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Rapid Report

First identification in Italy of SARS-CoV-2 Omicron BA.4 harboring KSF141_del: genomic comparison with omicron sub-variants

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Abstract: The rapid emergence and worldwide detection of the SARS-CoV-2 omicron variant underscore the importance of robust genomic surveillance systems and prompt information sharing among global public health partners. The Omicron variant has rapidly replaced the delta variant as a dominating SARS-CoV-2 variant because of natural selection, favoring the variant with higher infectivity and more strong vaccine breakthrough ability. Also known as B.1.1.529, Omicron has four sub-variants, BA.1, BA.2, BA.3, and BA.4. Among them, BA.1 is the currently prevailing sub-variant, BA.2 is found to be able to alarmingly re-infect patients initially infected by omicron BA.1. BA.3 sub-variants is a combination of mutations of BA.1 and BA.2, especially in the spike protein. Today emerging is the BA.4, herein described and first detected in Italy, harboring a new mutation, specifically a deletion in the ORF 1 ab gene, corresponding to KSF141_del in non-structural protein 1 (nsp1), a critical virulence factor able to suppress host translation. The bioinformatics comparison analysis with the other three sub-variants pointed out that the deletion was not present previously and was never reported until now. Therefore, we can speculate that omicron BA.4; will become a new dominating "variant of concern" and might also break vaccine protection . On the other hand, we show that other proteins are mutated in the BA.4 in particular, seven mutations are recognized in the nucleocapsid (N) protein, the capability of five different types of rapid antigenic tests to recognize it.

Keywords: SARS-CoV-2; Variant of Concern; BA.4; pandemic; KSF141_del; nsp1.

1. Introduction

A new variant of SARS-CoV-2, B.1.1.529 (Omicron) (1-2), was first reported to the World Health Organization (WHO) by South Africa on November 24, 2021, and designated as a variant of concern (3). We reported the first case in our region (Calabria, Italy) on December 5, 2021 (4). The variant carries an unusually high number of mutations, of which 32 are located within the spike (S) protein. The S protein in the key viral component determines the virus's infectivity and antigenicity. Furthermore, 15 of 32

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mutations are located at the receptor-binding region (RBD) of Spike protein that interacts with human cells before cell entry, possibly enhancing the transmissibility (5). Omicron (B.1.1.529) and four sub-lineages such as BA.1 (B.1.1.529.1), BA.2 (B.1.1.529.2), BA.3 (B.1.1.529.3), and BA. 4 (B.1.1.529.4) of Omicron are closely related variants with a common ancestor (6-8). The data reported in the literature confirm Omicron's high infectivity (9-10), high vaccine breakthrough rate (11-12), and severe antibody escape rate (13). Though all the three lineages have spread worldwide, the rate of spread of these three lineages is different. Of the Omicron sequences submitted to GISAID, the BA.1 lineage is approximate>98%, the BA.2 to approximately 1% sequence, and the BA.3 around 0.1% sequence (www.gisaid.org). Of these three lineages, only BA.1 dominates much more than the other lineages that have ousted Delta. This is likely due to differences in mutations in the spike protein required for virus transmission and host cell entry (14,15). Liu et al. report that of these four lineages, one appears to be the parental lineage of the Omicron (B.1.1.529) variant, and then the BA.1 lineage seems to be the closest to this lineage. Instead, BA.2 has significant diversity from the B.1.1.529 and BA.1 lineage, while BA.3 has the intermediate lineage to BA.1 and BA.2 (16). Today, any published data are reported in the literature about BA.4 lineage. In Italy, we first identified by sequencing this lineage on April 25 by depositing it in ICOGEN Platform by Istituto Superiore di Sanità (ISS). The omicron lineage BA.4 found in Calabria Region is studied compared to the other four lineages, together with the capability of five different types of Rapid Antigenic Tests (RATs) to recognize it.

