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SARS-CoV-2 MOLECULAR EVOLUTION AND HUMAN IMMUNE RESPONSE TO INFECTION

313.02. MEDICAL MICROBIOLOGY, VIROLOGY

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INTRODUCTION

Actuality and importance of the researched problem

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, in December 2019 and quickly spread globally, causing the COVID-19 pandemic [1]. s of March 11th, 2023, the pandemic has caused over 760.4 million confirmed cases and 6,8 million deaths globally [2]. Since the outbreak, the virus has undergone rapid evolution, leading to the emergence of several variants of concern (VOCs) that are more transmissible, virulent, and potentially resistant to immunity induced by natural infection or vaccination. Up to date, the following VOCs were detected: alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2) and omicron (B.1.1.529, BA.2, BA.4, BA.5) [3,4] the first variants have been de-escalated as no more circulating.

Understanding the molecular evolution of SARS-CoV-2 and the human immune response to infection is critical for developing effective strategies to combat the virus and protect public health [5,6].

Genomic sequencing allows real-time monitoring of viral transmission dynamics by tracking sequences that aggregate together in clusters and correlating them with clinical and epidemiological data [7,8]. These data are necessary to timely inform public health about the emergence of VOCs' allowing an efficacious response [9].

On 7 March 2020, the first confirmed case of SARS-CoV-2 infection was registered in the Republic of Moldova [10,11]. In one month, the number of infected people increased to 965, with 854 cases transmitted locally and 111 imported [11,12]. Up to date (July 17, 2023), there have been 620.758 confirmed COVID-19 cases and 12.124 deaths [13].

It is indeed tragic that the COVID-19 pandemic has caused so much loss of life worldwide, including in the Republic of Moldova. The development of vaccines and antiviral drugs, as well as the use of human-neutralising antibodies (nAbs) are all essential strategies to combat the virus [14].

In March 2021, 2000 doses of Sinopharm vaccine were donated to the Republic of Moldova, which were administered exclusively to students and professors at the Nicolae Testemitanu University of Medicine and Pharmacy [15].

While vaccination campaigns are important in preventing the spread of COVID-19, effective therapeutic solutions are still needed [16] to treat people who have already been infected with the virus, especially those at higher risk of developing severe disease.

Preliminary results from clinical trials have shown that the use of human neutralising antibodies targeting the ACE2 receptor binding domain (RBD) of SARS-CoV-2 can reduce disease severity and accelerate recovery in patients with COVID-19 [17,18]. Terapia cu plasmă convalescentă s-a dovedit a fi promițătoare în tratamentul pacienților cu COVID-19 în stare critică [19]. The FDA has also recommended that convalescent plasma with a neutralising antibody titre greater than 1:160 be used for therapeutic transfusions [20]. However, the use of convalescent plasma is limited by the availability of donors with high levels of neutralising antibodies.

Serological tests that detect neutralising antibodies to SARS-CoV-2 are essential for monitoring the effectiveness of vaccines and identifying people who may still be susceptible to the virus. Such tests can also be used to identify people who may have developed neutralising antibodies after being infected with SARS-CoV-2.

Neutralisation assays are considered the gold standard [21] for measuring the antiviral activity of antibodies, including nAbs, against SARS-CoV-2. However, conducting neutralisation assays with live SARS-CoV-2 virus requires the use of biosafety level 3 (BSL-3) containment facilities [22] , which can be expensive and difficult to access. In addition, the handling and manipulation of live virus samples requires highly trained personnel [15] and strict safety protocols to prevent accidental exposure. To overcome this limitation, pseudotyped viruses have been developed as alternatives to infectious viruses [21,23,24]. These viruses allow for the safe and efficient testing of donor plasma or serum for their ability to inhibit virus infection. Pseudotyped viruses are engineered viruses that contain the spike protein of SARS-CoV-2 on their surface but lack the ability to replicate and cause infection. These viruses can be handled safely in biosafety level 2 (BSL-2) containment facilities, making them more accessible and easier to use in research settings [24,25].

The **aim** of this research was to study the molecular evolution of SARS-CoV-2 and the humoral immune response among Sinopharm (BBIBP-CorV) vaccine recipients and COVID-19-recovered patients in Republic of Moldova.

Research objectives:

1. Isolation of SARS-CoV-2 RNA from nasopharyngeal swabs, full genome sequencing of the isolates with high viral load, identification of genomic variants and phylogenetic analysis of SARS-CoV-2 sequences.

2. Assessment of virus neutralisation titres in samples from COVID-19 convalescent plasma donors and from serum of vaccinated people.

3. Assessment of anti-Spike RBD IgG titre in convalescent plasma donors and in serum of vaccinated people.

4. Studying the correlation between neutralising activity and anti-Spike RBD IgG antibody titres among convalescent and vaccinated individuals.

The research methodology:

The research methodology for this study involved full genome sequencing of 19 SARS-CoV-2 isolates from patients with different clinical forms and from different geographical regions of the Republic of Moldova. The isolates were selected from the Biobank of the ALFA Diagnostica laboratory based on a Confidentiality Commitment, using RT PCR reports with a threshold cycle (Ct) value lower than 30. Viral RNA was isolated by the RT PCR method in the Alfa Diagnostica laboratory. Samples with possible new mutations were prioritized to be sequenced. After RNA isolation, samples were stored at -80°C and sent to the Molecular Virology Laboratory, ICGEB, Trieste, Italy, where full genome sequencing was performed. Afterwards, metadata was created, and the results were uploaded to the GISAID international repository. Mutations of each individual isolate were analysed, and a phylogenetic analysis was performed to understand the virus's evolutionary history in this region.

In addition to genome sequencing, it was developed a pseudotyped SARS-CoV-2 lentivirus and two neutralisation assays, one using flow cytometry and another using high content imaging, to investigate the effectiveness of the neutralising activity. An RBD ELISA test was also developed to study the level of IgG anti-Spike RBD antibodies in Sinopharm vaccinated individuals and convalescent patients. Convalescent plasma was taken from the Biobank of the National Blood Transfusion Center, and serum samples were collected from vaccinated patients. All samples were stored at -80°C, anonymized, and sent to the Molecular Virology laboratory, ICGEB, Trieste, Italy, where each sample was tested to assess the anti-SARS-CoV-2 RBD IgG antibody titre and neutralising antibody titre.

