy number by the standard curve for quantitative fluorogenic qPCR, allowing calculation of the correlation coefficient (R^2). Amplification efficiencies (E) of the reactions were calculated from the curves based on the equation: [45] [46]:

$$E = \left(10^{-1/\text{slope}} - 1\right) \times 100\% \quad (1)$$

Statistical analysis of S plasmid standard curves has helped us establish qPCR performance parameters, defining the LOD (Limit of Detection) and LOQ (Limit of Quantification) [47]. The limit of quantification (LOQ) is typically defined as the lowest concentration of a standard solution for which measurements

can be accurately made, usually with a measurement uncertainty of less than 20%. The limit of detection (LOD) was determined as the number of viral genome targets corresponding to the Ct, at which no more than 5% of truly positive samples tested negative (selectivity of 0.95). The standard curve was created from Ct values using linear regression, distinguished by slope (analytical sensitivity), y-intercept and confirmed by R^2. According to [48] [49], the Standard Error at the intercept (SE) was used to calculate the limit of detection (LOD = 3.3 × [standard deviation of intercept (SD)/slope]) and the limit of quantification (LOQ = 10 × [standard deviation of intercept (SD)/slope]).

To evaluate the performance of the qPCR assay, a total of 25 nasopharyngeal samples of known status, *i.e.* 19 positive and 6 negative for COVID-19, were used to determine sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) from the contingency table using XLSTAT version 2024 software.

2.6. Sequencing

The P1 segment of SARS-CoV-2 containing protein mutations at positions (415 - 490) including: K417N/T; N440K; G446S; N450K; L452R/Q; S477N; T478K; E484K/Q; V483A; and F490S was cloned for use in the HRM setup. Recombinant plasmids underwent sequencing with the BigDye™ Terminator v3.1 cyclic sequencing kit (Life Technologies, Austin, USA) following the manufacturer's protocol. A 20 µL reaction mix containing 2 µL of 5X sequencing buffer, 4 µL of BigDye™ Terminator v3.1 reaction mix, 1 µL of 4 µM of primer, and supplemented with H_2O was utilized. Amplification started at 96˚C for 30 s, followed by 25 cycles of 50˚C for 30 s and 60˚C for 4 min. Post-amplification, the Agencourt CleanSEQ kit (Beckman CoulTer, Indianapolis, USA) was employed for purifying the BigDye PCR products as per the manufacturer's guidelines. Subsequently, capillary electrophoretic analysis was conducted using the ABI 3500XL Genetic Analyzers 24-capillary system (Applied Biosystems, Hitachi, Japon).

2.7. Amplification Quantitative and High-Resolution Melting (HRM)

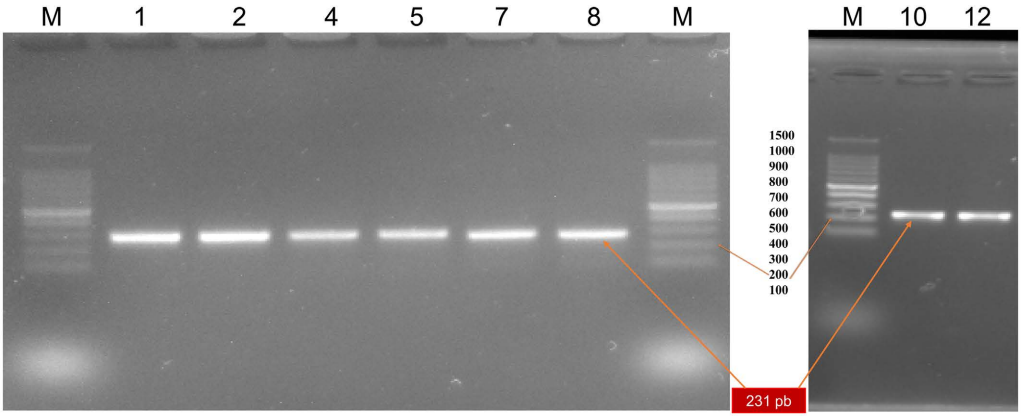
The amplicon melting temperature Tm was calculated using the OligoCalc tool [45] at Tm 86.86˚C. Validation of the qPCR-HRM amplification reaction was done using the Wuhan control strain from the Biosensor kit 20219-nCoV (Biosensor, Gyeonggi-do, Republic of Korea). PCR amplification took place in a StepOnePlus qPCR instrument (Applied Biosystems, Singapour). The reaction mixture included 10 µL of Master Mix MeltDoctor HRM 2X (Applied Biosystems, Foster, USA), 1 µL of each 5 µM primer, and molecular biology grade water to reach a final volume of 20 µL. Each sample underwent 3 consecutive repetitions. DNA concentration was standardized to 20 ng. The amplification program consisted of a step at 95˚C for 10 min to activate the enzyme, followed by 40 cycles at 95˚C for 30 s and 53˚C for 1 min to amplify the sequence. The melting cycle consisted of 95˚C for 10 s, 70˚C for 1 min, and 95˚C for 15 s. Regarding

the melting curve, the temperature increase gradient slope was 0.3% between 70˚C and 95˚C. The results were analyzed using High-Resolution Melting (HRM) analysis software from Applied Biosystems (Applied Biosystems, Waltham, USA).

3. Results

3.1. Amplification of the S Gene

The various nasophyngeal samples used to detect the S portion P1 gene resulted in the amplification of a 231 bp DNA band after electrophoresis on a 1.5% agarose gel (Figure 1). Several samples were positive in this period of year 2022.



M: 100 bp molecular weight marker (Promega, Madison, USA), 1: CoV801704 sample; 2: CoV815026 sample; 4: CoV801710 sample; 5: CoV799895 sample; 7: CoV800915 sample; 8: CoV801273 sample; 10: CoV820870 sample; 12: CoV799653 sample.

Figure 1. PCR amplification of the SARS-CoV-2 Portion P1 gene.

3.2. Clone Analysis

These plasmids, which contained the P1 region of the SARS-CoV-2 gene, were utilized for bacterial transformation and amplification. Following transformation, the culture of the modified bacteria on solid LB medium containing Xgal allowed us to distinguish between colonies that had integrated the plasmid (blue colonies) and those that had not (white colonies). The blue color of the colonies is a result of the presence of the Xgal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactoside), which is a characteristic of colonies lacking the insertion of the gene of interest. The white colonies are attributed to the existence of IPTG (isopropylthio-β-galactoside), an inducer of β-galactosidase activity in bacteria.

Blue colonies (Figure 2(b)) not having inserted the gene were discarded, while white colonies (Figure 2(a)) were selected for inoculation into LB liquid medium containing 100 µg/mL ampicillin at 37˚C for 24 hours in a 5% CO_2 environment. This led to the creation of 8 recombinant S gene plasmids named S1, S2, S4, S5, S7, S8, S10, and S12. Post-extraction analysis showed plasmid amounts ranging from 100 to 300 ng. The plasmid DNA obtained was subsequently employed for the HRM technique.