2. Materials and Methods

Sample collection and viral RNA extraction

A positive nasopharyngeal and oropharyngeal swab was collected in UTM™ and extracted for viral nucleic acid purification. The Real-Time (RT) PCR test was carried out with the TaqPath COVID-19 CE-IVD RT-PCR kit, which targets the following genes of SARS-CoV-2: i) open reading frame (ORF)1ab; ii) nucleocapsid (N) and iii) spike (S), coupled with QuantStudio 5 DX Thermo-Fisher Real-Time PCR (RT-PCR) as described in our previous study and according to manufacture instruction (17). Briefly, a total of 180 µl of the sample was used for RNA extraction by automated instrument (MGISP-100, MGI) using the MGIEasy Nucleic Acid Extraction Kit with superparamagnetic beads technology (MGI). Before RNA extraction, 10 µL of Proteinase K was added to each well in the King-Fisher™ Deep well 96 Plate. In addition, 10 µL of the MS2 Phage Control was added to all specimens together with 10 µL of magnetic beads. RNA extracted by the specimen underwent genomic characterization following two methodologies: the Sanger-based sequencing by the SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and whole-genome based on next-generation sequencing (NGS) by MiSeq System (Illumina, San Diego, CA, USA). The latter was performed by the Regional Center at the Microbiology and Virology Laboratory of Catanzaro. This workflow is followed in the routine practice by accredited laboratories in the clinical setting.

Library Preparation and Next-Generation Sequencing

RNA extracted from the specimen was used for the next library preparation step for next-generation sequencing (NGS) on the Illumina sequencing platform (MiSeq System, Illumina USA). CleanPlex SARS-CoV-2 FLEX Paragon Genomics Panel performed a Reverse transcription of the whole-genome and library preparation. The thermal-cycling or incubation reaction is followed by a library purification using magnetic beads (CleanMag Magnetic Beads). The workflow involves just three steps: i) the first step is cDNA synthesis and purification from purified RNA sample; ii) the second step is a multiplex PCR reaction that uses target-specific primers to amply targets of interest, thus covering the entire SARS-CoV-2 genome with the 2-pool design, at this stage, internal

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human (host) housekeeping RNA control primer pair was also added; iii) the third step is a digestion reaction to amply and add-sample level indexes to the generated libraries. These libraries were quantified using Qubit dsDNA HS Assay Kit (Invitrogen by Thermo Fisher Scientific). The quality of the library was checked using the DNA high sensitivity assay kit on Bio-analyser 2100 (Agilent Technologies, United States) and sequenced by the MiSeq platform providing 2x250 bp reads length data. The SOPHIA DDM Platform analyzed FASTQ reads. Clade analyses were obtained by ICOGEN Platform (ISS) and the GISAID database.

Alignment of FASTQ obtained by NGS data were performed SnapGene® software (from Insightful Science; available at snapgene.com). SNAP gene finder has been developed so far to be easily adaptable to a variety of genomes (18).

Rapid SARS-CoV-2 antigen detection assays

Rapid and accurate tests for SARS-CoV-2 screening are essential to expedite disease prevention and control. Five, Rapid Antigenic Tests (RATs) based on lateral flow immunoassay was carried out according to the manufacturer's instructions (read at 15 minutes, except for the one herein listed at #2 that was read at 10 minutes), including: 1-GeneFinder COVID-19 Ag Plus Rapid Test manufactured by OSANG Healthcare Co., Ltd, South Korea; 2-Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigen Detection Kit manufactured by Nanjing Vazyme Medical Technology Co., Ltd; 3-SARS-CoV-2 Antigenic Rapid Test Flowflex manufactured by ACON Biotech (Hangzhou) Co., Ltd, China; 4-SARS-CoV-2 Antigen manufactured by Lifotronic Technology Co, Ltd, China; 5-InstaView COVID-19 Antigen manufactured by SG Medical, Inc., South Korea.