The scientific novelty and the outcome of this research

Given the ongoing COVID-19 pandemic, this study's research problem is of the uttermost importance. The scientific novelty of this project lies in the sequencing and phylogenetic analysis of the SARS-COV-2 virus from the Republic of Moldova, which can provide valuable insights into the evolution and spread of the virus in the region. Additionally, developing a pseudotyped SARS-CoV-2 lentivirus, two neutralisation assays and RBD ELISA test can help study the humoral immune response and the efficacy of vaccines against the virus.

Overall, this research can contribute to developing better tools for sera analysis and a better understanding of the immune response to SARS-COV-2 infection and vaccination, which can ultimately aid in the control and management of the COVID-19 pandemic.

Furthermore, the results obtained from this study can also help identify potential individuals who may require booster doses of the vaccine or who may have a weaker immune response to the virus. This can aid in tailoring vaccination strategies and ensuring better protection against COVID-19.

The study was **reviewed and approved** by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 3/24.01.22).

The research was carried out at the Department of Preventive Medicine, Discipline Microbiology and Immunology of Nicolae Testemitanu State University of Medicine and Pharmacy, Chisinau, Republic of Moldova; ALFA Diagnostica Laboratory from Republic of Moldova; Molecular Virology Laboratory, ICGEB, Trieste, Italy; AREA Science Park of Trieste, Italy.

Approval of scientific results

Research results have been presented, discussed and approved at several national and international scientific forums: *Workshop "Strengthening epidemiological surveillance capacity to address COVID-19 and other epidemics*", A Republic of Moldova-Italy cooperation, Online event, 21-23 September 2021; *1st edition of the National Conference with International participation the One Health approach in a Changing World* , Online, 4-5 November, 2021; *Simpozionul Național:"110 ani de la nașterea savantului George Emil Palade, tradiție și continuitate in cercetarea medicala românească".* Târgu Mureș, România, 7-8 December, 2022; *XV International Summer School "Biology, Biotechnology and Biomedicine"*, Odesa, Ukraine, 29 June -10 July, 2020; *Noaptea Cercetătorilor Europeni 2020*. 15 November, 2020; *Virus Detection and Biosecurity - A Capacity-Building Course in the Framework of Article X of the BWC.* Trieste Italy, 14-16 June, 2023; *Tendințe actuale și provocări în medicina preventivă.* Chișinău, Republica Moldova, 8-9 June, 2023; *Congresul consacrat aniversării a 75-a de la fondarea USMF „Nicolae Testemiţanu*" din Republica Moldova, Chişinău, 21-23 Octomber, 2020; *Masa rotundă organizată între AŞM şi USMF*. https://usmf.md/ro/noutati/cercetatorii-usmf-nicolae-testemitanu-vin-cu-noi-date-privind-evolutia-covid-19-tara, Online, 04 September, 2020; *Medtraining-ul organizat de ASRM Asociaţia Studenţilor şi Rezidenţilor în Medicină din Moldova USMF*. Facebook, 26 November, 2020; Atelier de lucru: Strategii de diagnostic şi prevenire a infecţiei COVID-19. online. 09 February, 2021; *Interviu ICGEB: https://www.youtube.com/watch?v=Ve_6DXFZ8sM;*
Emisiune radio Spatiul Public 23.09.2020. ora 10.15; *Emisiune radio, Radio Vocea Speranței*. Facebook 24.11.2020, Mariana Ulinici - Covid 19 – provocarea anului 2020 - YouTube, 24 November, 2020; *Dialoguri interactive între cercetători în cadrul evenimentului Noaptea Cercetătorilor Europeni 2020.* https://usmf.md/ro/noutati/noaptea-cercetatorilor-europeni-la-

chisinau?fbclid=IwAR2OzEgIZsa_bqTAPlnEOFyfZmh2exjixQEZSMir-juS4j6ifOxY81vjEPA; Noaptea Cercetătorilor Europeni 2020 (privesc.eu) minutul -5:04:50, 27 November, 2020; *Emisiunea „Concret" despre vaccinurile anti-COVID și maratonul vaccinării*", TV Moldova1, 24 mai la 17:15 . 24 May, 2021; *Emisiunea „Miezul Zilei"*, TV Moldova1, 26 septembrie, 2021. *https://www.facebook.com/teleradiomoldova/videos/395699578798680,* 26 September, 2021*; Emisiunea „Fii sănătos cu Maria Marian",* Jurnal TV, 22.11.21, ora 18:00 *https://www.facebook.com/watch/?v=183617873984484&ref=sharing,* 22 November, 2021*;* Interviu Sănătate Info: Sănătate Info - Mariana Ulinici, doctorand: „Sunt pasionată de acest domeniu. Lucrul cu virusurile, cu bacteriile, cu microorganismele care se analizează la microscop mă face să meditez" (sanatateinfo.md)*;* Mesager TV Moldova 1, minutul 5:40. *https://fb.watch/aSBGXOXW_6/ https://trm.md/ro/social/noi-metode-de-diagnostic-pentru-virusul-sars-cov-2;* Emisiunea: Pro sănătate: https://youtube.com/playlist?list=PLX… Radio Vocea Speranței Republica Moldova | Facebook, ora 18:00, 23 June, 2022.

Publications on the thesis topic

Academic portfolio comprises 15 scientific publications. This includes four peer-reviewed articles that have been accepted and published in esteemed ISI and SCOPUS. Additionally, two articles have been published in national scientific journals of category B. The entity's active engagement with the scientific community is further evidenced by nine theses that have been presented at various scientific symposia, both at the national and international level.

Key words: SARS-CoV-2 molecular evolution, immune response, mutations, variants, phylogeny, neutralising antibodies.

THESIS CONTENT

1. UNDERSTANDING THE BIOLOGY AND DIAGNOSIS STRATEGIES OF SARS-CoV-2

The chapter reviews the biology of SARS-CoV-2 and associated diagnostic strategies. First, the taxonomy and epidemiology of SARS-CoV-2 are discussed, followed by its genomic organization. An important aspect is reviewed in detail, the S-glycoprotein (spike) of the virus, including its structure and receptor binding domain. The D614G and Omicron variants of the virus are also mentioned.

