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Next generation sequencing of multiple SARS-CoV-2 infections in the Omicron Era

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Since the emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the need for an effective vaccine has appeared crucial for stimulating immune system responses to produce humoral/cellular immunity and activate immunological memory. It has been demonstrated that SARS-CoV-2 variants escape neutralizing immunity elicited by previous infection and/or vaccination, leading to new infection waves and cases of reinfection. The study aims to gain into cases of reinfections, particularly infections and/or vaccination-induced protection. We conducted a retrospective descriptive study using data collected during the SARS-CoV-2 pandemic. This analysis involved Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) and Next Generation Sequencing (NGS). RT-qPCR was performed on 416,466 naso-oropharyngeal swabs, with 10,380 samples further analyzed using NGS technology. RT-qPCR identified 350 cases of reinfection, of which 228 were subjected to detailed analysis via NGS. Our findings revealed two interesting cases involving pediatric patients who were not vaccinated. Positive results were observed in these cases within a short interval (< 60 days) and the "nature" of the infection, whether attributable to Reinfection or Viral Persistence, was investigated. Specifically, we discuss a case involving an unvaccinated 18-month-old child, which may represent one of the earliest instances of BA.5/BA.5 reinfection identified worldwide.

Keywords SARS-CoV-2, NGS, Reinfection, Reactivation, Omicron, Vaccine

Abbreviations

CDC	Centers for Disease Control and Prevention
COVID-19	Coronavirus Disease 2019
Ct	Cycle threshold
DIBS	Defence Institute for Biomedical Sciences
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicines Agency
FDA	Food and Drug Administration
INMI	National institute for Infectious Disease
NGS	Next Generation Sequencing
ONT	Oxford Nanopore Technologies
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation

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SNPs	Single nucleotide polymorphisms
VOCs	Variants of concern
VOIs	Variants of interest
VUMs	Variants under monitoring
WHO	World Health Organization

The COVID-19 pandemic caused by SARS-CoV-2 began in China in December 2019. Since then, the virus has continuously mutated, causing the emergence of many variants around the world[1,2].

The World Health Organization (WHO) has assigned simple, easy-to-say and remember labels for key variants of SARS-CoV-2, using letters of the Greek alphabet to both variants of concern (VOCs) and variants of interest (VOIs). Similarly to VOIs, many VUMs, variants under monitoring, have been reported and checked[3].

Since September 2020, five VOCs have been identified worldwide: Alpha, Beta, Gamma, Delta and Omicron variants. The first four disappeared and were replaced by the Omicron VOC starting November 2021. In a few months, Omicron viruses, representing more than 98% of the sequences available since February 2022, acted as the genetic foundation for new SARS-CoV-2 variations[4]. It is also noteworthy that Omicron was the only variant that developed five different lineages (from BA.1 to BA.5) with very high genetic diversity[5]. New Omicron recombinant lineages, deriving from BA.2 were first identified in Southeast Asia and rapidly spread worldwide[3,4,6].

In March 2023, the WHO announced that Greek letters would only be assigned to VOCs, while VOIs and VUMs would be referred to using the nomenclature systems based on the hierarchical—like a family tree approach and the genetic relatedness according to the classification tools GISAID, Nextstrain and Pango[3]. To date, WHO reports no SARS-CoV-2 variants meeting the VOC criteria while designing a VOI named BA.2.86 and few VUMs[3].

Many papers explored the mechanisms that generated genetic variation in SARS-CoV-2, and underlying the importance of within-host and population-level processes. The emergence of the variants is a consequence of the high rate of genome mutations, creating an important antigenic distance among the different variants, even within Omicron itself[6,7], and events of recombination[8].

Thus, since the emergence of SARS-CoV-2, the need for an effective vaccine has appeared crucial for stimulating immune system responses to produce humoral/cellular immunity and activate immunological memory. Indeed, it has been demonstrated that vaccination contains viral spread and decreases the incidence of the virus, and the risk of serious complications and deaths. Vaccination against SARS-CoV-2 is based primarily on vaccines that induce immunity against the spike glycoprotein[9].

The EMA (European Medicines Agency) and FDA (Food and Drug Administration) approved different vaccination regimens that vary from country to country and include the administration of booster doses[10].

The administration of booster doses is recommended mainly for older adults (above 60 years of age) and vulnerable persons (elderly people above 80 years of age and immunocompromised individuals)[11–13].

It has been demonstrated that SARS-CoV-2 variants may escape neutralizing immunity elicited by previous infection and vaccination, leading to new infection waves[14–18] and resulting in reinfection events caused by the same or different SARS-CoV-2 variants[19–21].

Beta and Gamma were first associated with immune evasion, although they have no widespread diffusion. Otherwise, Alpha and Delta VOCs spread globally and were responsible for huge waves of infections, the former probably as a consequence of the low percentage of the vaccinated population, the latter because of the high number of single nucleotide polymorphisms (SNPs)[19–21].

The increased transmissibility has been explained by mutations in the polybasic cleavage site in the spike protein (H681 in Alpha and R681 in Delta) that, causing furin cleavage, increases virus entry into the cell. Published data point out that the Omicron is an immune escape variant also in patients vaccinated with 1 or 2 doses of vaccine, especially when antibody titers are waning[22]. Cases of reinfection due to Omicron lineages are widely reported[23–27]. The study of Burkholz et al.[24] suggests that SARS-CoV-2 non-Omicron infections may not generate the protective immunity necessary to defend against emerging Omicron sub-lineages. Moreover, Omicron/Omicron infections have been observed to occur in a shorter period if compared to non-Omicron/Omicron infections[21,24]. Cases of reinfection with the same Omicron lineage (BA.1/BA.1, BA.2/BA.2, and only one case of BA.5/BA.5) were also reported[22,27].

Even among the Omicron VOC subtypes the infection and/or vaccination-mediated cross-protection seems to be reduced, particularly for BA.4/BA.5 subtypes[28]. This evidence was supported also by a systematic review, including 28 studies and 11 million individuals, which investigated the real-world effectiveness and durability of protection conferred by primary course and booster vaccines against Omicron infection[29].

Nowadays, NGS technology is the best method to ensure the genomic tracking of emerging variants and allows for observing changes in viral infection rates and vaccine efficacy[24].

A retrospective descriptive study analysis based on RT-qPCR and NGS sequencing data, derived from samples collected by the Defence Institute for Biomedical Sciences (DIBS) during the pandemic, was performed to investigate the occurrence of reinfection about the variant and vaccination status. In particular, SARS-CoV-2 reinfection or reactivation has been discussed for two pediatric unvaccinated cases referred to patients presenting a short-time reinfection (i.e. < 60 days), thus highlighting the importance of the antibody-mediated neutralization induced by COVID-19 vaccination.