3. Results and Discussion

The uncertainty of the following SARS-CoV-2 variant will be and remains a significant cause of concern for the World Health Organization (WHO). The viral genome consists of several genes encoding non-structural, structural, and accessory proteins. In the viral genome, genes for four structural proteins, the spike surface glycoprotein (S), the envelope (E), the membrane (M), and nucleocapsid (N), and several accessory proteins. For diagnostic and screening monitoring, E, N, S, and Open Reading Frame (ORF) 1 ab genes are the targets most frequently used by the RT-PCR method. ORFs are conserved in all coronavirus genomes and code for structural proteins that create the viral envelope. Omicron is currently dominant worldwide, and currently, sub-variants are tracked as well as their sister- BA.2.12.1, BA.5 and BA.4. This latter was sequenced by Sanger sequencing as the first level of screening based on S-gene that was performed using a standard protocol with 12 commercial primer pairings described by Paden et al. as first-level genetic screening and full-genome sequencing of SARS-CoV-2 (19). Data analysis obtained by SeqScape software revealed the omicron variant. The NGS approach provided 2x250 bp read length data. The SOPHIA DDM Platform analyzed FASTQ reads. Then, sub-variants information was described using the Pangolin nomenclatures (20), and the omicron variant sequences were deposited in the ICOGEN Platform on the April, 25 2022 and GISAID on the April, 26 2022. Clade analysis revealed 2 deletions and 28 mutations on the S gene corresponding to the following amino acids on the translated protein: T19I; LPPA24S_del; IHV68I_del; G142D; V213G; S371F; S373P; S375F; T376A; D405N; R408S; K417N; N440K; L452R; T478K; E484A; F486V; Q498R; N501Y; Y505H; D614G; S640F; H655Y; N679K; P681H; N764K; D796Y; Q954H; N969K; D1146 of which some in common to other omicron, interestingly missense mutation L452R is present only in this sub-variant, as shown in Table 1.

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Table 1. Amino acidic mutations on omicron sub-variants obtained by complete genome sequence. The missense mutations in green and the new deletion in red.

	Mutations									
	ORF1ab	**S**	**ORF3a**	**E**	**M**	**ORF6**	**ORF7a**	**ORF7b**	**ORF8**	**N**	**ORF10**
BA. 1.1.529	A555; K38R; F106; SL1265I_del; T492I; P132H; LSGF105F_del; I189V; V57; P323L; N600;	A67V; IHV68I_del; T95I; GVYY142D_del; NL211I_del; D215EPED; R346K; S371L; S373P; S375F; K417N; N440K; G446S; F456; T547K; D614G; H655Y; N679K; P681H; N764K; N856K; Q954H; N969K; D1146	T64		D3G; Q19E; A63T;			L18		P13L; GERS30G_del; R203K; G204R;	
BA.1	E563D; K38R; F106; SL1265I_del; T492I; P132H; LSGF105F_del; I189V; V57; P323L; N600	A67V; IHV68I_del; T95I; GVYY142D_del; NL211I_del; D215EPED; S371L; S373P; S375F; K417N; N440K; G446S; T547	T64	T9I	D3G; Q19E; A63T	R20		L18		P13L; GERS30G_del;R203K;G 204R	
BA.2	S135R; T24I; F106; G489S; A534; A1526V; L264F; V290; T327I; T492I; D48; R131; P132H; SGF106_del; F251L; R252T; Y253S; I65; S11; P323L; L758; I258; R392C; I42V; T112I; E145	T19I; LPPA24S_del; G142D; V213G; D405N; R408S; K417N; N440K; S477N; T478K; E484A; Q493R; Q498R; N501Y; Y505H; T547K; D614G; H655Y; N679K; P681H; N764K; D796Y; Q954H; N969K; D1146	T64; T223I	T9I	Q19E; A63T; F112	R20; D61L		L18		P13L; GERS30G_del; R203K; G204R	
BA.3	R27C; K38R; F106; L264F; V290; T327I; R131; P132H; SGF106_del; P323L; L758; R392C; I42V; E145	T19I; LPPA24S_del; G142D; S371F; S373P; S375F; T376A; N440K; F456; D614G; H655Y; N679K; P681H; D796Y; Q954H	T64	T9I	F112	D61L		L18		P13L	
BA.4	**S135R; KSF141_del**; T24I; F106; G489S; A534; L264F; V290; T327I; L417; T492I; D48; R131; P132H; SGF106_del; E23; I65; S11; P323L; L758; R392C; I42V; T112I; E145	T19I; LPPA24S_del; IHV68I_del; G142D; V213G; S371F; S373P; S375F; T376A; D405N; R408S; K417N; N440K; **L452R**; T478K; E484A; F486V; Q498R; N501Y; Y505H; D614G; S640F; H655Y; N679K; P681H; N764K; D796Y; Q954H; N969K; D1146	T64; T223I	T9I	Q19E; A63T; F112	R20; D61L		**L11F**; L18		P13L; GERS30G_del; **P151S**; R203K; G204R; **S413R; S416L**	