Next, the humoral immune response in SARS-CoV-2 infection is examined. Strategies used for the diagnosis of SARS-CoV-2 infection are presented, with emphasis on the role and challenges of serological testing. An important aspect of diagnostic strategies is the detection of neutralizing antibodies. These tests assess the ability of antibodies to neutralise the virus and are used to evaluate the efficacy of vaccines or convalescent plasma therapy. The importance and unresolved issues surrounding testing in the fight against COVID-19 are highlighted.

2. MATERIALS AND METHODS

The following biological samples were used: 96 Negative Donor Plasma collected before the announcement of the COVID-19 pandemic in the Republic of Moldova; Convalescent Plasma collected from patients who tested negative for COVID-19 in a PCR test and who are 14 days post clinical recovery (n=100); Serum from Sinopharm vaccinated individuals who are 14 days post second dose of COVID-19 vaccine (n=100) and 25 SARS-CoV-2 RNA samples for sequencing collected in the Republic of Moldova between June 2020 and September 2021.

To obtain the genome sequences, several steps were performed. Nucleic acid extraction was performed using a manual DNA/RNA extraction kit (Vector-Best). Illumina MiSeq was used for sequencing according to the standard protocol for 150-base paired-end reads. Raw data quality control was performed using FastQC software. For adaptor removal and read trimming, the Primerclip trimming tool and Swift Biosciences Accel-Amplicon panel adaptor sequences were used. Genome assembly was performed using dedicated Swift docker data analysis guides.

Phylogenetic analysis was performed using the Nextstrain platform. A sub-sampling strategy was used at the Moldavian level in the Nextregions/Europe dataset. Evolutionary history was inferred using the Maximum Likelihood method and the General Time Reversible model. 542 nucleotide sequences were analysed and 3309 positions were included in the final dataset. The phylogenetic tree was constructed using MEGA7 software and edited using FigTree.

Cell cultures (HEK293T, expressing SV40 T antigen) and SARS-CoV-2 pseudovirus were used to study the humoral immune response. Huh7 and HEK293 cell lines expressing the ACE2 receptor, which were obtained by transduction of a lentivector expressing human ACE2, were also used. The expression level of ACE2 on the HEK293-ACE2 cell line was verified by flow cytometry.

A second-generation lentivector system expressing green fluorescent protein (GFP) was used to prepare the SARS-CoV-2 pseudovirus. HEK-293T cells were transfected with three plasmids. We used the lentiviral transfer plasmid *plVTHM*, which encodes for the reporter gene (eGFP), the HIV packaging plasmid *psPAX2* (which encodes for gag-pol) and a plasmid carrying the sequence coding for the Spike glycoprotein structure, *pcDNA3-SARS-CoV-2-Spike D614GΔ19*. *E. coli DH5α* was used for plasmid propagation.

We performed titration experiments to ensure the accuracy of SARS-CoV-2 lentivirus transduction. VSV lentivirus was used as a positive control because it can infect both HEK 293-ACE2 and HEK 293 cell lines, whereas SARS-CoV-2 lentivirus can only infect HEK 293 expressing the ACE2 receptor on the membrane [15].

Two methods were developed to assess the neutralization of SARS-CoV-2 pseudovirus: one based on flow cytometry (FC) and one based on high-content screening microscopy (HCI). For the FC method, HEK293-ACE2 cells were infected with the pseudovirus and incubated for 72 h. Cells were then harvested and analyzed by flow cytometry to calculate the percentage of infection reduction and neutralization titer. For the HCI method, Huh7-hACE2 cells were infected with pseudovirus and after 48 hours, cells were fixed and digital images were obtained to determine the percentage of transduction and neutralization.

An ELISA test was also developed using RBD Spike SARS-CoV-2 to detect specific antibodies.

Statistical analysis was carried out using R software and correlations and differences between titers obtained by different methods and between groups of participants were assessed.

3. GENOMIC VARIANTS AND PHYLOGENETIC ANALYSIS OF SARS-CoV-2 SEQUENCES FROM REPUBLIC OF MOLDOVA

To get a general idea of the SARS-CoV-2 variants circulating in the Republic of Moldova between March 2020 and May 2022, we studied the phylogenetic relationships between the viral cases in the Republic of Moldova and the main SARS-CoV-2 lineages in Europe.

For this we performed phylogenetic analysis using the Nextstrain bioinformatics platform, whereby 5 clusters were identified, namely 19A, 20A, 20B, 20I/501Y.V1 and 21D from March 2020 to September 2021.

Figure 1 shows the genetic relationship between the complete genome sequences of SARS-CoV-2 in Moldova in the context of the Nextregions/Europe dataset. A country-level sub-sampling strategy was developed at the level of the Republic of Moldova using the reference strain hCoV-19/Wuhan/WH01/2019 (GISAID accession number EPI_ISL_402125) as the initial root [11].

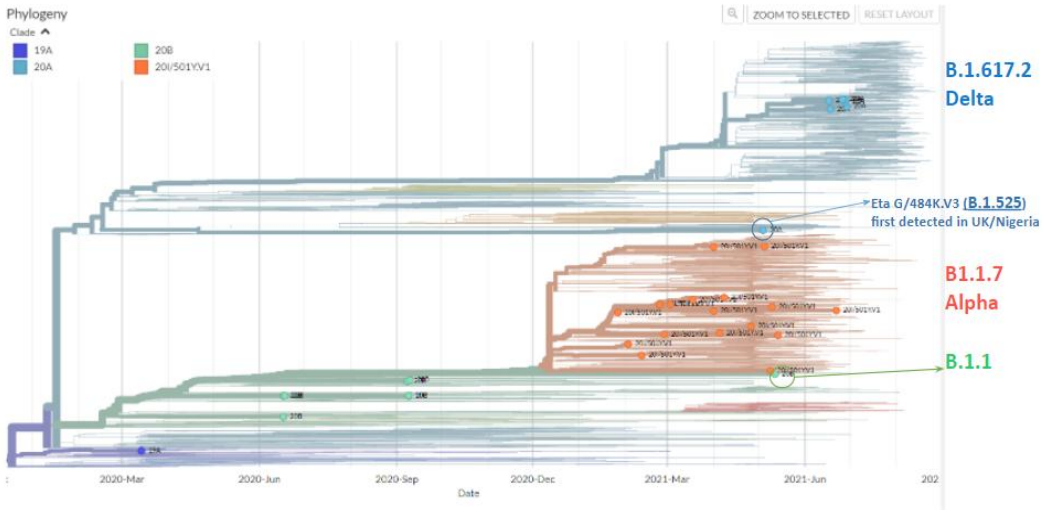


Figure 1. **Genomic evolution of SARS-CoV-2 in Republic of Moldova** [11]