Results

SARS-CoV-2 related activities

Since the first days of the SARS-CoV-2 pandemic in Italy, from February 5, 2020 to March 20, 2024, the DIBS laboratory has screened 416,466 SARS-CoV-2 swabs by RT-qPCR, referred to 168,481 patients [119,622 (71%)

	Total Study population	RT-qPCR positive population	RT-qPCR reinfections
n° (%)	168,481 (100)	16,384 (9.7)	350 (0.2)
Males n° (%)	119,622 (71)	10,158 (62)	217 (62)
Females n° (%)	48,859 (29)	6,226 (38) *	133 (38)
Mean Age Males years ± SD	42 ± 20	40.4 ± 17	42 ± 15
Mean Age Females years ± SD	42 ± 22	41.5 ± 23	36 ± 16 **

Table 1. SARS-CoV-2 related activities. Patients screened for SARS-CoV-2 by DIBS from February 5, 2020 to March 20, 2024. The * symbol indicates statistically significant data (p-value < 0.0000001) carried out by two-tail, Yates corrected, χ^2 test. The ** symbol indicates statistically significant data (p-value < 0.0000001) carried out by Student's t test. SD = standard deviation; RT-qPCR = reverse transcriptase quantitative polymerase chain reaction.

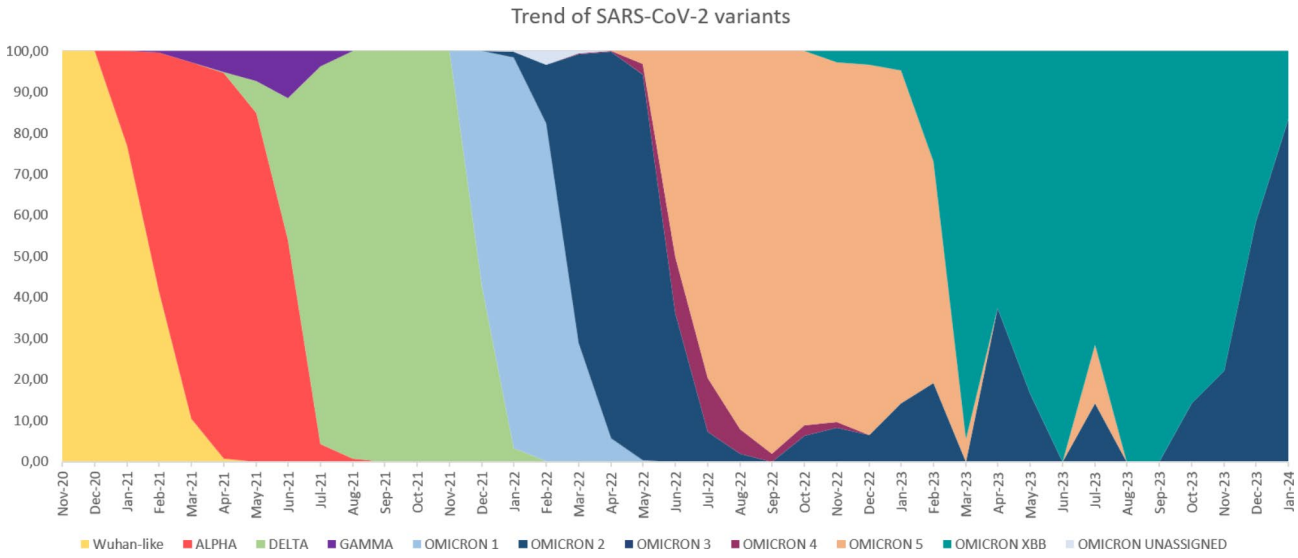


Fig. 1. Trend of identified SARS-CoV-2 variants at DIBS from November 2020 to January 2024.

men, mean age 42 years ± 22 SD; 48,859 (29%) women, mean age 42 years ± 22 SD] (Table 1). From the beginning of the second wave (November 2020) our Institute massively collected and sequenced all samples tested positive with an RT-qPCR Ct < 30.

RT-qPCR identified 35,088 positive samples (8.4%) referred to 16,384 patients (9.7%), 10,158 men (mean age 40.4 years ± 17 SD) and 6,226 women (mean age 41.5 years ± 23 SD). The analysis highlighted 350 cases of possible reinfection [217 (62%) men, mean age 42 years ± 15 SD; 133 (38%) women, mean age 36 years ± 16 SD], 228 of which were further characterized by NGS [136 (60%) men, mean age 41.5 years ± 14 SD; 92 (40%) women, mean age 34.5 years ± 16 SD] (Table 1).

Indeed, all first positive samples and samples from patients retested positive (with at least one intermediate negativity) reached the number of 10,380, referring to 10,031 patients [6,069 (60.5%) men, mean age 39 years ± 18 SD; 3962 (39.5%) women, mean age 37 years ± 19 SD]. All of them were successfully analyzed by NGS.

Despite the female percentage of the total analyzed population is 29%, the female percentage of the SARS-CoV-2 positive people is significantly higher (38%, p < 0.0000001). No significant difference was instead observed between the female percentage of the total positive subjects and those who made reinfections.

A t-test for two independent samples was used to compare the mean ages of two groups (total and positive populations), separately for males and females. The mean age of males who tested positive for SARS-CoV-2 is significantly different from the mean age of the male total population (p-value < 0.0000001). The difference between the mean age of females in the total population and the mean age of females who tested positive for SARS-CoV-2 is not statistically significant.

Figure 1 shows the global trend of the 10,380 identified SARS-CoV-2 variants reported at DIBS.

On December 14, 2020 the first Italian case of the Alpha variant, from a UK traveler, was detected at the DIBS, and then the Alpha variant started to spread across the Country. In 2021, our casuistry registered 27.8% of Alpha variant in late January, 82.8% in April and only 0.4% in August, while few cases of Gamma VOCs were registered in April (0.5%) and May (12.5%). The decrease of Alpha was inversely proportional to the increase in Delta cases first identified in April (0.7%). Delta reached 99% of the cases in December (99%) when the first case of Omicron VOC (BA.1) was found. In 2022, BA.1, representing 95% of the cases at the end of January, was replaced in the following months by BA.2 (over 90% between March and April). BA.4 was 13% in July and

finally disappeared in December. BA.5 became prevalent in July (80%), while Omicron XBB sub-lineage was first detected in November (3%). During the winter season both BA.2 and XBB grew up, reaching in February 2023 the percentage of 19% and 54%, respectively. In the same period, BA.5 gradually decreased (90% in December, 81% in January and 54% in February). In March the Omicron lineages registered were BA.5 (6%) and XBB (94%); in April BA.5 disappeared, BA.2 was detected again with a percentage of 38% and XBB counted 62% of the cases. XBB continued to grow and in January 2024, when the last positive sample was detected, it was still the most common sub-lineage.

Retrospective analysis of cases with double instances of infection

Three hundred forty-four out of 350 cases were characterized by double instances of infection (Fig. 2).

Figure 2A shows the dynamics of SARS-CoV-2 reinfections (right axis) compared to the non-omicron and omicron epidemics (left axis).

Three hundred forty-two cases out of 344 (99%) referred to patients with samples tested positive > 60 days, with at least one intermediate negative RT-qPCR test.

Among the 342 cases, 127 (37%) registered a first positivity infection in 2020: 18/127 (14%) resulted again positive in 2021, 104/127 (82%) in 2022 and 5/127 (4%) in 2023. In 2021, 120 (35%) reported a first infection: 11/120 (9%) were reinfected in 2021, 107/120 (89%) in 2022 while 2/120 (1.7%) in 2023. In 2022, 92 (27%) reported a first infection: 54/92 (59%) were positive twice in 2022, 36/92 (39%) were positive in 2023 and 2/92 (2%) in 2024. Only three cases (1%) were positive twice in 2023 (Fig. 2B).