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As expected, the NGS data pointed out other mutations in additional gene regions of the virus herein also reported for ORF1 ab protein: S135R; KSF141_del; T24I; F106; G489S; A534; L264F; V290; T327I; L417; T492I; D48; R131; P132H; SGF106_del; E23; I65; S11; P323L; L758; R392C; I42V; T112I; E145 as shown in Table 1. In addition, the report pointed out in ORF1 ab gene a mutation in S135R, already reported in Africa, less than 20 days ago (on 4th April 2022) see figure 1 (GISAID EPI ISL: EPI_ISL_12243764 for verification) and the deletion KSF141_del that instead result as new.

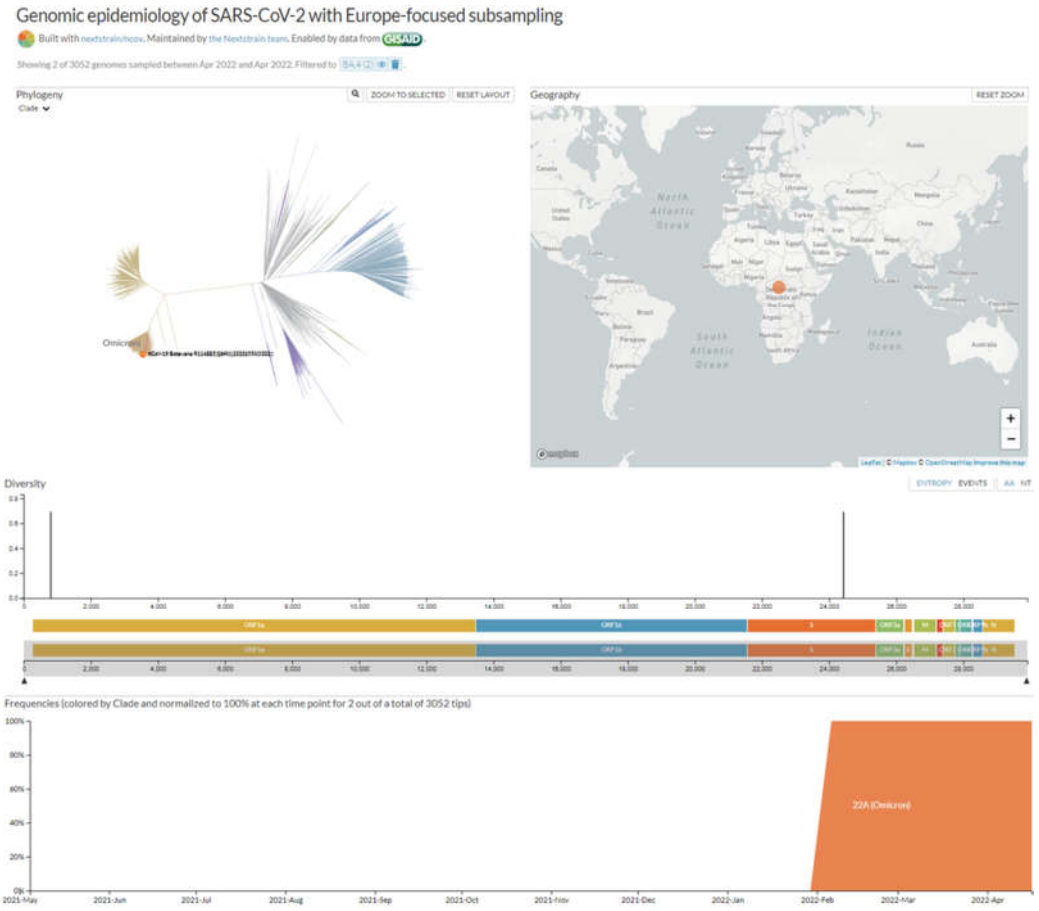

Figure 1. Next strain GSAID Built with next train/ncov Accessed April 25, 2022 11:46 pm