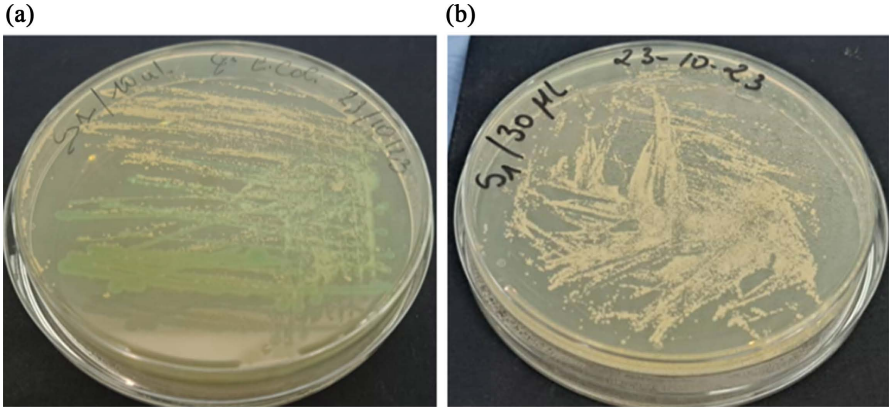


Figure 2. *E. coli* cells transformed with the vector integrating the S portion P1 gene of SARS-CoV-2 after 24 h incubation at 37˚C with 5% CO_2. White colonies represent transformed bacteria containing the plasmid, while blue colonies indicate non-transformed bacteria.

3.3. qPCR Efficiency, Sensitivity and Specificity

3.3.1. qPCR Efficiency

The plasmid containing the viral target was serially diluted ten-fold and tested to investigate the dynamic range of qPCR plasmids. Figure 3 shows the standard curve obtained from the qPCR assay. A linear correlation was observed between Ct values and log10 viral copy number. The R^2, E and slope of the standard curve for plasmid S from the qPCR assay are measured and reported in Table 1. To ensure accurate and reproducible quantification, the acceptance criteria were: correlation coefficient R^2 = 0.9771; efficiency E = 99.29% (the slope of the regression curve was between −3.1 and −3.6). The standard curve was used to determine the limit of detection LOD of 5.30 Copies/µL and the limit of quantification LOQ of 16.93 Copies/µL.

3.3.2. Sensitivity and Specificity

Analysis of nasopharyngeal samples enabled us to assess sensitivity, which was 83.33% with a CI confidence interval (41.6% to 98.4%), and specificity, which

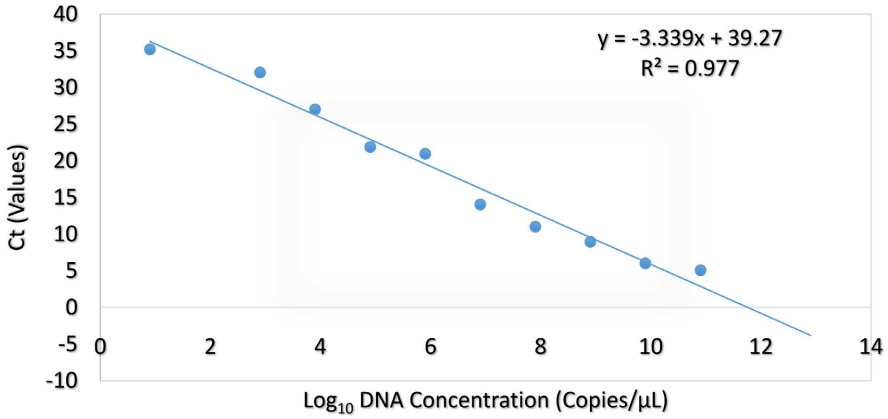


Figure 3. Standard curve for recombinant plasmid S portion P1 using serial dilutions of 10.

Table 1. Analytical sensitivity of plasmid S.

Standard curve		Plasmid qPCR
Copies/µL	Log_{10} Concentration	Plasmid S Average Ct ± Standard Deviation
10^{10}	10	5.07 ± 0.36
10^{9}	9	6.02 ± 0.01
10^{8}	8	8.97 ± 0.24
10^{7}	7	11.03 ± 0.43
10^{6}	6	14.06 ± 0.18
10^{5}	5	20.96 ± 0.31
10^{4}	4	21.89 ± 0.22
10^{3}	3	27.02 ± 0.30
10^{2}	2	32.05 ± 0.48
10^{1}	1	35.20 ± 0.23
R^2		0.9771
Pente (a)		−3.339
E (%)		99.29
b (Intercept)		39.27
	LOD (Copies/µL)	LOQ (Copies/µL)
Plasmid S	5.30	16.93

R^2: Correlation coefficients (R) of standard curves by Ct values of reactions versus log_{10} values of concentrations.a: Slope of standard curves by Ct values of reactions versus log_{10} values of concentrations. E: Amplification efficiency (E) of the assay.

Table 2. Nasopharyngeal samples contingency table.

	Sick	No Sick
Positive test	18	01
Negative test	01	05

This table presents the results of 25 nasopharyngeal swabs according to their original status, 19 of which were positive and 6 negative. From the sensitivity and specificity, we deduced the positive and negative predictive values.

was 94.70% with a CI confidence interval (73.2% to 100%). This also enabled us to determine a positive predictive value (PPV) of 83.33% with a CI of 53.5% to 100%, and a negative predictive value (NPV) of 94.70% with a CI of 84.70% to 100% Table 2.

3.4. Sanger Sequencing Analysis

The sequences obtained through the Sanger method were aligned with the plasmid S2 sequence, chosen as our reference. When comparing plasmids S8 and

S10 with our reference S2 (Figure 4(a)), no mutations were detected. Sequence alignment of plasmids S4, S5, and S12 with reference S2 revealed mutations