Two pediatric patients (2/344; 0.58%) were found to have two events of infection with a time interval of less than 60 days (46 days each). The first one was positive in 2021 and then in 2022, the second one experienced the two infections in 2022 (Fig. 2B).

Figure 3 shows the results obtained for the 224/344 (65%) double infections that were sequenced. Specifically, among the 108/224 (48%) patients initially infected with the Wuhan-like virus, 2/108 (2%) were reinfected with the Delta variant, 67/108 (62%) with Omicron BA.1, 28/108 (26%) with Omicron BA.2, 5/108 (5%) with Omicron BA.4, 5/108 (5%) with Omicron BA.5, and 1/108 (1%) with Omicron XBB. Among the 14/224 (6%) Alpha cases, 8/14 (57%) patients tested positive again for Omicron BA.1 and 6/14 (43%) for BA.2. Among the 3/224 (1.3%) patients previously infected with the Gamma variant, 3/3 (100%) were reinfected with Omicron BA.1. Finally, of the 38/224 (17%) Delta cases, 12/38 (32%) were reinfected with Omicron BA.1 (including one referred to as the first pediatric case), 10/38 (26%) with BA.2, and 16/38 (42%) with BA.5.

As regards Omicron's first infected cases, 38/224 (17%) Omicron BA.1 cases experienced a relapse: 7/38 (18%) with BA.2, 1/38 (2%) with BA.4, 24/38 (63%) with BA.5 and 6/38 (16%) with XBB. Eighteen out of 224 (8%) Omicron BA.2 cases were found positive: 9/18 (50%) with BA.2, 4/18 (22%) with BA.5 and 5/18 (28%) with XBB. Five out of 224 (2%) were with Omicron BA.5: 1/5 (20%) with BA.2, 1/5 (20%) showed reinfection with the same Omicron sub-lineage, BA.5 (one referred to the second pediatric case) and 3/5 (60%) with XBB.

Retrospective analysis of cases with three instances of infection

Six patients tested positive three times, with time intervals (Δt) always exceeding 60 days, were classified as having experienced triple infections (Table 2). Among these six patients, two (2/6; 33%) had their first infection in 2020, followed by a second and third infection in 2022. One out of six (1/6; 17%) was first infected in 2020, then again in 2021 and 2022; another one (1/6; 17%) tested positive initially in 2021, with reinfections occurring in 2022. Similarly, one (1/6; 17%) had a first infection in 2021, a second in 2022, and a third in 2023; and finally, one patient (1/6; 17%) was first and second infected in 2022, with a third infection occurring in 2023. Sex and age are reported in Table 2.

Four of these six cases were sequenced. Case 1 was initially infected with Delta, followed by two reinfections with Omicron BA.2. Case 2 first tested positive in January 2022, but the Ct value was too high for sequencing; in May 2022, this patient was reinfected with Omicron BA.2, and again in March 2023 with Omicron XBB. Case 3 had a Delta infection, followed by a second Omicron BA.5 infection in July 2022, and a third infection with Omicron BA.2 in December 2023. Finally, Case 4 experienced a first infection with the Wuhan-like strain, followed by reinfections with Omicron BA.1 and Omicron BA.5.

The other two cases, 5 and 6, both show no amplification of the S gene in RT-qPCR (S gene dropout) during the second infection event, while the gene was amplifiable in the first and third events (data not shown).

Cases of dual infections occurring within a 60-day period

Focusing on the two cases characterized by two events of infection with a time interval of less than 60 days, both were unvaccinated and pediatric cases (Fig. 2).

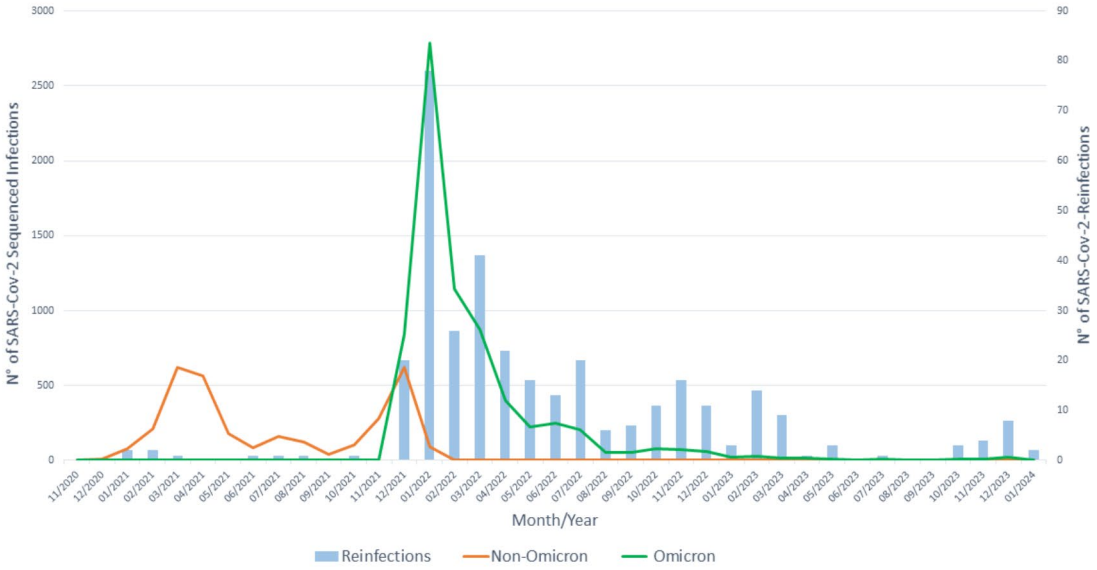
The first pediatric case regards an unvaccinated 7-year-old boy resulted positive twice 46 days apart, on December 2021 and January 2022. The boy was tested on December 10, 2021 together with his mother (40-year-old, unvaccinated) and resulted positive for SARS-CoV-2 (Table 3). On December 17, he became negative while his mother turned negative on December 23. The boy was positive again on January 25, 2022 and then negative on February 5, his mother performed a molecular test only on February 5 2022, resulting negative.

The positive samples were sequenced reaching high coverage (> 99%) and revealing the presence of Delta infection in both samples of December (boy EPI_ISL_8014526; mother EPI_ISL_8014553) and an Omicron BA.1.1 infection in the January sample (EPI_ISL_18585079) (Table 3).

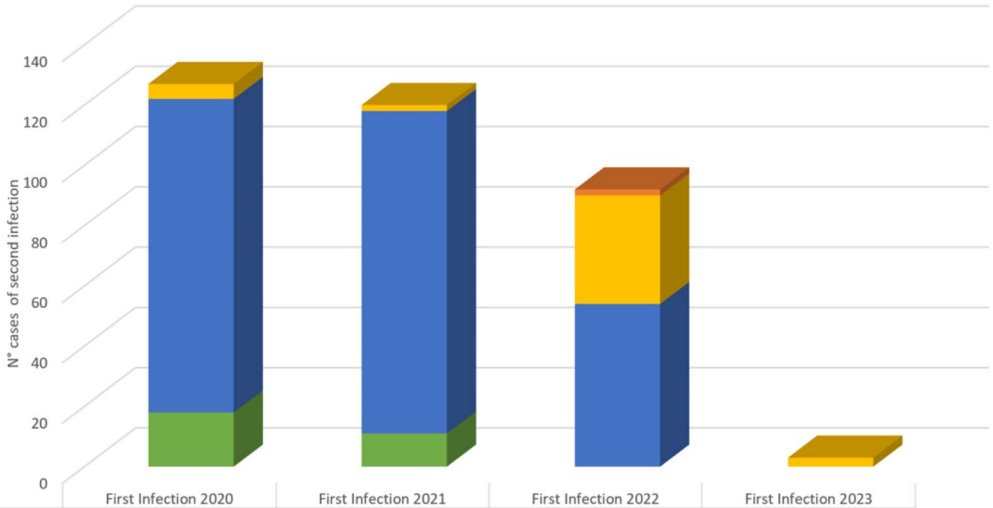
Data are compatible with RT-qPCR results (i.e. the S gene dropout in the sample of January). The boy had a complete rescue after the first infection, confirmed by a negative molecular swab performed on December 17, 2021.