The ~30,000 nucleotide (nt) of virus genome is organized as follows (21), 5' to 3': replicase ORF1ab (~21,000 nt), S, ORF3a (ORF3a protein), E, M, ORF6 (ORF6 protein), ORF7a (ORF7a protein), ORF7b (ORF7b protein), ORF8 (ORF8 protein), N, and ORF10 (ORF10 protein) as it is shown in Figure 2 panel A. The importance and novel results come out form our NGS data, is the evidence of a deletion on ORF1 ab gene in KSF141_del. ORF1 ab is the biggest gene, in the virus genome thew deletion identified as KSF141_del, could be peculiar of this variant, classified as BA.4 and discovered in Italy. The KSF141_del involve the nsp1 which is a critical virulence factor suppressing host translation (21), redirecting the host translation machinery to increase the synthesis of viral proteins, similarly to SARS-CoV (22). The protein nsp1 is usually highly conserved viral protein for the fundamental biochemical function described above. Furthermore, it impairs the host immune response directly, inducing low levels of the antiviral interferons (INFs), types I and III syntheis and release from cell, animal models and patients (23,24), reflecting the effective viral counteraction of host innate immune responses. It is worth to note, that the mutant virus strain harboring an engineered nsp1 with mutations in a critical specific

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amino acidic region (aa 122-130 and aa 155-165) induced more robust antiviral responses and was substantially attenuated in the ability of replication compared to the wild type virus (25). The deletion KSF141_del of the protein, identified here, between these fundamental regions. The KSF141_del nucleic acid deletion was also verified by SNAPgene nucleotides alignment with the other three omicron variant in which nucleotides in this genetic region is missing, as shown in Figure 2 panel B. It has been highlighted in numerous studies as a target for both the development of antivirals and the design of live-attenuated vaccines (26).