We then decided to focus our phylogenetic analysis on the study of the genomic region corresponding to the spike protein gene, since this protein is one of the main antigenic determinants of this virus. In this context, initially more than 2,900,000 complete SARS-CoV-2 genomes were downloaded from the GISAID database, filtered by European geographic location. Using proprietary scripts, all genomes containing non-deleted nucleotides (NNN) were removed. Since the dataset was still large, a random selection was performed to reduce the number of sequences without losing population variability. The final dataset contains a total of 542 nucleotide sequences from several European countries. Of these 505 sequences, 25 correspond to the samples analysed in this study.

The main SARS-CoV-2 lines are highlighted in the phylogenetic tree (figure 2). As can be seen in the figure, strains from the Republic of Moldova (painted in red) mainly cluster with strains from the Alpha lineage, denoting a close genetic relationship between these sequences. However, the phylogeny also shows somewhat close genetic relationships between circulating variants from Moldova and sequences from the Eta, Lambda and Delta lines. On the other hand, no phylogenetic relationship was observed between the Moldovan and Omicron strains until 26.05.2022.

This shows the great diversity of viral variants that have circulated in Moldova. This result was expected because pandemic events of this style are characterized by a rapid evolution in which many viral variants are generated.

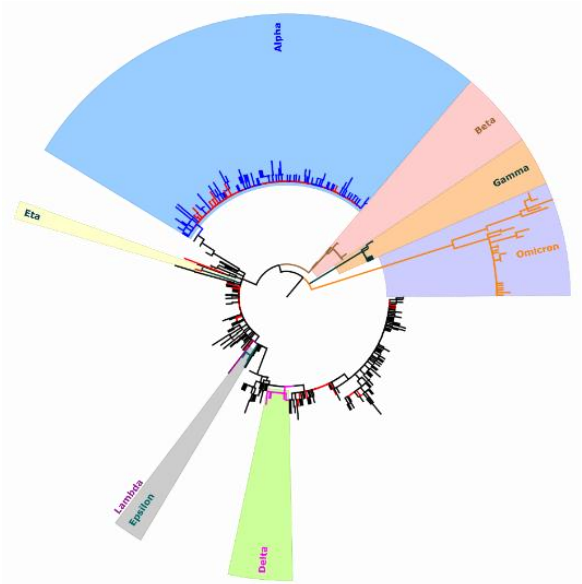


Figure 2. **Maximum-likelihood phylogenetic tree analysis of the spike gene of SARS-CoV2 circulating in Moldova during COVID19 pandemic (March 2020-May 2022).** Looking in an anti-clockwise direction, Delta is highlighted in green, Epsilon (grey), Lambda (magenta), Eta (yellow), Alpha (sky-blue), Beta (pink), Gamma (orange) and Omicron (lilac). The branches of the tree corresponding to sequences from different European countries are coloured in black. Moldova's circulating sequences are highlighted in red.

4. HUMORAL IMMUNE RESPONSES TO SARS-COV-2 IN SINOPHARM VACCINATED AND CONVALESCENT INDIVIDUALS

4.1. Generation of SARS-CoV-2 pseudotyped lentivirus

We produced a SARS-CoV-2 lentivirus carrying the D614G mutation, accompanied by the deletion (Δ19) of the last 19 amino acids at the C-terminal domain, corresponding to the ER-retention motif.

4.2. Titration of the SARS-CoV-2 lentivirus

Table 1. **Transduction efficiency and titre of lentiviral preparations at various volumes**

Lenti SARS-CoV-2		**Lenti VSV**	
Virus volume	% of transduction	Virus volume	% of transduction
200 µL	**54%**	200 µL	52,46%
100 µL	**32%**	100 µL	63,93%
50 µL	**16%**	50 µL	55,1%
25 µL	13,5%	25 µL	35,11%
12.5 µL	12%	**12.5 µL**	**30,2%**
6 µL	10%	**6 µL**	**18,35%**
3 µL	9%	**3 µL**	**11,58%**
NT	0,2%	NT	0,5%
Titre: 3 x 10^5 TU/ml		**Titre**: 6,69 x 10^6 TU/ml	

72 hours after transduction, the quality of the virus produced was assessed. Infection ratios were calculated by observing the presence of GFP protein in infected cells. The percentage of GFP-positive infected cells (highlighted in table 1) was used to estimate the quantity of virus that was produced, while the values that showed the most linearity along the 2-fold dilution curve were chosen to calculate the titre. VSV lentivirus was used as a positive transduction control, as it can infect both HEK 293-ACE2 and HEK 293. In contrast, SARS-CoV-2 lentivirus can only infect HEK 293 because it expresses the ACE2 receptor on the membrane.

Based on this analysis, the SARS-CoV-2 lentivirus preparation had a titre of approximately $3x10^5$ TU/mL, as measured in five independent experiments.

4.3. SARS-CoV-2 neutralising antibodies after natural infection or following vaccination

Our next objective was to examine the ability of antibodies produced by either the Sinopharm vaccine or natural infection to neutralise the virus. In order to achieve this goal, we established two separate assays that utilize SARS-CoV-2 pseudotyped lentiviruses. One of these assays involved the use of HCI microscopy, while the other employed FC.

By employing two different techniques, it was feasible to categorize all the examined samples into three distinct groups: weakly neutralising serum, moderately neutralising serum and strongly neutralising serum. Neutralisation assays showed that only 20% of the samples efficiently neutralised the pseudotyped SARS-CoV-2 at titres above 1:250. In fact, for 50% of sera, the response was weak or absent when determined either by flow cytometry or high content imaging, with titres ranging between 1:10-1:50.