The second case referred to an unvaccinated 18-month-old child, tested with her family for SARS-CoV-2 and Influenza viruses on May 19, 2022, concomitant with a SARS-CoV-2 outbreak at her nursery.

A)

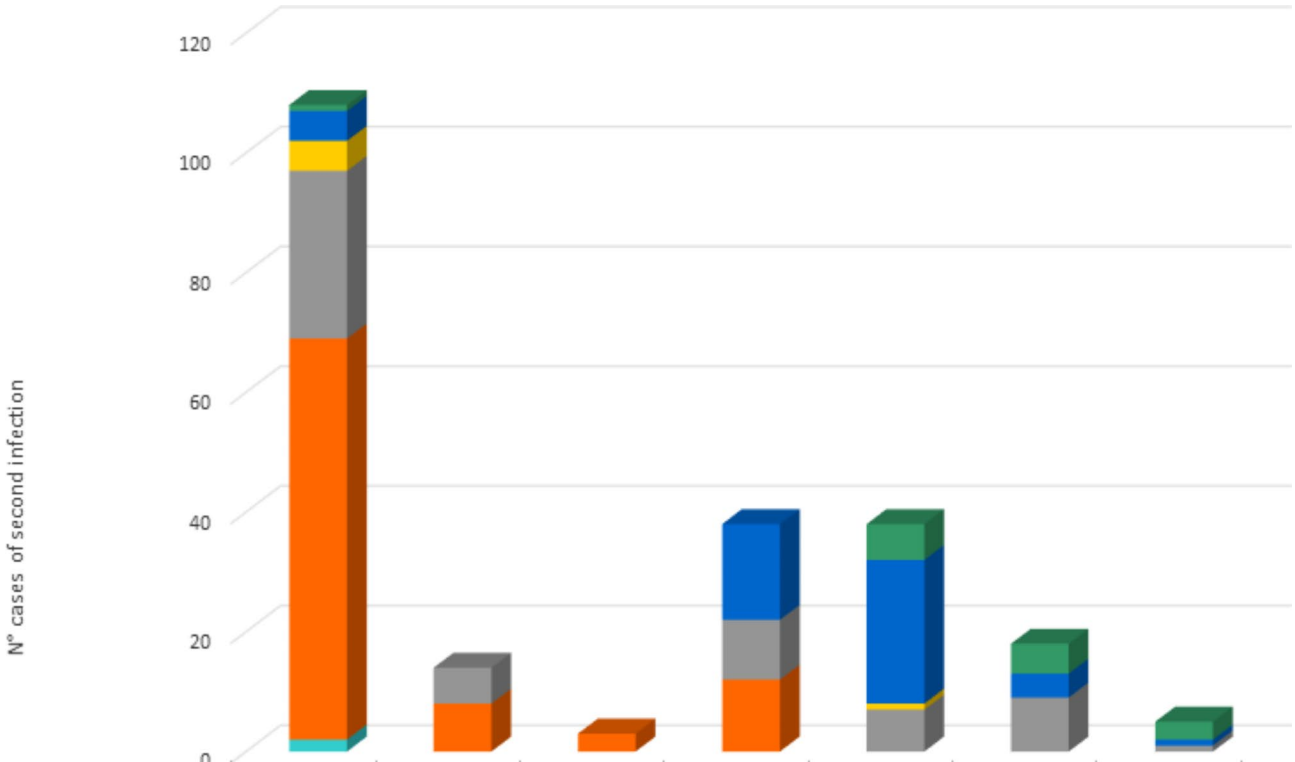


B)



	First Infection 2020	First Infection 2021	First Infection 2022	First Infection 2023
Second Infection 2024			2	
Second Infection 2023	5	2	36	3
Second Infection 2022	104	107 +1*	54 +1*	
Second Infection 2021	18	11		
TOT samples	127	120 +1*	92 +1*	3

Fig. 2. Double instances of infection. (**A**) Dynamics of SARS-CoV-2 reinfections (blue columns, right axis) compared to the non-omicron and omicron sequenced cases (orange and green lines, left axis). (**B**) Double infection cases assessed by RT-qPCR: 344 cases of double infections, two of which characterized by two events of infection with a time interval of less than 60 days and highlighted with Symbol *. The X-axis reports the year of the first infection; the Y-axis shows the number of cases, and each column depicts the number of second infection cases with different colors).



	Wuhan-like	ALPHA	GAMMA	DELTA	OMICRON 1 (BA.1)	OMICRON 2 (BA.2)	OMICRON 5 (BA.5)
OMICRON XBB	1				6	5	3
OMICRON 5 (BA.5)	5			16	23+1*	4	1*
OMICRON 4 (BA.4)	5				1		
OMICRON 2 (BA.2)	28	6		10	7	9	1
OMICRON 1 (BA.1)	67	8	3	12			
DELTA	2						
TOT SAMPLES	108	14	3	38	38	18	5

Fig. 3. Reinfection cases evaluated by NGS: 224 cases of double infections sequenced. Symbol * indicates the pediatric cases presenting two infection events with a time interval of less than 60 days (46 days each). The X axis reports the first infection, the Y axis reports the number of second infection cases, each column depicts by different colors the subtypes of SARS-CoV-2 found at the second infection.

The child and her mother were both pauci-symptomatic (37.5°C fever and mild headache, respectively) while the father was asymptomatic. The parents were both vaccinated three times [two doses of BNT162b2-Pfizer-BioNTech (Comirnaty) and one of Spikevax mRNA-1273 (Moderna)].

The child (EPI_ISL_14149323) and her parents (mother EPI_ISL_14149325; father EPI_ISL_14149324) resulted negative for Flu A/B and positive for SARS-CoV-2. The positive samples were sequenced reaching high coverage (>99%) and revealing the presence of Omicron BA.5.2.1 sub-lineage, compatible with the S gene dropout found in all samples by RT-qPCR test.

After a week, on May 26, the parents resulted negative. The child remained persistently positive until May 30, even with a low viral load (34 Ct, equal to the limit of detection of the method). On June 1st the child resulted negative by an antigenic rapid test and this result was confirmed on 3rd June (RT-qPCR).

Consequently, to a second outbreak at the child's nursery, the toddler was tested again on July 4 and resulted positive for SARS-CoV-2 (EPI_ISL_13826896), 46 days after the first infection. The child experienced mild respiratory symptoms, compatible with the infection and the molecular test showed the S gene dropout. The parents were also tested, resulting both negative. One week later, on July 11, the child's swab resulted negative.