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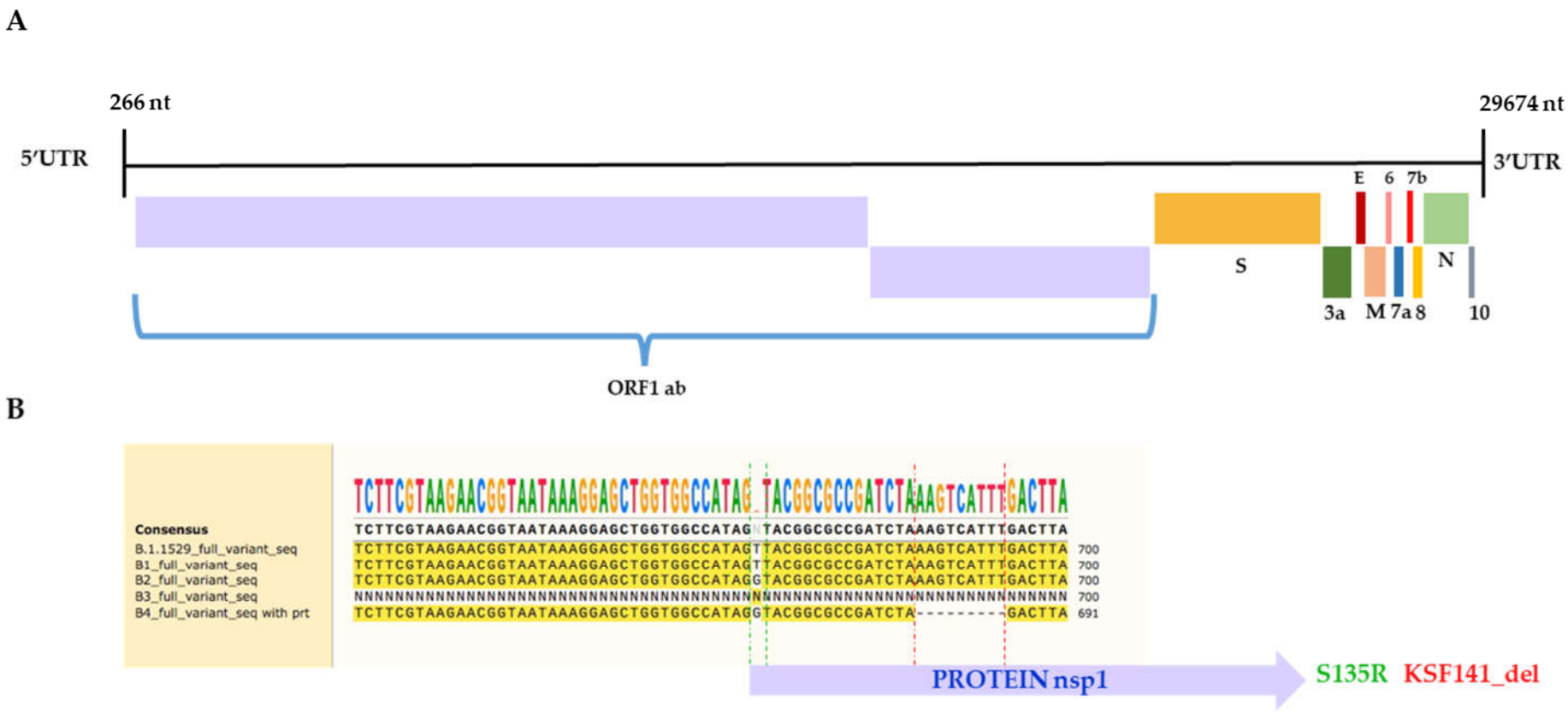

Figure 2. A) Schematic rapresentation of SARS-CoV-2 full genome. B) Pictogram of SNAPgene alignment among omicron lineages.

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In the multiple nucleic acids substitution corresponding, of course, to protein mutations, on the omicron BA.4 the presence of seven mutations in the N protein, attracted our attention. Compared to other lineage BA.1, BA.2 and BA.3 which instead harbors four mutations for BA. 1 and BA. 2 and one for BA. 3, this more abundance of mutations represents a red flag that could compromise the diagnostic utility of antigen detection rapid tests.

The diagnostic utility of antigen detection rapid diagnostic tests for Covid-19 was recently the object of studies in an elegant systematic review with the meta-analysis by Ghasemi et al. (27). The main goal of their study was to determine the accuracy of RATs showing that they can be used to identify the suspected patients even in the early stage of the disease without concluding if the accuracy of RATs can affect the spread of the COVID-19 virus or not (28). Moreover, the authors push for more research to determine the efficacy of RATs in detecting the various types of COVID-19 viruses, known as variant of concerns (VOCs). Therefore, given the emergence of novel SARS-CoV-2 variants of concern, the performance of available diagnostics for these new variants starts being investigated (29,30). RATs offer quick, cheap, and laboratory-independent results at the point of care. Although sensitivity is lower compared with RT-PCR, these tests enable consistent detection of high viral loads associated with the presence of infectious viral particles, making them essential public health tools. Therefore, a small aliquot of the collected specimen was processed through RATs device based on lateral flow immuno-chromatographic assay. We tested five RATs reporting the capability of all of them to recognizing BA.4 new variants harboring on the nucleocapsid (N) protein the following mutations: P13L; GERS30G_del; P151S; R203K; G204R; S413R; S416L. The capability of lateral flow immunoassay are present in figure 3 and are shown as following: 1-GeneFinder COVID-19 Ag Plus Rapid Test manufactured by OSANG Healthcare Co., Ltd, South Korea; 2-Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigen Detection Kit manufactured by Nanjing Vazyme Medical Technology Co., Ltd; 3-SARS-CoV-2 Antigenic Rapid Test Flowflex manufactured by ACON Biotech (Hangzhou) Co., Ltd, China; 4-SARS-CoV-2 Antigen manufactured by Lifotronic Technology Co, Ltd, China; 5-InstaView COVID-19 Antigen manufactured by SG Medical, Inc., South Korea.