Table 2. **Comparison of quantitative antibody titres between studied groups and subgroups using median and range across methods** [15]

	Overall titre	Titre in convalescent	Titre in vaccinated	Titre in vaccinated (+prior infection)	Titre in vaccinated (naïve)
ELISA IgG RBD; median (range)	1678 (1-13565)	1239 (1-13565)	1742 (152-7184)	1936 (524-6978)	1731 (152-7184)
FC; median (range)	35.8 (1-2182)	27.6 (1-1819)	40.1 (1-2182)	43.9 (1-896)	39.6 (1-2182)
HCI; median (range)	41.3 (1-11051)	24.6 (1-11051)	60.9 (1-10451)	67 (1-3940)	57 (1-10451)

Nonlinear regression analysis was used to determine neutralization titers at half maximum (NT50). When comparing the two methods used for neutralisation assays (table 5), flow cytometry showed a median NT50 of 27.6 (95% CI: 13.6 - 31.8) (range: 1-1819) in convalescent patients, while high content imaging showed a median titre of 24.6 (95% CI: 11.4 - 29.4) (range: 1-11051). The mean convalescent titre was 357 (95% CI: 0 - 623) when tested by HCI and 123 (95% CI: 61.5 - 175) when determined by FC. This indicates that both methods are comparable in terms of detecting neutralising antibodies in convalescent patients.

Nonlinear regression analysis was used to determine half-maximal neutralisation titres (NT50), which were found to be 40.1 (95% CI: 27.8 - 47.4) with range values of 1 to 2182 for vaccinated individuals using the FC method. Similarly, for the HCI method group, the study found that the NT50 was 60.9 (95% CI: 40.2 - 78.6) and the range value of 1 to 10451 (range: 1-10451) (table 2). This indicates that high-content imaging may be slightly more sensitive than flow cytometry in detecting neutralising antibodies in vaccinated individuals.

Overall, these results suggest that both convalescent patients and vaccinated individuals may have a suboptimal humoral immune response to SARS-CoV-2. This may have implications for the durability of protection against the virus and the potential need for booster vaccinations against SARS-CoV-2 in the future.

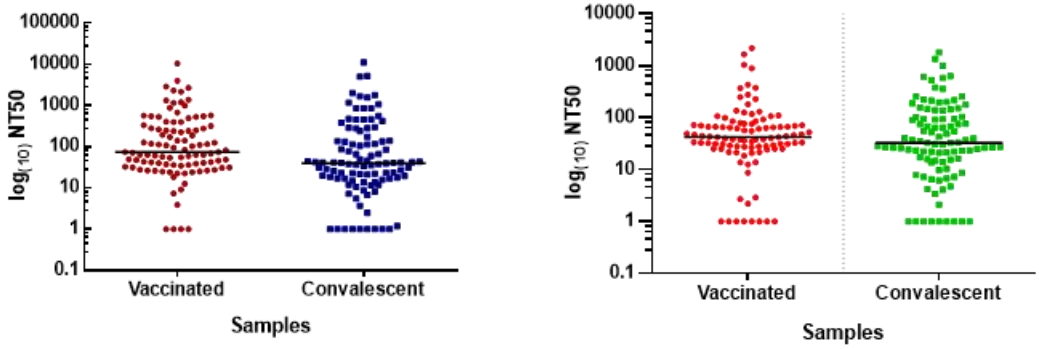


Figure 3. **Neutralising antibody titres in Sinopharm vaccinated cohort, and COVID-19 recovered patients measured by (a) HCI and (b) FC** [15]

Figure 3 shows the neutralising antibody titres in the Sinopharm vaccinated cohort and COVID-19-recovered patients, as measured by high-content screening microscopy (HCI) and flow cytometry (FC). The black line indicates the median titres. The scatter plot displays the results obtained by both methods. For visual clarity, the 25 extreme values are not shown in the plot but were included in the calculations. The sera from healthy donors were tested at a single 1:12,5 dilution, and all showed an $NT_{50} < 0,94$.

4.4. Comparison of SARS-CoV-2 RBD-specific IgG antibody levels in vaccinated individuals and convalescent patients using ELISA

The RBD-specific ELISA test was conducted to measure the level of IgG anti-Spike RBD antibodies in Sinopharm vaccinated individuals and convalescent patients. The OD threshold for the test was 0.0819, and the IgG titres were defined as the reciprocal of the last dilution at which the OD450 was above the threshold.

The results showed that both groups had potent and specific serological activity towards RBD binding compared to pre-pandemic healthy controls (figure 4). However, the Sinopharm vaccination induced a stronger humoral immune response than natural infection, as the vaccinated individuals had significantly higher anti-RBD IgG antibody levels compared to convalescent patients, with median titres of 1742 versus 1239 (table 2). These findings are consistent with previous studies that have reported higher antibody titres in vaccinated individuals compared to those who have recovered from COVID-19 [15].

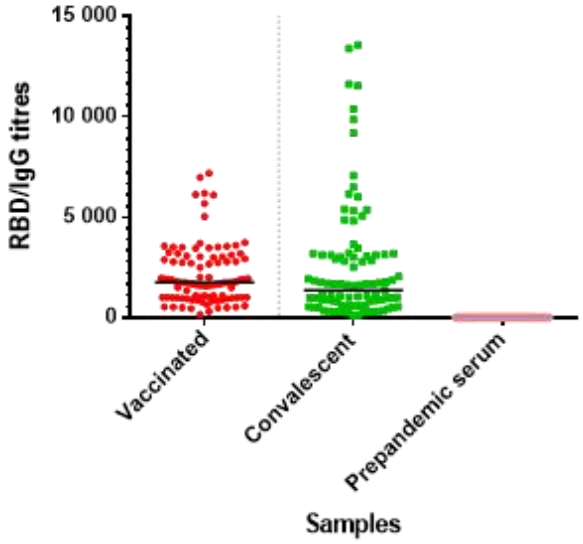


Figure 4. **Comparison of Anti-SARS-CoV-2 RBD IgG antibody levels in Sinopharm-vaccinated, COVID-19 recovered and seronegative individuals**

The overall titre (measured in 200 samples) had a mean titre of 2322 (CI: 1991-2653) and a median titre of 1678 (CI: 1598-1863). It exhibited a higher standard deviation (2342) and a wider interquartile range (2151) compared to the convalescent and vaccinated titres. The range of values observed for the overall titre was 1 to 13565.