The positive sample from July underwent high-coverage NGS analysis (>99%), leading to the identification of a BA.5.2.1 sub-lineage (EPI_ISL_13826896) (Table 4).

A genomic sequence alignment, clade assignment, mutation calling, phylogenetic placement, and quality checks for SARS-CoV-2 were performed for all family sequences using the Nextclade tool[30]. The toddler's sequences comparison (Fig. 4A) showed a total of 8 mutations: five nucleotide substitutions in July that were absent in the sequence of May; in particular 3 were located in ORF1a (C1912T; A3859G; T6979G), 1 in ORF1b

Case	Date	NGS result	Time between 1st and 2nd infection (days)	Time between 2nd and 3rd infection (days)	Sex	Age (years)
1	13/12/2021	DELTA	145	170	M	29
	07/05/2022	OMICRON 2 (BA.2)				30
	24/10/2022	OMICRON 2 (BA.2)				30
2	07/01/2022	NA	136	305	M	39
	23/05/2022	OMICRON 2 (BA.2)				39
	24/03/2023	OMICRON XBB				40
3	22/07/2021	DELTA	356	507	M	58
	13/07/2022	OMICRON 5 (BA.5)				59
	02/12/2023	OMICRON 2 (BA.2)				59
4	13/10/2020	Wuhan-like	504	115	M	54
	01/03/2022	OMICRON 1 (BA.l)				55
	24/06/2022	OMICRON 5 (BA.5)				55
5	12/09/2020	NA	507	140	F	48
	31/01/2022	NA				48
	20/06/2022	NA				49
6	04/11/2020	NA	268	239	M	46
	29/07/2021	NA				48
	24/03/2022	NA				48

Table 2. Cases of triple infections evaluated by RT-qPCR and NGS. NA = not evaluable, M = male, F = female.

Patient	Age, Sex	Date	Infection	Symptomatology	COVID-19 RT-qPCR (viral load Ct) *N/ORF1ab/S*	GISAID accession ID	Virus variant	Time between episodes	1st Dose of COVID-19 vaccine	2nd Dose of COVID-19 vaccine	3rd Dose of COVID-19 vaccine
Boy	7, M	10/12/2021	First	Paucisymptomatic	27/27/27	EPI_ISL_8014526	AY.103	46 days	None	None	None
		25/01/2022	Second	Symptomatic	21/21/Neg	EPI_ISL_18585079	BA.5.1.1				
Mother	40, F	10/12/2022	First	Symptomatic	28/28/28	EPI_ISL_8014553	AY.103		None	None	None

Table 3. Delta/Omicron case family's data. Resume of the data related to the 7-year-old boy (EPI_ISL_8014526 and EPI_ISL_18585079) and his mother (EPI_ISL_8014553). Ct = cycle threshold; Neg = negative; F = female; M = male.

Patient	Age, sex	Date	Infection	Symptomatology	COVID-19 RT-qPCR (viral load Ct) *N/ORF1ab/S*	GISAID accession ID	Virus variant	Time between episodes	1st Dose of COVID-19 vaccine	2nd Dose of COVID-19 vaccine	3rd Dose of COVID-19 vaccine
Child	2, F	19/05/2022	First	Paucisymptomatic	29/29/Neg	EPI_ISL_14149323	BA.5.2.1	46 days	None	None	None
		04/07/2022	Second	Symptomatic	28/28/Neg	EPI_ISL_13826896	BA.5.2.1				
Mother	44, F	19/05/2022	First	Paucisymptomatic	18/18/Neg	EPI_ISL_14149325	BA.5.2.1		Pfizer-BioNTech	Pfizer-BioNTech	Spikevax
		04/07/2022			Neg						
Father	55, M	19/05/2022	First	Asymptomatic	22/22/Neg	EPI_ISL_14149324	BA.5.2.1		Pfizer-BioNTech	Pfizer-BioNTech	Spikevax
		04/07/2022			Neg						

Table 4. Omicron BA.5/BA.5 case family's data. Resume of the data related to the 18-month-old child (EPI_ISL_14149323 and EPI_ISL_13826896); her mother (EPI_ISL_14149325); her father (EPI_ISL_14149324). Ct = cycle threshold; Neg = negative; F = female; M = male.

(G16181A), 1 in ORF3a (C25626T); only the point mutation in ORF1b region was missense causing an amino acid change (R905K). An additional difference was reported, with a low coverage, in the S gene (A22786C). Moreover, 2 SNPs were found in ORF9b in the sequence of May that were not present in the one of July (G28361T, G28371T).

Moreover, both child viral genomic sequences were analyzed by querying the EpiCoV database to retrieve the most similar sequences using the Audacity-Instant tool integrated in GISAID[31]. Among the sequences collected in the same month of May, eight homologous sequences were found, including one from Belgium that was the closest geographically (EPI_ISL_13204456; Fig. 4B). The comparison between these two sequences showed only two different nucleotide substitutions present in the Belgium sequence (T22917G in the S gene; A27038G in the M gene).