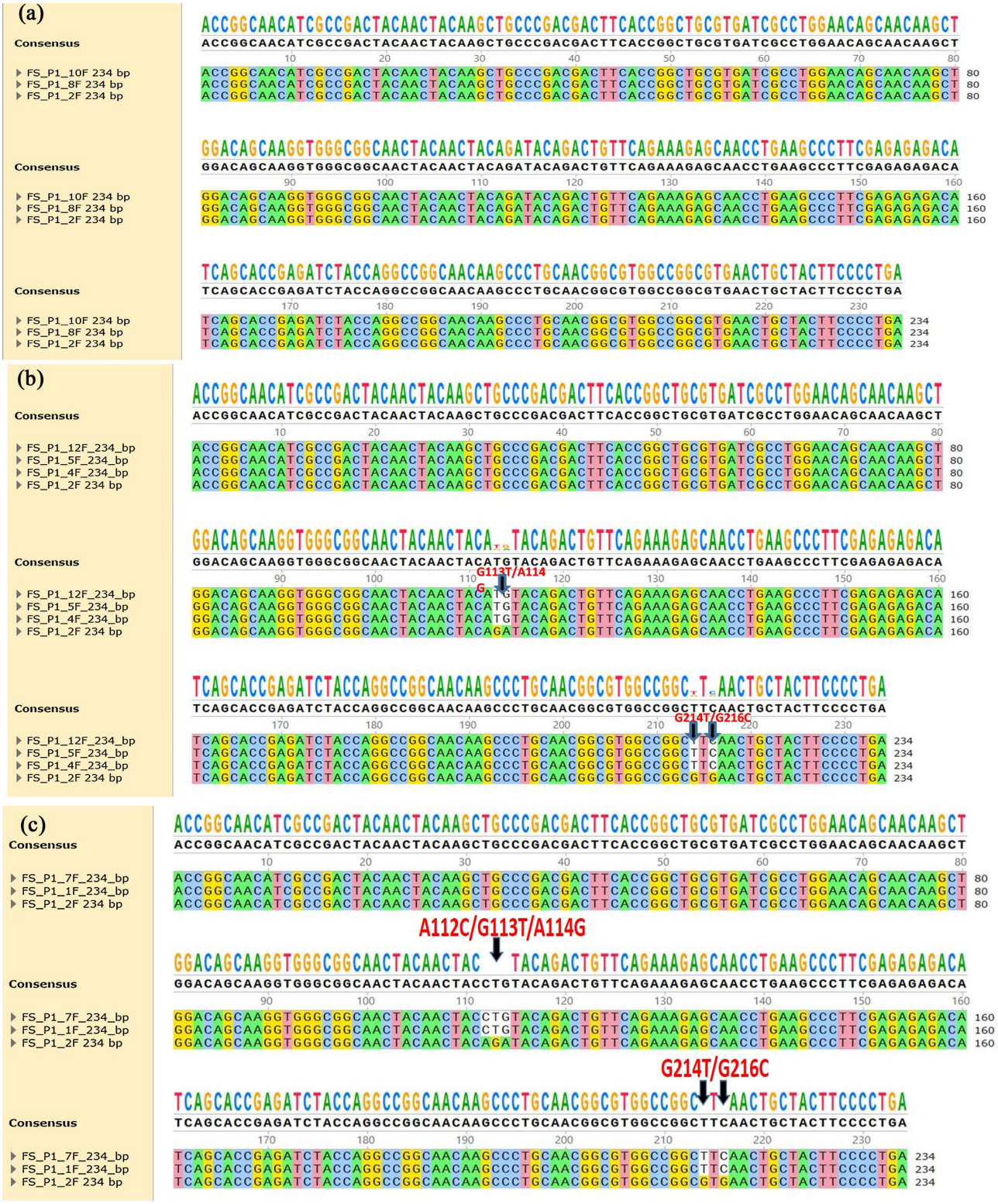


Figure 4. Sequence alignment with the reference control (plasmid S2). (a) Nucleotide sequences of plasmids S2, S8, and S10, (b) Nucleotide sequences of plasmids S2, S4, S5, and S12, (c) Nucleotide sequences of plasmids S2, S1, and S7.

G113T, A114G, G214T, and G216C in these plasmids (Figure 4(b)). Comparison of plasmids S1 and S7 sequences with the reference revealed mutations A112C, G113T, A114G, G214T, and G216C (Figure 4(c)).

In total, we identified 3 different variants among the samples. These variants were confirmed by analyzing the corresponding protein sequences: one variant matched our reference sequence (plasmids S2, S8, and S10), another variant had the R452L and V486F mutations (plasmids S1 and S7), and the third variant contained the R452M and V486F mutations (plasmids S4, S5, and S12) (Figure 5).

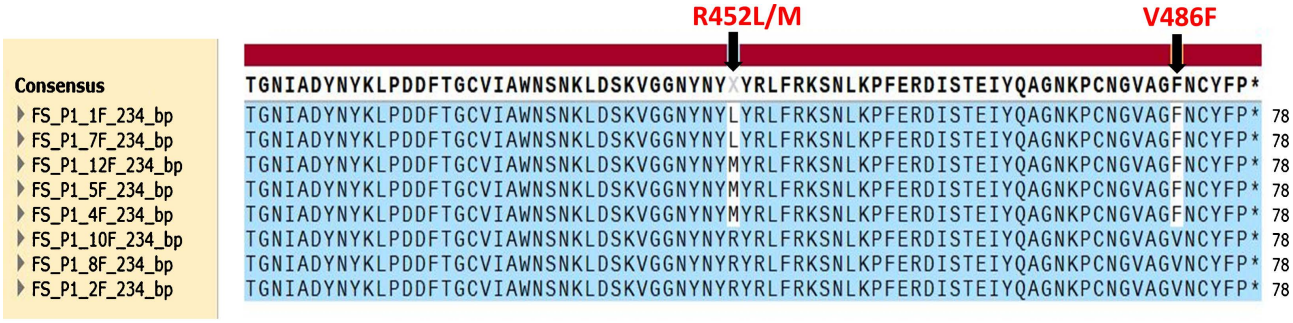


Figure 5. Alignment of plasmid protein sequences with the reference control.

3.5. HRM Profile of Plasmids and Samples

For the qPCR-HRM analysis of recombinants, we utilized the S2 plasmid as a reference, similar to the approach taken for Sanger sequencing analysis. Examination of the "Derivative melt curve" (Figure 6(a)) indicates that the primers employed are highly specific to the targeted region across all plasmids, displaying a single melt peak. The "Aligned melt curve" (Figure 6(b)) reveals three distinct populations, and a more in-depth analysis using the differential curve (Figure 6(c)) validates these findings. The analysis of the data reveals that the initial population, exhibiting a melt curve closely resembling that of the reference, consists of plasmids S8 and S10. The second population, displaying a +10 variance in relative fluorescence (RFU), encompasses plasmids S4, S5, and S12. Lastly, the third population, with a −7.5 RFU difference, includes plasmids S1 and S7. Analysis of nasopharyngeal samples by PCR-HRM shows a profile common to all samples. This profile differs from that of the referent controls (plasmids S1, S2 and S4) used. The differential curve (Figure 7) shows a clear distinction between the referent control in red and the samples in green. These outcomes suggest that recombinant plasmids have the potential to serve as substitutes for genomic DNA as positive controls in identifying mutations within two viral or bacterial populations.

4. Discussion

Côte d'Ivoire has been affected by the COVID-19 pandemic, as has almost the entire world. The Institut Pasteur de Côte d'Ivoire is the reference center for