In the convalescent group (measured in 100 samples), the mean titre was 2519 (CI: 1906-3069) and the median titre was 1239 (CI: 745-1486). It had the highest standard deviation (2959) and interquartile range (2521), with a range of values from 1 to 13565.

For the vaccinated group (measured in 100 samples), the mean titre was 2126 (CI: 1830-2409) and the median titre was 1742 (CI: 1565-1882). It had the lowest standard deviation (1481) and interquartile range (1930), with a range of values from 152 to 7184.

Additionally, SARS-CoV-2 RBD-specific IgG antibodies were present in the sera of all vaccinated subjects, while one convalescent individual (60CP) had undetectable titre values. This individual appears to be a healthy non-responder who did not produce antibodies after recovering from COVID-19 [15].

4.5. Correlation between neutralising and anti-RBD SARS-CoV-2 IgG antibodies

The spike receptor-binding domain (RBD) of the SARS-CoV-2 virus is a critical component in triggering the production of neutralising antibodies (nAbs) that can help protect against the virus. The RBD also significantly impacts the activation of T-cell immune responses, further emphasizing its importance in the body's defence against COVID-19 [27].

The results of the correlation analysis between the neutralising antibodies obtained by FC or HCI assays and anti-RBD SARS-CoV-2 IgG antibodies as measured by ELISA are shown in the table 3.

Table 3. **Correlation between SARS-CoV-2-specific antibody responses and neutralisation titres using different methods**

Group	method_1	method_2	correlation_coefficient (spearman)	p_val
All	ELISA_RBD	FC	0.64	< 0.001
Convalescent	ELISA_RBD	FC	0.68	< 0.001
Vaccinated	ELISA_RBD	FC	0.58	< 0.001
All	HCI	ELISA_RBD	0.52	< 0.001
Convalescent	HCI	ELISA_RBD	0.45	< 0.001
Vaccinated	HCI	ELISA_RBD	0.53	< 0.001
All	FC	HCI	0.55	< 0.001
Convalescent	FC	HCI	0.51	< 0.001
Vaccinated	FC	HCI	0.58	< 0.001

There was found moderate to strong positive correlation between the neutralising and anti-RBD SARS-CoV-2 IgG antibodies, with correlation coefficients ranging from 0.45 to 0.68 and p-values less than 0.001 for all comparisons. This suggests that higher levels of anti-RBD SARS-CoV-2 IgG antibodies are associated with a stronger neutralising response to the virus.

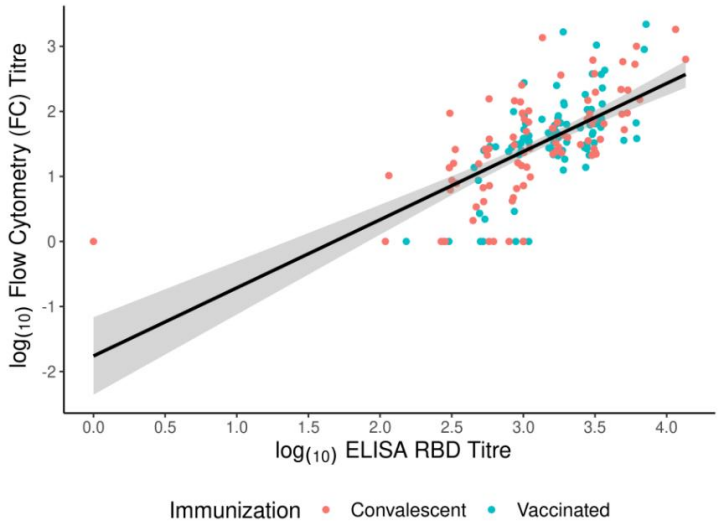


Figure 5. **Comparison of overall log10 transformed ELISA RBD and FC titres** [15]

Specifically, the correlation coefficients between ELISA RBD and FC assays were 0.64 for all participants, as shown in figure 5, while between ELISA RBD and HCI assays, the correlation coefficients were the lowest (ρ=0.52) for all participants, the results being presented in figure 6.

Furthermore, when we compared the correlation coefficients between the tests in convalescent and vaccinated groups separately, we found similar results, suggesting that the correlation was not influenced by the type of immune response (i.e., natural infection *vs.* vaccination). The correlation coefficients between ELISA RBD and FC assays were 0.68 for convalescent patients, and 0.58 for vaccinated individuals (figure 6).

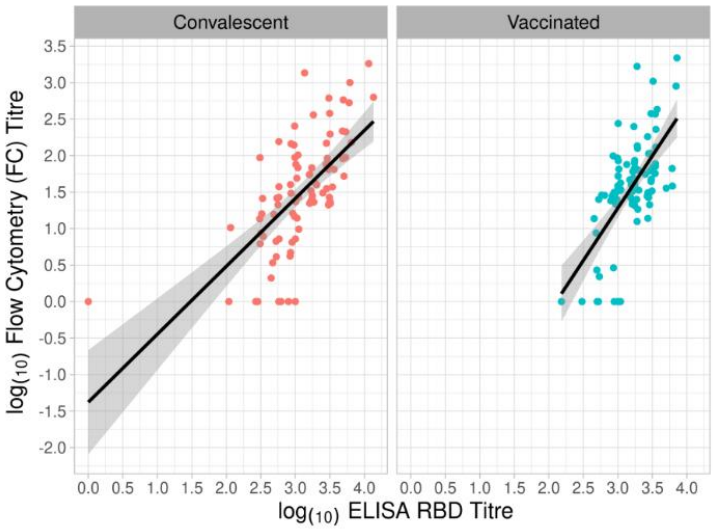


Figure 6. **Correlation between log10 transformed ELISA RBD and FC titres in convalescent versus vaccinated individuals** [15]

The correlation coefficients between neutralising antibodies detected by HCI and the anti-SARS-CoV-2 Spike RBD antibodies titres determined by ELISA assay were slightly higher in the vaccinated individuals (ρ=0.53) than in the convalescent patients (ρ=0.45). We found a statistically significant difference between the groups only in the HCI assay ($p < 0.001$), with the median titre of vaccinated individuals being significantly higher than that of convalescent patients.