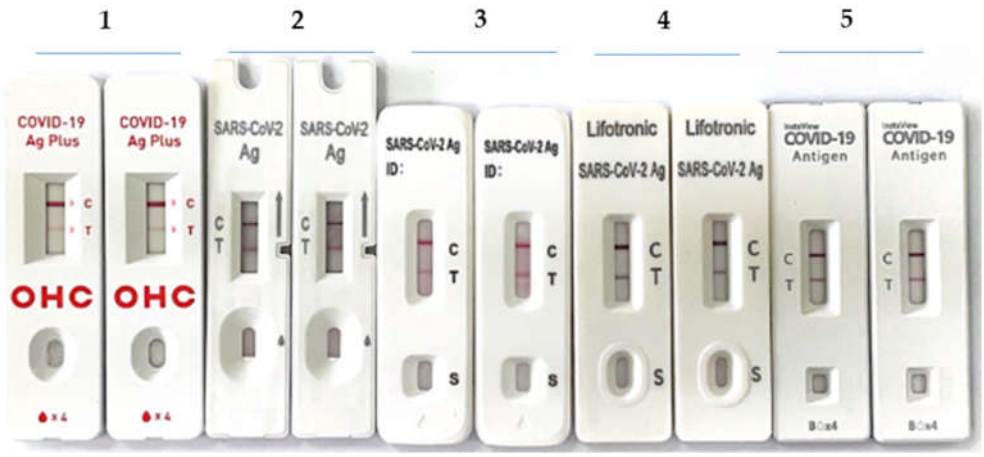

Figure 3. Capability of RATs to recognize omicron BA.4

The N protein from SARS-CoV-2 is recognized by capturing antigen-conjugate gold particle complexes. They migrate across a reaction area coated by antibodies to nucleocapsid proteins. Positive results display two-color related to control (C) and test (T) lines, while only one line in the C area is present for the negative one. The colored test (T) line's intensity depends on the amount of SARS-CoV-2 N antigen presented in the sample.

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The devices showed positive results, indicating how RATs with high sensitivity and specificity represent an excellent screening method, especially in high prevalence areas, also for the omicron BA.4 variants, as it is in this case with mild symptoms (31,32). Although, as with all variants, a lag exists between infection and more severe outcomes, and symptoms would be expected to be milder in vaccinated persons and those with previous SARS-CoV-2 infection than in unvaccinated persons (32,33). Although the vaccine produces a whole array of antibodies against RBD-S spike protein, many unknown mutations are associated with the Omicron variant; therefore, partial immune escape may be expected (34,35). Lastly, more studies are needed to better understand Omicron transmissibility, clinical presentation, immunity escape potential, disease severity, and the role of other available diagnostic and therapeutic countermeasures.

4. Conclusions

The clinical presentation of COVID-19 is non-specific. To improve and limit the spread of the SARS-CoV-2 virus, accurate diagnosis with a robust method is needed, even in light of reinfection due to the viral genome's continuous evolution that leads to proteins mutation. Herein, we show a new deletion in the ORF1 ab gene linked to nsp1 protein that was indicated as an exciting target for developing live attenuated vaccines with deletions in critical regions of nsp1. The BA 4. sub-variant identified here, harboring the KSF141_del, could be a light at the end of the dark. This viral strain should be studied in vitro and might be used to produce a live attenuated vaccine.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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Informed Consent Statement: The study was conducted using residual specimens. According to Italian health public law, this type of study did not require specific, informed consent and/or ethics committee approval.

Data Availability Statement: Data supporting reported results can be found at the Department of Microbiology and Virology, Pugliese Ciaccio's Hospital, Catanzaro, Italy and at Unit of Microbiology and Virology, North Health Center ASP 5, Reggio Calabria, Italy

Conflicts of Interest: The authors declare no conflict of interest.

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