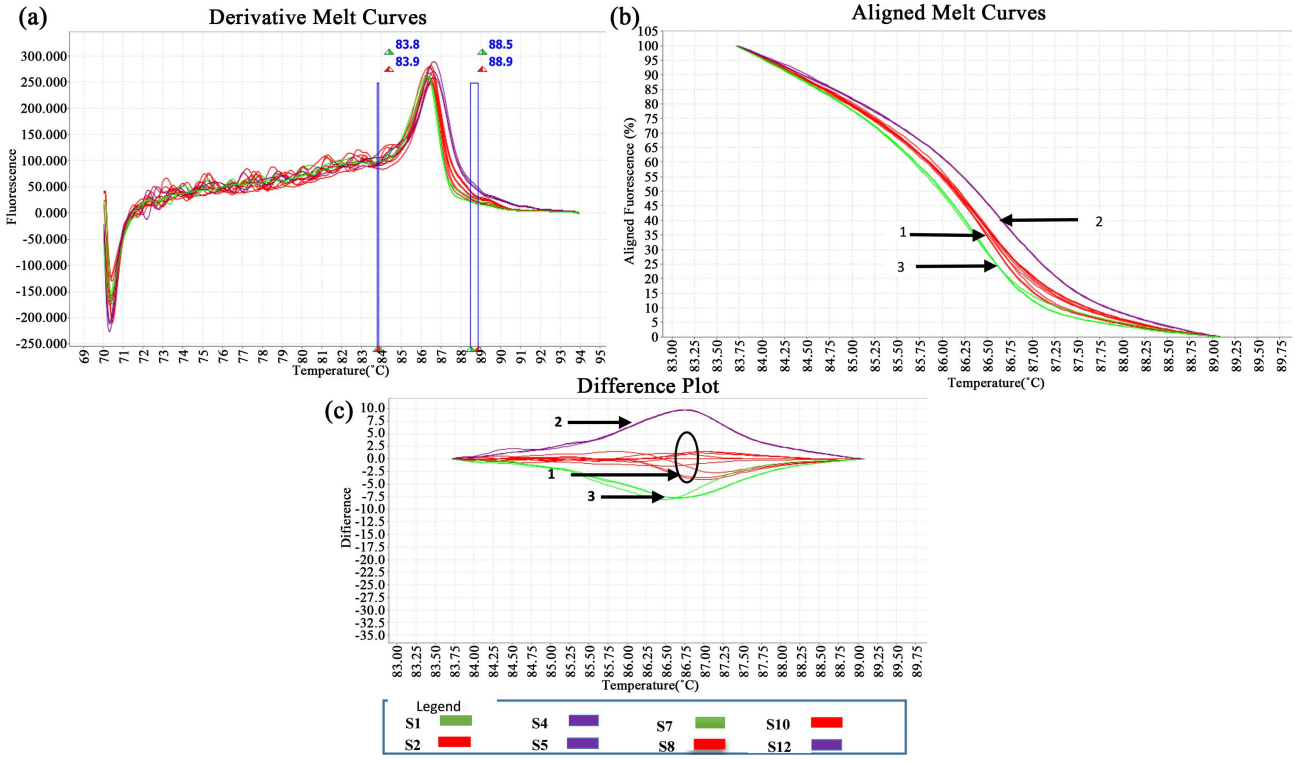


Figure 6. HRM Profile Presentation Post qPCR Analysis. (a) depicts the melting curve based on the melting temperature of various variants, while (b) illustrates the normalization of the melting curve. (1) The melting curve of plasmid S2 serves as a reference, with plasmids S10 and S8 highlighted in red representing variant 1. (2) Plasmids S4, S5, and S12, shown in purple, correspond to variant 2 in the melting curve. (3) Plasmids S1 and S7, depicted in green, signify variant 3. (c) shows the differentiation in melting temperatures.

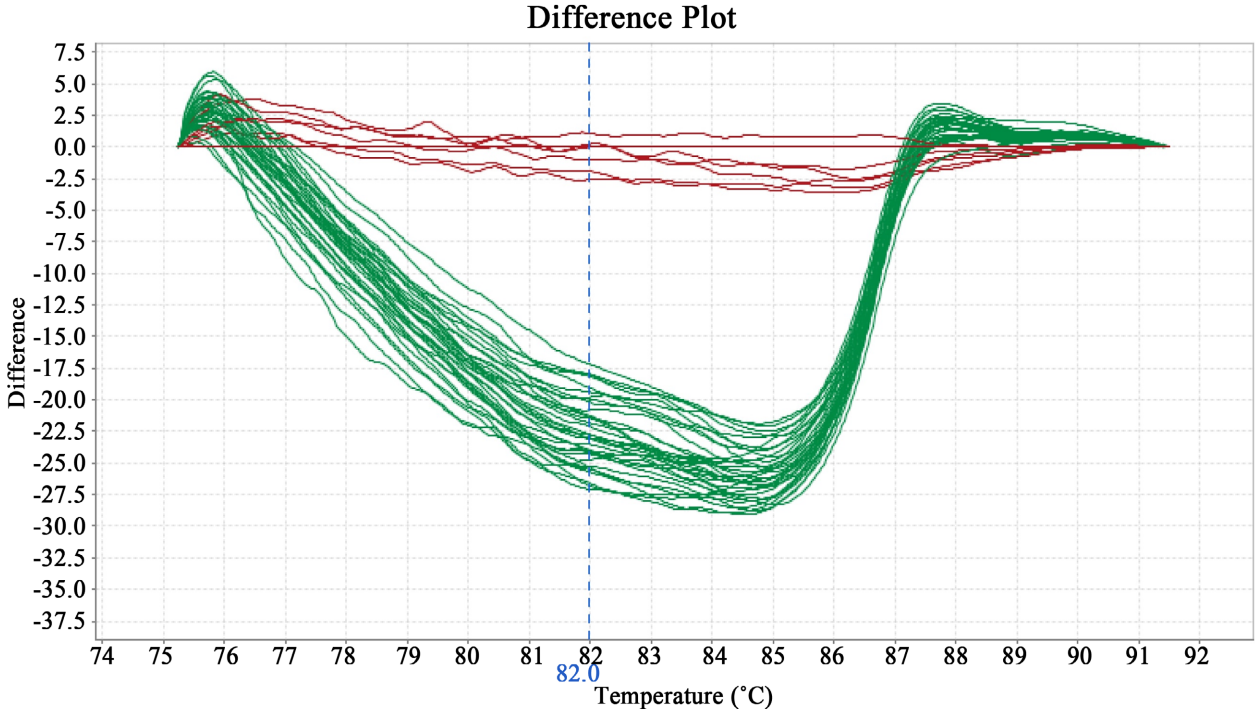


Figure 7. Analysis of post-qPCR HRM profiles of nasopharyngeal samples.

COVID-19 diagnosis. To reduce the cost of NGS sequencing, we have developed recombinant plasmids based on a P1 portion of the SARS-CoV-2 Spike protein. These plasmids are used as positive controls in the qPCR-HRM method for variant detection. Our aim is to work with SARS-CoV-2 positive samples from COVID-19 diagnostic tests to identify possible mutations for whole genome sequencing. The qPCR analysis revealed excellent linearity, sensitivity and specificity. This indicates that the primers designed to detect the P1 portion of the fragment are highly sensitive with adequate specificity. The P1 portion S plasmids enabled detection with a limit of detection (LOD) of 5.30 copies/µL and a limit of quantification (LOQ) of 16.93 copies/µL. The results of our study differ from those of [50], who obtained a detection limit of 7 copies/µL for the E484K and L452R mutations, and 14 copies/µL for the E484A mutation, with a sensitivity and specificity of 100%. In contrast, our results are almost similar to those of [51]. In our study, the detection limit was 5.67 Copies/µL for the N501Y mutation and 5.30 Copies/µL for the wild-type N501. This difference in detection limits can be attributed to the nature of the samples, the experimental conditions, the calculation method used and the type of qPCR instrument employed for detection. In addition, clinical performance was assessed using 25 nasopharyngeal samples of known COVID-19 status. Cross-reactivity with other pathogens and reproducibility were not determined.