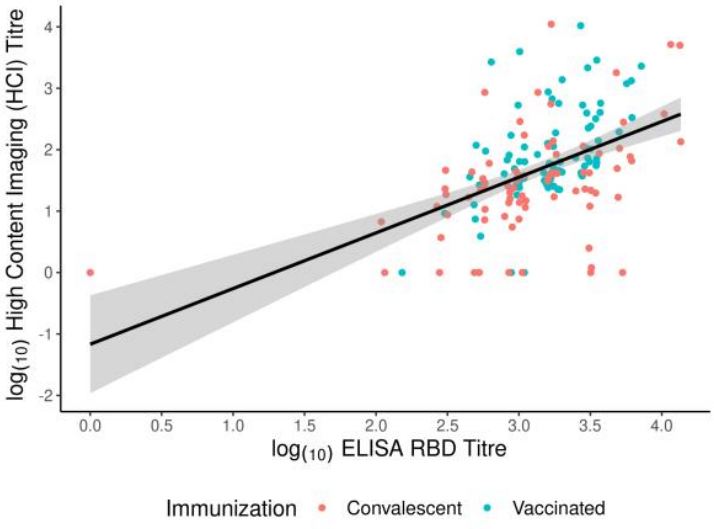


Figure 7. **Correlation between anti-RBD IgG antibodies and HCI neutralising titres in human sera** [15]

Lastly, the correlation coefficients between FC and HCI assays were 0.55 for all participants, 0.51 for convalescent patients, and 0.58 for vaccinated individuals.

The plot in figure 8 illustrates the relationship between anti-RBD IgG and neutralising antibody levels in convalescent and vaccinated subjects. The graph includes different colour

points indicating convalescent (red) and vaccinated (blue) participants. The black line represents the best estimate of the relationship between these two variables, while the grey band represents the range of values the genuine relationship will likely fall within. The fact that there is a best-fit linear regression line with a corresponding confidence interval suggests a correlation between the levels of anti-RBD IgG and neutralising antibodies in these individuals.

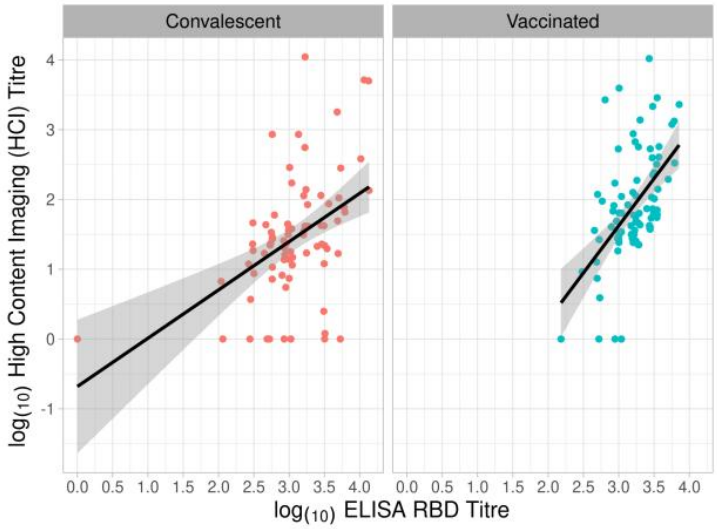
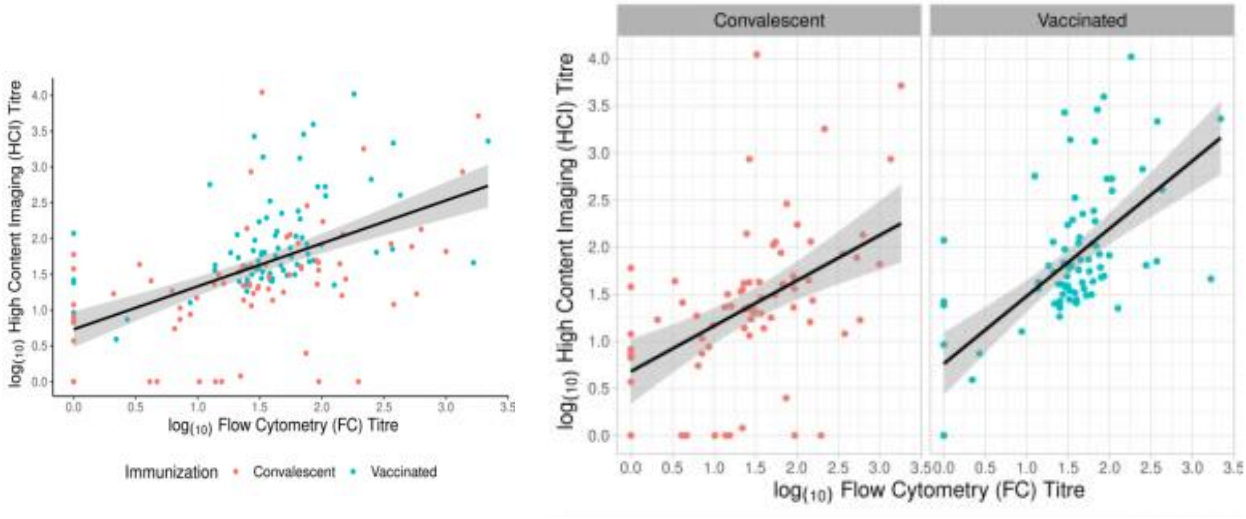


Figure 8. **Anti-RBD IgG antibodies in human sera and its correlation with NT_{50} determined by HCI**

Interestingly, we observed that there were some samples with undetectable nAbs that still had binding antibodies, indicating that a large proportion of antibodies do not neutralize the virus. However, the higher the binding titre, the more likely that neutralisation is detected, suggesting that binding and neutralisation do correlate to some extent. Our findings suggest that measuring neutralising and binding antibodies is important for comprehensively evaluating the humoral immune response to SARS-CoV-2.