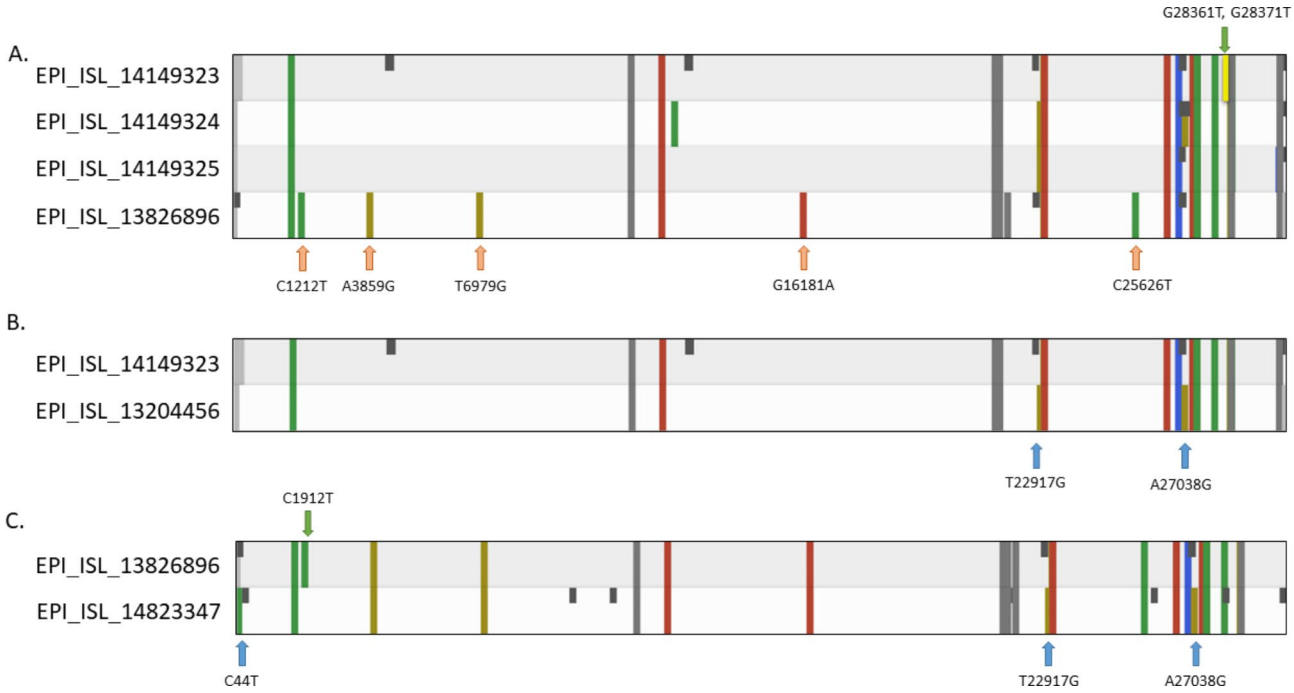


Fig. 4. Genome comparison among the sequences of the girl family VS the most similar ones identified in GISAID using SARS-CoV-2 (BA.2) as reference genome (reference: Wuhan-Hu-1 with BA.2 SNPs; dataset name: nextstrain/sars-cov-2/BA.2). (**A**) Genome comparison among EPI_ISL_14149323 (child in May), EPI_ISL_14149324 (father), EPI_ISL_14149325 (mother), EPI_ISL_13826896 (child in July). The orange arrows indicate the five SNPs present in the July sequence of the child [in ORF1a (C1912T; A3859G; T6979G), in ORF1b (G16181A) and in ORF3a (C25626T)] and the green one indicates the two SNPs present in the May sequence (G28361T and G28371T in Orf9b). (**B**) Genome comparison between EPI_ISL_14149323 (child in May), EPI_ISL_13204456 (sequence from Belgium). The blue arrows indicate the two different SNPs, present in the Belgium sequence (T22917G in the S gene; A27038G in the M gene). (**C**) Genome comparison between EPI_ISL_13826896 (child in July), EPI_ISL_14823347 (sequence from INMI L. Spallanzani I.R.C.C.S). The blue arrows indicate the three SNPs present in the INMI L. Spallanzani I.R.C.C.S sequence (C44T in Orf1a; T22917G in the S gene; A27038G in the M gene) and the green one indicates the SNP present in the child (C1912T in ORF1a).

Conversely, the sequence with the highest similarity to the one from July was submitted by the National Institute for Infectious Diseases (INMI) L. Spallanzani I.R.C.C.S. and was collected from a patient in Rome (EPI_ISL_14823347; Fig. 4C). The sequences comparison showed three SNPs present in the INMI L. Spallanzani I.R.C.C.S sequence (C44T in Orf1a; T22917G in the S gene; A27038G in the M gene) and one present in the child (C1912T in ORF1a).

Discussion

Immune escape and SARS-CoV-2 reinfections

SARS-CoV-2 originated in Wuhan (China) in early December 2019 and rapidly spread worldwide. To date, five VOCs determined new waves of the pandemic and from the first one, the Alpha wave, cases of reinfection with different or the same SARS-CoV-2 virus have been reported globally. Since the initial outbreak, SARS-CoV-2 has undergone significant mutation and the emergence of new variants and lineages have been considered as the main cause of reinfections[32]. Although early reinfections of SARS-CoV-2 are nowadays frequent, ECDC (European Centre for Disease Prevention and Control) still defines them as a positive PCR or rapid antigen test > 60 days after the previous positive test (RT-qPCR, rapid antigen or serologic test) since before the Omicron wave, rapid reinfection with SARS-CoV-2 was highly unlikely within a very short period[1]. CDC (Centers for Disease Control and Prevention) reduced this time to 45 days, however rapid reinfections with Omicron sub-lineages in less than 45 days have already been reported[26,27,33].

Most of the cases reported in the literature lack sequencing data and are based only on a RT-qPCR test, resulting in the inability to establish if those cases could be due to prolonged shedding, repeat infection or erroneous PCR results[26]. Many ECDC reports underline the importance of using integrated genomic sequencing methods to distinguish reinfections from persistent infections[1].

Since the emergence of Omicron, in mid-November 2021[1] an immune escape compared to previous variants and a significant growth of reinfections appeared evident[22,34].

Despite in the literature the sex responsibility for the higher risk of SARS-CoV-2 infection has not been ascertained yet[35] the percentage of female subjects SARS-CoV-2 positive in this study is 38%, significantly higher

than the percentage of female subjects of the whole analyzed population (29%, $p < 0.0000001$). Moreover, no significant difference was instead observed in the female sex percentage between the positive population and the 350 individuals who made reinfections, in contrast with what has been previously observed[36], where reinfections were significantly more likely in females.

Regarding the age, the mean age of the male studied population is significantly higher than the mean age of SARS-CoV-2 positive subjects. Besides, the mean age of SARS-CoV-2 female subjects is significantly higher than the mean age of the females who made reinfections. These observations, even though apparently in contrast with the literature data[37], should not be surprising, because in the current study demographic data only are available and they may not be associated with clinical data (vaccination status, symptomatology, comorbidities), which are unavailable instead; besides, the risk factors for infections/reinfections are dynamic according to the circulating viral variants and should periodically be updated[38].

Numerous studies have reported that BA.1 and BA.2 could effectively reinfect COVID-19 patients[24,26,27,39,40]. SARS-CoV-2 Omicron lineages, carrying additional mutations in their spike protein, further evade neutralizing antibodies and compromise the efficacy of COVID-19 vaccines[20,41,42]. A recent study showed that both BA.1 and BA.2 escape antibody-mediated neutralization elicited by vaccination, previous infection with SARS-CoV-2, and monoclonal antibodies[43]. A survey study, carried out in Denmark, pointed out that the BA.2 strain is more transmissible than the BA.1 and highlighted an increase in transmissibility among unvaccinated individuals when infected by BA.2[44]. Wang and colleagues evaluated the antigenic properties of some Omicron lineages, finding that BA.5 is more resistant to sera from vaccinated and boosted individuals than BA.2[45]. BA.4 and BA.5 omicron subgroups have a greater ability to escape from the immune system, reducing current vaccines' effectiveness[46]. Cao et al. found that BA.4/5 have stronger humoral immune evasion than previous variants[41]. Cases of reinfection with the same Omicron lineage were also reported[19–21,24,27].

Molecular surveillance of SARS-CoV-2: DIBS experience

Starting from the advent of Alpha VOC, the molecular surveillance of SARS-CoV-2 has proved crucial for monitoring the spread of the virus[1], therefore our laboratory increased SARS-CoV-2 whole genome sequencing to identify, monitor and assess the virus spread and evolution. At the DIBS our group has screened 416,466 SARS-CoV-2 swabs by RT-qPCR and sequenced all the positive cases found by virtue of a massive sequencing activity. NGS analysis was applied to 10,380 samples, including samples that were collected for research and epidemiological purposes and derived from patients tested positive a second time < 60 days from the first positivity. Globally, samples from 10,031 patients were analyzed. Sequencing resulted in the complete SARS-CoV-2 genomes from 228 patients, the majority occurring with Omicron reinfection (Fig. 3).

Our casuistry evidenced 9 cases of putative reinfection involving the BA.2 lineage (Fig. 3), as already widely described in the literature[19–21,24,27]. The bioinformatics analysis of the sequencing data generated for these nine cases revealed the same lineage but different sub-lineages. Consequently, these cases could be considered as true viral reinfections.

In addition, two pediatric cases were observed, which suggest the possibility of early reinfection.