The results obtained by HRM (High-Resolution Melting) for detecting of SARS-CoV-2 virus variants were confirmed and validated by sequencing, making it a more accurate and reliable method. We can therefore rely on the results obtained by HRM to identify the different variants of the SARS-CoV-2. Firstly, the results showed that the primers used are specific to the targeted region and could be used to diagnose SARS-CoV-2. In addition, the method has the advantage of simultaneously tracking the pathogen's genomic evolution. Its primers were used to validate the HRM approach as a tool for genomic surveillance. Analysis of the PCR-HRM profiles of the nasopharyngeal samples showed a different profile to the reference controls used. This is due to the sample selection period, which is the year 2020. This period in Côte d'Ivoire was dominated by the first wave of the COVID-19 pandemic, and the circulating virus variant was lineage (A.19; A.18) [52]. The A.19 lineage is also known as the Alpha variant, which was first identified in the U.K. [53] and the A.18 lineage is known as the Beta variant, which was first identified in South Africa [54]. In contrast, the plasmid controls used in the PCR-HRM method came from strains from the 2022 period, which is dominated by the Omicron variant.

For this study, we focused on the P1 portion of the Spike S protein, which is known to contain mutations characteristic of the Omicron variant. These mutations are: K417N/T; N440K; G446S; N450K; L452R/Q; S477N; T478K; E484K/Q; V483A and F490S compared with the Wuhan reference strain of SARS-CoV-2 [55].

Sequence analysis of the plasmids revealed variants containing the L452R,

L452M, and F486V mutations derived from the nucleotide mutations observed above. These mutations were found in the Omicron variant, specifically in the Omicron sub-variants BA.2, BA.2.3 and BA.4. These results are supported by the work of [56], which states that the Omicron variants that appeared in 2022 contain mutations in the receptor-binding domain (RBD), notably L452M in BA.2.13 and L452R/F486V in BA.4. Several recent studies have investigated the potential effects of L452R and other mutations. It has been shown that L452R gives the virus a cytotoxic T lymphocyte-mediated evasion of human leukocyte antigen (HLA)-restricted cellular immunity [57] [58]. Finally, it was predicted that mutations in residue L452, located near the RBD-ACE2 interaction interface, would result in at least slightly higher receptor-binding affinity and thus an increased rate of human cell infectivity[58] [59]. In addition, numerous important changes in the Spike RBD, such as D405N, R408S, K417N, N440K, L452R, S477N, T478K, E484A, F486V, Q498R, N501Y and Y505H were observed, as well as the detection of other frequent Omicron mutations, linked to increased transmission, contagiousness and virulence of Omicron variants [60].

One of the limitations observed is that if we use the Wuhan strain as a reference in the HRM analysis, the sub-populations will be grouped, making discrimination between them more difficult. It is therefore advisable to use one of the mutated sequences in addition to the non-mutated sequence, to refine the analysis and discriminate between the different variants. The HRM profiles of these plasmids made it possible to discriminate 3 population groups according to wild type strain. For HRM analysis, the use of the Wuhan strain as a referent gave a considerable discrepancy with recombinant plasmids from different Omicron sub-variants. The Wuhan reference strain contains no mutations and discrimination between recombinant plasmids becomes very difficult. This led us to use the S2 plasmid as a reference strain. The 3 distinctive groups in the HRM analysis revealed melting temperatures Tm of 86.3˚C, 86.5˚C and 86.6˚C, with a mean deviation of 0.2˚C. The limited number of samples and diversity are weaknesses noted during this study, there is a need to work with more samples but also to diversify the collection period according to the appearance of epidemic waves. Other limitations to the use of HRM have been illustrated by [61], namely 1) the need for skilled technicians to analyze melting curve profiles to distinguish variants, and 2) the challenge of differentiating closely related variants with high mutation rates. According to [62], typing tests can give unexpected results for emerging variants due to new mutations in primer binding sites, which can reduce amplification efficiency, or between primers, leading to poorer detection of variants. With the emergence of new variants, new tests need to be developed and improved before being integrated into the diagnostic algorithm.

The work of [63] highlighted the function of these mutations at residues L452 and F486 in Omicron BA.2 and BA.4, which are involved in the escape of certain class 2 and 3 antibodies [64], and class 1 and 2 antibodies respectively. L452R/M mutations are also found in Delta [65] and Lambda [66] variants.

5. Conclusions

Recombinant plasmids can be used by the PCR-HRM method as positive standards for the discrimination of SARS-CoV-2 variants. Its plasmids have great stability and the PCR-HRM method is inexpensive. Additionally, since HRM analysis is reflected in single-nucleotide polymorphisms, an unexpected mutation in the target regions would affect the melting temperature, whereby single-nucleotide polymorphisms can merge into the target regions.

It is necessary to use both wild type and mutants as positive controls in order to identify SARS-CoV-2 variants using HRM analysis. HRM analysis can be applied for high-throughput screening of new, unknown mutations although Sanger sequencing analysis remains necessary to identify the nature of mutations. Our results suggest that the current technique based on HRM analysis is a powerful high-throughput tool for determining SARS-CoV-2 variants. This could be used for other pathogens which are the subject of international surveillance due to their epidemic risk.

In this study, HRM profile analysis can be easily adapted to accurately detect mutations based on the requirements of each region or country and the pathogen under surveillance. Genetic mutations frequently linked to variants can be used to create libraries of recombinant plasmids that can be maintained for a long time. The *E. coli* strain may be cultured in low-cost media, making it simple to maintain these libraries.

Acknowledgements

We thank the Fund for Science, Technology and Innovation (FONSTI) and the Strategic Support Program for Scientific Research (PASRES) of Côte d'Ivoire for financial support of this study for COVID-Grant 2020.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Hoffman, S.A. and Maldonado, Y.A. (2024) Emerging and Re-Emerging Pediatric Viral Diseases: A Continuing Global Challenge. *Pediatric Research*, **95**, 480-487. https://doi.org/10.1038/s41390-023-02878-7

[2] Williams, R., Kemp, V., Porter, K., Healing, T. and Drury, J. (2024) Major Incidents, Pandemics and Mental Health: The Psychosocial Aspects of Health Emergencies, Incidents, Disasters and Disease Outbreaks. Cambridge University Press. https://doi.org/10.1017/9781009019330

[3] Dabbu Kumar, J., Jian, L., Rong, H. and Hua, Z. (2018) Emerging and Reemerging Human Viral Diseases. *Annals of Microbiology and Research*, **2**, 31-44. https://doi.org/10.36959/958/567

[4] Gedif Meseret, A. (2020) Emerging and Re-Emerging Viral Diseases: The Case of Coronavirus Disease-19 (COVID-19). *International Journal of Virology and AIDS*,

7, 67-80. https://doi.org/10.23937/2469-567X/1510067

[5] Van Duin, D. and Paterson, D.L. (2020) Multidrug Resistant Bacteria in the Community: An Update. *Infectious Disease Clinics of North America*, **34**, 709-722. https://doi.org/10.1016/j.idc.2020.08.002

[6] Marques, R.Z., Da Silva Nogueira, K., de Oliveira Tomaz, A.P., Juneau, P., Wang, S. and Gomes, M.P. (2024) Emerging Threat: Antimicrobial Resistance Proliferation during Epidemics—A Case Study of the SARS-CoV-2 Pandemic in South Brazil. *Journal of Hazardous Materials*, **470**, Article 134202. https://doi.org/10.1016/j.jhazmat.2024.134202

[7] Gagnaire, J., Verhoeven, P., Denis, C., Grattard, F., Carricajo, A., Pozzetto, B. and Berthelot, P. (2015) Prise En Charge Des Bactéries Multirésistantes Aux Antibiotiques Dans Les Établissements De Santé. *Feuillets De Biologie*, **322**, 13-20.

[8] Maamar, B., Abdelmalek, R., Messadi, A.A. and Thabet, L. (2019) Étude Épidémio-Clinique Des Infections à Entérobactéries Productrices De CarbapénéMases Chez Les Brûlés. *Annals of Burns and Fire Disasters*, **32**, 10-16. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6588333/

[9] Choi, Y.K. (2021) Emerging and Re-Emerging Fatal Viral Diseases. *Experimental & Molecular Medicine*, **53**, 711-712. https://doi.org/10.1038/s12276-021-00608-9

[10] Nordmann, P. and Poirel, L. (2014) Résistances aux antibiotiques émergentes et importantes chez les bactéries Gram négatif: épidémiologie, aspects théoriques et détection. *Revue Médicale Suisse*, **10**, 902-907. https://doi.org/10.53738/REVMED.2014.10.427.0902

[11] Kieny, M.P., Rottingen, J.-A. and Farrar, J. (2016) The Need for Global R&D Coordination for Infectious Diseases with Epidemic Potential. *The Lancet*, **388**, 460-461. https://doi.org/10.1016/S0140-6736(16)31152-7

[12] Zumla, A., Dar, O., Kock, R., Muturi, M., Ntoumi, F., Kaleebu, P., Eusebio, M., Mfinanga, S., Bates, M., Mwaba, P., Ansumana, R., Khan, M., Alagaili, A.N., Cotten, M., Azhar, E.I., Maeurer, M., Ippolito, G. and Petersen, E. (2016) Taking Forward a 'One Health' Approach for Turning the Tide Against the Middle East Respiratory Syndrome Coronavirus and Other Zoonotic Pathogens with Epidemic Potential. *International Journal of Infectious Diseases*, **47**, 5-9. https://doi.org/10.1016/j.ijid.2016.06.012

[13] Mcentire, C.R.S., Song, K.-W., McInnis, R.P., Rhee, J.Y., Young, M., Williams, E., Wibecan, L.L., Nolan, N., Nagy, A.M., Gluckstein, J., Mukerji, S.S. and Mateen, F.J. (2021) Neurologic Manifestations of the World Health Organization's List of Pandemic and Epidemic Diseases. *Frontiers in Neurology*, **12**, 1-22. https://doi.org/10.3389/fneur.2021.634827

[14] Dortet, L., Bonnin, R. and Naas, T. (2017) Impact du Séquençage D'adn à Haut Débit sur la Surveillance des Épidémies de Bactéries Multi-Résistantes aux Antibiotiques. *Feuillets de Biologie*, **354**, 1-13.

[15] Beviere, M., Reissier, S., Penven, M., Dejoies, L., Guerin, F., Cattoir, V. and Piau, C. (2023) The Role of Next-Generation Sequencing (NGS) in the Management of Tuberculosis: Practical Review for Implementation in Routine. *Pathogens*, **12**, 978-1001. https://doi.org/10.3390/pathogens12080978

[16] Marchand, S., Rodriguez, C., and Woerther, P.-L. (2024) Séquençage à haut débit pour le diagnostic en maladies infectieuses: exemple de la métagénomique shotgun dans les infections du système nerveux central. *La Revue de Médecine Interne*, **45**, 166-173. https://doi.org/10.1016/j.revmed.2023.05.002

[17] Quick, J., Loman, N.J., Duraffour, S., *et al.* (2016) Real-Time, Portable Genome

Sequencing for Ebola Surveillance. *Nature*, **530**, 228-232. https://doi.org/10.1038/nature16996

[18] Faria, N.R., Azevedo, R.D.S.D.S., *et al.* (2016) Zika Virus in the Americas: Early Epidemiological and Genetic Findings. *Science*, **352**, 345-349. https://doi.org/10.1126/science.aaf5036

[19] Faria, N.R., Quick, J., Claro, I.M., *et al.* (2017) Establishment and Cryptic Transmission of Zika Virus in Brazil and the Americas. *Nature*, **546**, 406-410. https://doi.org/10.1038/nature22401

[20] Adelino, T.É.R., Giovanetti, M., *et al.* (2021) Field and Classroom Initiatives for Portable Sequence-Based Monitoring of Dengue Virus in Brazil. *Nature Communications*, **12**, 2296-2308. https://doi.org/10.1038/s41467-021-22607-0

[21] Grubaugh, N.D., Faria, N.R., Andersen, K.G. and Pybus, O.G. (2018) Genomic Insights into Zika Virus Emergence and Spread. *Cell*, **172**, 1160-1162. https://doi.org/10.1016/j.cell.2018.02.027

[22] Aoki, A., Adachi, H., Mori, Y., Ito, M., Sato, K., Okuda, K., Sakakibara, T., Okamoto, Y. and Jinno, H. (2022) Discrimination of SARS-CoV-2 Omicron Sublineages BA.1 and BA.2 Using a High-Resolution Melting-Based Assay: A Pilot Study. *Microbiology Spectrum*, **10**, e0136722.

[23] WHO COVID-19 dashboard. https://COVID19.Who.Int

[24] Wu, H.S., Zhang, Q., Wu, H.L., Tian, F., Cui, B., Qi, Z., Xu, X., Zhang, X. and Wang, H. (2020) Tackling COVID-19: Insights from the Qinghai Province Plague Prevention and Control (PPC) Model. *Biosafety and Health*, **2**, 187-192. https://doi.org/10.1016/j.bsheal.2020.08.001

[25] Ganguli, A., Mostafa, A., Berger, J., Aydin, M.Y., Sun, F., de Ramirez, S.A.S., Valera, E., Cunningham, B.T., King, W.P. and Bashir, R. (2020) Rapid Isothermal Amplification and Portable Detection System for SARS-CoV-2. *Proceedings of the National Academy of Sciences of the United States of America*, **117**, 22727-22735. https://doi.org/10.1073/pnas.2014739117

[26] Sun, L., Xiu, L., Zhang, C., Xiao, Y., Li, Y., Zhang, L., Ren, L. and Peng, J. (2022) Detection and Classification of SARS-CoV-2 Using High-Resolution Melting Analysis. *Microbial Biotechnology*, **15**, 1883-1894. https://doi.org/10.1111/1751-7915.14027

[27] Wang, R., Hozumi, Y., Yin, C. and Wei, G.-W. (2020) Mutations on COVID-19 Diagnostic Targets. *Genomics*, **112**, 5204-5213. https://doi.org/10.1016/j.ygeno.2020.09.028

[28] Xiao, M., Liu, X., *et al.* (2020) Multiple Approaches for Massively Parallel Sequencing of SARS-CoV-2 Genomes Directly from Clinical Samples. *Genome Medicine*, **12**, 57-72. https://doi.org/10.1186/s13073-020-00751-4

[29] OMS (2021) Séquençage Génomique du SRAS-CoV-2: Un Guide de Mise en Œuvre Pour un Impact Maximal sur la Santé Publique.