Insights into the pediatric cases

The first pediatric case regards an unvaccinated 7-year-old boy who resulted positive twice 46 days apart, on December 2021 and January 2022. The boy's samples were sequenced showing Delta and BA.1.1 infections, compatible with RT-qPCR results (the S gene dropout in the sample of January).

In the case of the 7-year-old boy, both the timing and characteristics of the identified SARS-CoV-2 variants strongly support the "rapid reinfection" hypothesis. Many studies, including a large one reporting 41% of the occurrence of Delta/BA.1 reinfection, support this hypothesis[24]. In our case, the NGS results are of high quality, and the identification of two distinct variants leaves no room for doubt. Moreover, similar Delta/BA.1 reinfection cases showing a very short time-lapse between the two infections, have been already reported in the literature[21].

While this first case appears to be a genuine reinfection, the case of the unvaccinated 18-month-old girl is more challenging to define since two very similar BA.5 sequences were detected. On one hand, the immature immune system and the lack of vaccination may promote the hypothesis of viral persistence; on the other hand, the characteristics of the sequences suggest a BA.5/BA.5 reinfection.

The existence of two negative swabs in June (antigenic on 1st and molecular on 3rd) which may apparently stay against the interpretation of viral persistence, may indeed be observed in these cases[47].

Nevertheless, it is not possible to exclude viral persistence and intra-host evolution probably favored by both the child's immature immune system and the unvaccinated status due to her age (< 5 years). The most accredited theory on the origin of SARS-CoV-2 variants refers to the persistent infection in immunocompromised patients, with the viral RNA mutating under the pressure of an immune system unable to clear the virus[48].

Finally, the sequences' comparison highlighted eight SNPs, in particular, the BA.5.2.1 sequence of July showed five high coverage and one low coverage nucleotide substitutions; the BA.5.2.1 sequence of May showed two nucleotide substitutions that resulted absent in the one of July. The lack of mutations in the Spike region and the relatively short time for the appearance of mutations may themselves be interpreted as elements against the viral persistence[6,49]. However, the reaction of the immune system to a SARS-CoV-2 infection is not only against the spike protein, in contrast to the reaction to vaccination, thus able to modify viral antigens other than the spike protein[50]. Concerning the relatively short time, it has to be underlined that 8 mutations have been observed in 46 days (from May 19 to July 4), a not unrealistic data, considering that 31 mutations have been observed in 152 days in an immunocompromised patient[51] and 23 mutations in 101 days in another patient[49,52].

Otherwise, the rate found in the child's sequences seems to be higher than the one due to the natural evolution of the virus. According to Wang and colleagues, who calculated the rate of mutation of the SARS-CoV-2 virus as

equal to 6.677E-4 nucleotide substitution per site per year[53], in 46 days we should have maximum 3 SNPs while we found 8 SNPs.

Another finding in support to the hypothesis of BA.5/BA.5 reinfection is the results obtained by the comparison of the sequences of the studied family and those deposited in GISAID in the same period. As shown by Fig. 4, we found a Belgian sequence with high similarity to the girl-child's May one (only two SNPs different), while the sequence of July showed the highest similarity with one submitted by the INMI L. Spallanzani I.R.C.C.S., in the same period and in the same geographic area (Rome) and showing only 4 differences in terms of SNPs. Thus, the presence of circulating sequences in Europe and Italy, similar to those of the family under study, suggests that the July sequence is not the result of a viral evolution from the May infection, but rather of a new infection originating from circulating strains.

Noteworthy to mention that the age of the patient and her unvaccinated status are compatible with the findings reported in the literature[26]. Few studies are focused on the neutralization capacity of antibodies against Omicron after a prior SARS-CoV-2 infection in children and adolescents[54]. Chen et colleagues demonstrated a reduced susceptibility of Omicron variant to serum antibodies from children who recovered from COVID-19 infection or those who have received two doses of the BNT162b2 vaccine[55]. Tang et al. evaluated virus-neutralizing capacity against the five SARS-CoV-2 VOCs of pediatric samples from patients hospitalized and convalescent. They showed less than 10% neutralizing antibody titers against Omicron and some loss of cross-neutralization against all variants, with the most pronounced loss against Omicron[54]. Conversely, Dowell et al. observed a good response in children, comparable to that observed in adults, thus emphasizing the variability of immune response to SARS-CoV-2 in unvaccinated pediatric patients[56].

Regardless of interpretation, either reinfection or viral persistence, this case is equally interesting, since only one BA.5/BA.5 reinfection has been so far described[21] and early appearance of slight mutations for BA.5 sub-variant in consequence of viral persistence has not been described yet, to our knowledge.

However, in both cases the pivotal role of vaccination for protection is emphasized, considering the lack of reinfection in the parents compared to the girl-child.

Moreover, few weeks after the second infection, the child came in close contact with the grandmother tested positive for SARS-CoV-2 and she was not reinfected. Thus, probably, the girl-child was protected by efficient neutralizing antibodies, induced by the recent SARS-CoV-2 infections that acted as a booster dose of vaccine.

Conclusions

This study reports a wide casuistry of naso-oropharyngeal swabs tested by RT-qPCR for SARS-CoV-2 and evaluated by NGS. Our data revealed two interesting pediatric cases characterized by short-time reinfection (i.e. < 60 days).

The most interesting case regards an unvaccinated 18-month-old child, compatible with either a rapid reinfection or a viral persistence. The first scenario would depict one of the first cases of BA.5/BA.5 reinfection so far identified worldwide. On the other hand, the age of the child and her unvaccinated status may support the intra-host evolution of the virus.

The importance of the antibody-mediated neutralization elicited by the COVID-19 vaccine appeared clear considering the immune protection developed by her parents as a consequence of the Omicron infection of May that probably acted as a vaccine booster. Whatever the origin of the infection in July, either reinfection or viral persistence, the effectiveness of vaccination and of a mature immune system is underlined by the different outcome in the same family, where the parents remained protected during the reinfection or persistent infection of their girl-child. The usefulness of NGS sequencing in public health, both for interventions and recommendations, is highlighted in this paper and we believe that NGS sequencing could support public health interventions for the purposes of longitudinal monitoring of SARS-CoV-2 or research, even in young populations.

Materials and methods

Study design

The present study is a retrospective descriptive study: we analyzed, by molecular biology techniques, over 416,466 naso-oropharyngeal swabs from February 5, 2020 until March 20, 2024.

Ethics declarations

The study was conducted in accordance with the Declaration of Helsinki.

All the data in this study were anonymized by deleting all sensitive information. All study participants provided informed consent, as required by Article 40 of Decree-Law No. 23 dated April 8, 2020, in Italy.

Ethical Committee of National Institute of Infectious Diseases (INMI) Lazzaro Spallanzani—IRCCS approved the study (approval number 2/2020).

Samples

All the study subjects have read and signed the information note, containing personal data as age, sex, residence, symptoms, occupation, risk factors for severe infection, date and types of vaccination. However, while demographic information is available for the whole population, clinical information has inconstantly been collected, thus it could not be further processed and discussed. Swabs were collected from both symptomatic and asymptomatic subjects respectively for diagnostic and surveillance purposes.

All first positive samples and samples from patients retested positive and showing at least one intermediate negativity were NGS sequenced. Three different NGS platforms were utilized: Illumina (San Diego, CA, USA), MGI Tech Co (Shenzhen, PRC) and Oxford Nanopore Technology (Oxford, UK).

Nucleic acid extraction

RNAs from naso-oropharyngeal swabs underwent RT-qPCR were extracted automatically by KingFisher™ Flex Purification System performed on 96 PCR head (Thermo Scientific™, Massachusetts, USA) or Pre-Filled 96 prep MagaBio plus Virus DNA/RNA Purification (Magnetic particles) on GenePure Pro 96 fully automatic Nucleic Acid purification System (Bioer Technology, Hangzhou, PRC), according to the manufacturer's instructions. The RNAs tested positive in RT-qPCR were used for amplicon sequencing, while RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to obtain RNAs for shotgun sequencing. To this purpose, genomic RNA was reverse-transcribed using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Double-stranded DNA was subsequently synthesized by using the Klenow enzyme (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

A one-step RT-qPCR multiplex assay was performed to investigate the viral infection by SARS-CoV-2 (ORF1ab and N genes) and/or Flu A/B (M and NP genes respectively) viral infection (SARS-CoV-2 and Flu A/B Virus Multiplex Nucleic Acid Diagnostic Kit, Sansure Biotech Inc., Changsha, PRC). The RT-qPCR was performed on QuantStudio 5 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions; reactions were performed aliquoting 15 µL of PCR-Master Mix into PCR reaction tube with 10 µL of RNA sample. Reporter dye detectors were FAM, HEX, ROX for SARS-CoV-2, Flu A, and Flu B respectively. The thermal cycling protocol was reverse transcription 50°C for 5', pre-denaturation 95°C for 1', and 41 cycles of PCR denaturation 95°C for 10" and annealing/extension and fluorescence collection 60°C for 20". To discriminate between Omicron lineages, the presence/absence of S gene of SARS-CoV-2 in positive samples were detected by TaqPath COVID-19 CE-IVD RT-PCR Kit (Life Technologies Corporation, Pleasanton, CA, USA), analyzing N, ORF1ab and S genes. The RT-qPCR was performed on QuantStudio 5 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions; reactions were performed aliquoting 15 µL of Reaction Mix (5.0 µL TaqPath TM 1-Step Multiplex Master Mix, 1.0 µL COVID-19 Real Time PCR Assay Multiplex, 4.0 µL Nuclease-free Water) plus 10 µL of RNA sample. Reporter dye detectors were FAM, VIC, ABY and JUN for ORF1ab, N gene, S gene, MS2 respectively. The thermal cycling protocol was incubation 25°C for 2' reverse transcription 53°C for 10', activation 95°C for 2', 40 cycles of PCR denaturation 95°C for 3" and annealing/extension 60°C for 30".

Library preparation and next-generation sequencing

Upon RT-qPCR evaluation, samples having cycle threshold (Ct) values < 25 and < 30 were selected for multiplexed shotgun and amplicon-based sequencing approaches., respectively Depending on the availability of reagents, three different NGS platforms were employed: Illumina (San Diego, CA, USA), MGI Tech Co (Shenzhen, PRC) and Oxford Nanopore Technology (Oxford, UK). Library preparation was attempted by using different kits: Nextera XT and COVIDSeq protocols for Illumina Technology platforms (MiSeq, MiSeqDx, NextSeq 500, NextSeq 2000) (San Diego, CA, USA), ATOPLEX for MGI (DNBSEQ-G400) (Shenzhen, PRC), NEB-Artic (New England Biolabs, Massachusetts, USA) for Oxford Nanopore Technologies (MinION) (Oxford, UK).

Illumina

Libraries created using positive samples with Ct values < 25 were sequenced with Nextera XT (San Diego, CA, USA), an approach based on enzymatic fragmentation. The recommended amount of cDNA was 1 ng. The Illumina COVIDSeq (San Diego, CA, USA) protocol was used for samples with Ct values < 30. QuantiFluor dsDNA system (Promega, Wisconsin, USA) was used for library quantification and High Sensitivity D1000 kit on 4200 TapeStation (Agilent, California, USA) was used to evaluate size quality. Sequencing was performed using one of these kits and instruments: MiSeq Reagent Kit v2 (300 Cycles) or v3 (600 Cycles) on the Illumina MiSeqDx instrument; NextSeq 500/550 High and Mid Output Kit v2.5 (300 Cycles) on the Illumina NextSeq 500 instrument; NextSeq 1000/2000 P2 and P3 Reagents (300 Cycles) on the Illumina NextSeq 2000 instrument (San Diego, CA, USA).

ONT (oxford nanopore technologies)

The ONT Native Barcoding Expansion Kits were used to prepare amplicons created using PCR-tiling for Nanopore sequencing, in accordance with the manufacturer's instructions. Libraries were run on the MinION-IT MK1B (Oxford, UK) and multiplexed on the FLO-MIN106 Flow Cells (R9.4.1).

MGI Tech

Amplicon libraries were generated via RT and two-step PCR from viral RNA by using the ATOPLEX RNA Library Prep Set (Shenzhen, PRC). Libraries were quantified by QuantiFluor system (Promega, Wisconsin, USA) and FCL and FCS flow cells were chosen based on the number of samples loaded on the DNBSEQ-G400 sequencer (Shenzhen, PRC).

Sequence analysis

Concerning the bioinformatic analysis, we applied a reference-based genome assembly approach consisting of the following main steps: data quality control, reference-based mapping, variant calling, consensus generation and lineage definition. The pipeline for Illumina and MGI sequencing includes as main software: sickle pe (v1.33) for quality control and removal of short reads or low-quality bases (min length > = 100; qscore > = 20); BWA-MEM[57] for reads alignment against the reference genome of SARS-CoV-2 isolate Wuhan-Hu-1 (Genbank acc. n.: MN908947); Samtools for handling SAM and BAM files (v1.15), FreeBayes (v1.3.6)[58] for variant calling (-p 1 –min-coverage 10), BCFtools (v1.15) with vcffilter -f AO/DP > 0.7 for consensus generation and pangolin (v4.1)

for the lineage identification. MinION sequencing output was analyzed through the ARTIC nCoV-2019 specific protocol[59]. Consensus sequences containing > 5% of ambiguous nucleotides (N) were detected and removed from the dataset. Clade assignment, mutation calling, and sequence quality checks were performed with the Nextclade tool[30]. The genomic sequences were submitted in the GISAID EpiCoV database[31].

Statistical analysis

Statistical analysis of percentages was carried out by two-tail, Yates corrected, χ^2 test, whereas parametric values expressed as mean ± standard deviation (SD) were analyzed by Student's t test.

Data availability

All the genomes sequenced in the present study were deposited in the GISAID (https://www.gisaid.org), the global science initiative for genomic data of flu and COVID-19 viruses, as well as all the other genomes in silico analyzed. Access to this dataset can be granted by request to GISAID; in particular, assembled virus genomes for the two case family's data are available at GISAID by typing the following IDs: EPI_ISL_8014526, EPI_ISL_18585079 and EPI_ISL_8014553 for the Delta/Omicron case family's data and EPI_ISL_14149323, EPI_ISL_13826896, EPI_ISL_14149325, EPI_ISL_14149324, EPI_ISL_13204456 and EPI_ISL_14823347 for the Omicron BA.5/BA.5 case family's data. Other IDs of interest can be provided upon request by the corresponding author.

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Declarations

Competing interests
The authors declare no competing interests.

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