[30] Wang, J., Hawken, S.E., *et al.* (2022) Collaboration between Clinical and Academic Laboratories for Sequencing SARS-CoV-2 Genomes. *Journal of Clinical Microbiology*, **60**, e01288-21. https://doi.org/10.1128/jcm.01288-21

[31] Helmy, M., Awad, M. and Mosa, K.A. (2016) Limited Resources of Genome Sequencing in Developing Countries: Challenges and Solutions. *Applied & Translational Genomics*, **9**, 15-19. https://doi.org/10.1016/j.atg.2016.03.003

[32] Diaz-Garcia, H., Guzmán-Ortiz, A.L., Angeles-Floriano, T., Parra-Ortega, I., López-Martínez, B., Martínez-Saucedo, M., Aquino-Jarquin, G., Sánchez-Urbina, R., Quezada, H. and Granados-Riveron, J.T. (2021) Genotyping of the Major SARS-CoV-2

Clade by Short-Amplicon High-Resolution Melting (SA-HRM) Analysis. *Genes*, **12**, 531-541. https://doi.org/10.3390/genes12040531

[33] Koshikawa, T. and Miyoshi, H. (2022) High-Resolution Melting Analysis to Discriminate Between the SARS-CoV-2 Omicron Variants BA.1 and BA.2. *Biochemistry and Biophysics Reports*, **31**, 101306-101311. https://doi.org/10.1016/j.bbrep.2022.101306

[34] Vossen, R.H.A.M., Aten, E., Roos, A. and Den Dunnen, J.T. (2009) High-Resolution Melting Analysis (HRMA): More than Just Sequence Variant Screening. *Human Mutation*, **30**, 860-866. https://doi.org/10.1002/humu.21019

[35] Tamburro, M. and Ripabelli, G. (2017) High Resolution Melting as a Rapid, Reliable, Accurate and Cost-Effective Emerging Tool for Genotyping Pathogenic Bacteria and Enhancing Molecular Epidemiological Surveillance: A Comprehensive Review of the Literature. *Annali di Igiene: Medicina Preventiva e di Comunità*, **29**, 293-316.

[36] Coulibaly, T.G.S., Gbonon, V.M., Osseni, A., Diplo, F.B., Coulibaly, D.N., Sylla, A., *et al.* (2022) First Detection of KRAS Mutation in Colorectal Cancer Patients in Côte d'Ivoire. *European Journal of Biomedical Research*, **1**, 16-20. https://doi.org/10.24018/ejbiomed.2022.1.5.30

[37] Aoki, A., Mori, Y., Okamoto, Y. and Jinno, H. (2021) Development of a Genotyping Platform for SARS-CoV-2 Variants Using High-Resolution Melting Analysis. *Journal of Infection and Chemotherapy*, **27**, 1336-1341. https://doi.org/10.1016/j.jiac.2021.06.007

[38] Sacks, D., Ledwaba, J., Morris, L. and Hunt, G.M. (2016) Rapid Detection of Common HIV-1 Drug Resistance Mutations by Use of High-Resolution Melting Analysis and Unlabeled Probes. *Journal of Clinical Microbiology*, **55**, 122-133. https://doi.org/10.1128/JCM.01291-16

[39] Kiani, S.J., Ramshini, M., Bokharaei-Salim, F., Donyavi, T., Eshrati, B., Khoshmirsafa, M., Ghorbani, S., Tavakoli, A., Monavari, S.H., Ghalejoogh, Z.Y. and Abbasi-Kolli, M. (2023) High Resolution Melting Curve Analysis for Rapid Detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Variants. *Acta Virologica*, **67**, 91-98. https://doi.org/10.4149/av_2023_109

[40] Silva, J.L.D., Leite, G.G.S., Bastos, G.M., *et al.* (2013) Plasmid-Based Controls to Detect rpoB Mutations in Mycobacterium Tuberculosis by Quantitative Polymerase Chain Reaction-High-Resolution Melting. *Memorias do Instituto Oswaldo Cruz*, **108**, 106-109. https://doi.org/10.1590/S0074-02762013000100017

[41] Yi, C., Sun, X., Ye, J., Ding, L., Liu, M., Yang, Z., Lu, X., Zhang, Y., Ma, L., Gu, W., Qu, A., Xu, J., Shi, Z., Ling, Z. and Sun, B. (2020) Key Residues of the Receptor Binding Motif in the Spike Protein of SARS-CoV-2 That Interact with ACE2 and Neutralizing Antibodies. *Cellular & Molecular Immunology*, **17**, 621-630. https://www.nature.com/articles/S41423-020-0458-Z

[42] Candido, K.L., Eich, C.R., *et al.* (2022) Spike Protein of SARS-CoV-2 Variants: A Brief Review and Practical Implications. *Brazilian Journal of Microbiology*, **53**, 1133-1157. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8994061/

[43] Mittal, A. and Verma, V. (2021) Connections between Biomechanics and Higher Infectivity: A Tale of the D614G Mutation in the SARS-CoV-2 Spike Protein. *Signal Transduction and Targeted Therapy*, **6**, Article No. 11. https://www.nature.com/articles/s41392-020-00439-6

[44] Sylla, A., Kakou-Ngazoa, S., Bla, B.K., Coulibaly, T.G.S., Ouattara, Z., Ouattara, Y.K., Addablah, A.A.Y., Kouamé-Sina, M.S., Kouakou, V.-L., Coulibaly, D.N. and Dosso, M. (2023) Amplification of SARS-CoV2 Viral Markers in Côte d'Ivoire.

European Journal of Biomedical Research, **2**, 8-13.
https://doi.org/10.24018/ejbiomed.2023.2.4.80

[45] Rutledge, R.G. and Stewart, D. (2008) Critical Evaluation of Methods Used to Determine Amplification Efficiency Refutes the Exponential Character of Real-Time PCR. *BMC Molecular Biology*, **9**, 96-107.
https://bmcmolbiol.biomedcentral.com/articles/10.1186/1471-2199-9-96

[46] Laamiri, N., Aouini, R., Marnissi, B., Ghram, A. and Hmila, I. (2018) A Multiplex Real-Time RT-PCR for Simultaneous Detection of Four Most Common Avian Respiratory. *Virology*, **515**, 29-37.
https://www.sciencedirect.com/science/article/pii/S0042682217304002

[47] Nutz, S., Döll, K. and Karlovsky, P. (2011) Determination of the LOQ in Real-Time PCR by Receiver Operating Characteristic Curve Analysis: Application to qPCR Assays for *Fusarium verticillioides* and *F. proliferatum*. *Analytical and Bioanalytical Chemistry*, **401**, 717-726. https://doi.org/10.1007/s00216-011-5089-x

[48] De Brun, M.L., Cosme, B., Petersen, M., Alvarez, I., Folgueras-Flatschart, A., Flatschart, R., Panei, C.J. and Puentes, R. (2022) Development of a Droplet Digital PCR Assay for Quantification of the Proviral Load of Bovine Leukemia Virus. *Journal of Veterinary Diagnostic Investigation*, **34**, 439-447.
https://doi.org/10.1177/10406387221085581

[49] Fertig, T.E., Chitoiu, L., Marta, D.S., Ionescu, V.-S., Cismasiu, V.B., Radu, E., Angheluta, G., Dobre, M., Serbanescu, A., Hinescu, M.E. and Gherghiceanu, M. (2022) Vaccine MRNA Can Be Detected in Blood at 15 Days Post-Vaccination. *Biomedicines*, **10**, Article 1538. https://www.mdpi.com/2227-9059/10/7/1538

[50] Gomez-Martinez, J., Henry, S., Tuaillon, E., Van de Perre, P., Fournier-Wirth, C., Foulongne, V. and Brès, J.-C. (2022) Novel Lateral Flow-Based Assay for Simple and Visual Detection of SARS-CoV-2 Mutations. *Frontiers in Cellular and Infection Microbiology*, **12**. https://doi.org/10.3389/fcimb.2022.902914

[51] Abdulnoor, M., Eshaghi, A., Perusini, S.J., Broukhanski, G., Corbeil, A., Cronin, K., Fittipaldi, N., Forbes, J.D., Guthrie, J.L., Kus, J.V., Li, Y., Majury, A., Mallo, G.V., Mazzulli, T., Melano, R.G., Olsha, R., Sullivan, A., Tran, V., Patel, S.N., Allen, V.G. and Gubbay, J.B. (2022) Real-Time RT-PCR Allelic Discrimination Assay for Detection of N501Y Mutation in the Spike Protein of SARS-CoV-2 Associated with B.1.1.7 Variant of Concern. *Microbiology Spectrum*, **10**, E00681-21.

[52] Anoh, E.A., Wayoro, O., Monemo, P., Belarbi, E., Sachse, A., Wilkinson, E., San, J. E., Leendertz, F.H., Diané, B., Calvignac-Spencer, S., Akoua-Koffi, C. and Schubert, G. (2023) Subregional Origins of Emerging SARS-CoV-2 Variants during the Second Pandemic Wave in Côte d'Ivoire. *Virus Genes*, **59**, 370-376.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10023306/

[53] Shi, S., Zhu, H., Xia, X., *et al.* (2019) Vaccine Adjuvants: Understanding the Structure and Mechanism of Adjuvanticity. *Vaccine*, **37**, 3167-3178.
https://www.sciencedirect.com/science/article/abs/pii/S0264410X19305298

[54] Mohammadi, M., Shayestehpour, M. and Mirzaei, H. (2021) The Impact of Spike Mutated Variants of SARS-CoV-2 Alpha, Beta, Gamma, Delta, and Lambda on the Efficacy of Subunit Recombinant Vaccines. *Brazilian Journal of Infectious Diseases*, **25**, 101606-101614.
https://www.scielo.br/j/bjid/a/dYxfThwdjJcqkbySTcx6xsq/?lang=en

[55] Aoki, A., Mori, Y., Okamoto, Y. and Jinno, H. (2022) Simultaneous Screening of SARS-CoV-2 Omicron and Delta Variants Using High-Resolution Melting Analysis. *Biological and Pharmaceutical Bulletin*, **45**, 394-396.
https://doi.org/10.1248/bpb.b21-01081

[56] Cao, Y., Yisimayi, A., Jian, F., *et al.* (2022) BA.2.12.1, BA.4 and BA.5 Escape Antibodies Elicited by Omicron Infection. *Nature*, **608**, 593-602. https://doi.org/10.1038/s41586-022-04980-y

[57] Motozono, C., Toyoda, M., Zahradnik, J., *et al.* (2021) SARS-CoV-2 Spike L452R Variant Evades Cellular Immunity and Increases Infectivity. *Cell Host & Microbe*, **29**, 1124-1136.E11. https://linkinghub.elsevier.com/retrieve/pii/S1931312821002845

[58] Tchesnokova, V., Kulasekara, H., *et al.* (2021) Acquisition of the L452R Mutation in the ACE2-Binding Interface of Spike Protein Triggers Recent Massive Expansion of SARS-CoV-2 Variants. *Journal of Clinical Microbiology*, **59**. https://journals.asm.org/doi/full/10.1128/jcm.00921-21

[59] Deshpande, A., Harris, B.D., Martinez-Sobrido, L., Kobie, J.J. and Walter, M.R. (2021) Epitope Classification and RBD Binding Properties of Neutralizing Antibodies Against SARS-CoV-2 Variants of Concern. *Frontiers in Immunology*, **12**, 1-14. https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2021.691715/full

[60] Almalki, S.S., Izhari, M.A., Alyahyawi, H.E., Alatawi, S.K., Klufah, F., Ahmed, W.A. and Alharbi, R. (2023) Mutational Analysis of Circulating Omicron SARS-CoV-2 Lineages in the Al-Baha Region of Saudi Arabia. *Journal of Multidisciplinary Healthcare*, **16**, 2117-2136. https://www.tandfonline.com/doi/full/10.2147/JMDH.S419859

[61] de Souza Andrade, A., Freitas, E.F., de Castro Barbosa, E., *et al.* (2023) Potential Use of High-Resolution Melting Analyses for SARS-CoV-2 Genomic Surveillance Utilisation Potentielle D'Analyses DE Fusion à Haute RÉSolution Pour La Surveillance GÉNomique Du SRAS-CoV-2. *Journal of Virological Methods*, **317**, 114742-114748. https://www.sciencedirect.com/science/article/pii/S0166093423000678

[62] Diotallevi, A., Buffi, G., *et al.* (2023) Rapid Monitoring of SARS-CoV-2 Variants of Concern through High-Resolution Melt Analysis. *Scientific Reports*, **13**, 21598-21607. https://www.nature.com/articles/s41598-023-48929-1

[63] Wang, Q., Guo, Y., Iketani, S., Nair, M.S., *et al.* (2022) Antibody Evasion by SARS-CoV-2 Omicron Subvariants BA.2.12.1, BA.4 and BA.5. *Nature*, **608**, 603-608. https://doi.org/10.1038/s41586-022-05053-w

[64] Barnes, C.O., Jette, C.A., *et al.* (2020) SARS-CoV-2 Neutralizing Antibody Structures Inform Therapeutic Strategies. *Nature*, **588**, 682-687. https://doi.org/10.1038/s41586-020-2852-1

[65] Planas, D., Veyer, D., *et al.* (2021) Reduced Sensitivity of SARS-CoV-2 Variant Delta to Antibody Neutralization. *Nature*, **596**, 276-280. https://doi.org/10.1038/s41586-021-03777-9

[66] Kimura, I., Kosugi, Y., *et al.* (2022) The SARS-CoV-2 Lambda Variant Exhibits Enhanced Infectivity and Immune Resistance. *Cell Reports*, **38**, 110218-110225. https://doi.org/10.1016/j.celrep.2021.110218