(**a**) (**b**)

Figure 9. **Correlation of neutralising antibody titres in convalescent and vaccinated groups, determined by FC and HCI**

Individuals who have had both previous SARS-CoV-2 infection and have received a vaccine showed higher antibody levels compared to those who only had one or the other. The difference was statistically significant only in the HCI titres between convalescent and vaccinated individuals ($p<0.05$). Interestingly, there was no statistical significance in the comparison between those who had recovered from the infection and those who had received the vaccine but never contracted the virus. Furthermore, convalescent individuals and those who were both previously exposed to the virus and vaccinated had significantly higher antibody levels compared to those who had only received the vaccine ($p < 0.001$).

Figure 10 provides a detailed comparison of antibody levels between these different subgroups of participants, and shows the distribution of antibody titres for each group.

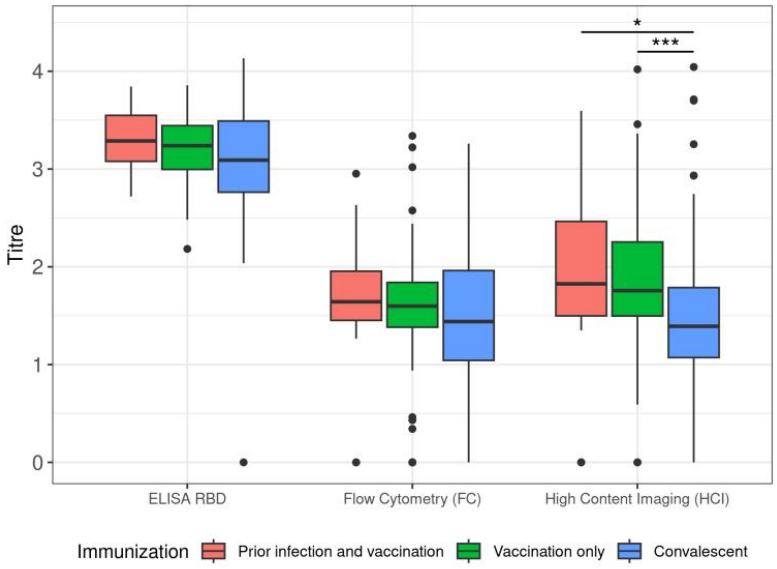


Figure 10. **The relationship between SARS-CoV-2 exposure and vaccine-induced antibody responses**

Our study provides important insights into the correlation between neutralising and anti-RBD SARS-CoV-2 IgG antibodies. These findings have important implications for developing effective treatments and vaccines against COVID-19, as they suggest that a strong humoral immune response, including neutralising and binding antibodies, may be necessary for protection against the virus. However, further research is needed to clarify the exact nature of the relationship between binding and neutralising antibodies, confirm this relationship, evaluate the long-term protective immunity conferred by these antibodies, and determine the most effective methods for measuring the humoral immune response to SARS-CoV-2.

CONCLUSIONS

1. SARS-CoV-2 undergoes a continuous molecular evolution, as evidenced by the presence of several amino acid substitutions in viral proteins in the samples analysed from the Republic of Moldova. Some of these mutations are frequent and may have implications on the transmissibility, virulence and immune escape of the virus, such as Spike D614G, N G204R, N R203K, L452R and P681H mutations.

2. Ongoing monitoring of the molecular evolution of SARS-CoV-2 is necessary to identify emerging mutations and their potential impact on the effectiveness of vaccines and treatments.

3. The SARS-CoV-2 pseudotype lentivirus was successfully produced using a 2nd generation HIV-LV system, which could be used to identify antibodies that can detect the spike protein in its natural form.

4. The neutralisation assays showed that only 20% of the samples in population under study efficiently neutralised the pseudo-type SARS-CoV-2 at titres above 1:250, indicating that a significant portion of convalescent and vaccinated individuals possibly did not have a developed strong humoral immune response to the virus.

5. The Sinopharm COVID-19 vaccine induces a robust and specific immune response in vaccinated individuals, as shown by the high levels of anti-RBD IgG antibodies detected in serum samples.

6. The humoral immune response induced by the Sinopharm vaccine is stronger than the response observed in individuals who have recovered from COVID-19, as indicated by the higher levels of anti-RBD IgG antibodies detected in vaccinated individuals compared to convalescent patients.

7. There is a moderate positive correlation between the levels of anti-RBD SARS-CoV-2 IgG antibodies and the neutralising response to the virus. This suggests that higher levels of anti-RBD SARS-CoV-2 IgG antibodies are associated with a stronger neutralising response to the virus.

8. The study demonstrated the significance of developing diagnostic tools and conducting studies on the humoral immune response to fight against the transmission of SARS-CoV-2 and mitigate its impact on public health effectively. This is especially important for the Republic of Moldova, as such efforts can provide invaluable insights into the molecular epidemiology of the virus and contribute to the global fight against the pandemic.

RECOMENDATIONS

1. It is crucial to strengthen and enhance the effectiveness of surveillance systems to monitor the molecular evolution of SARS-CoV-2. Regular monitoring should be conducted to track the prevalence of mutations, especially those with implications for transmissibility, virulence, and immune escape.

2. Scientific research should continue to develop diagnostic tools that can accurately measure the humoral immune response to SARS-CoV-2. By measuring both neutralising and binding antibodies, a comprehensive evaluation of the immune response can be achieved. This will provide valuable insights into the effectiveness of vaccines, identify individuals with weak immune responses, and assist in the development of targeted therapies.

3. Given the global impact of the pandemic, regional and international collaboration and sharing of scientific data and findings are crucial. The Republic of Moldova should actively participate in global initiatives to combat the transmission of SARS-CoV-2. Sharing insights from studies conducted in the country can contribute to the global fight against the pandemic and assist in the development of effective containment and treatment strategies.

4. It is recommended to implement the developed protocols and tools from this research project into the teaching and practical curriculum of the microbiology department. This will provide students with hands-on experience and exposure to cutting-edge research methodologies,

preparing them for future scientific endeavours and equipping them with knowledge and skills to be prepared and response to potential future pandemic.

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LIST OF SCIENTIFIC PUBLICATIONS AND EVENTS

at which the results of the researches for the doctoral thesis in medical sciences with the topic **„SARS-CoV-2 molecular evolution and human immune response to infection"** were presented

- **Articles in ISI, SCOPUS journals and other international databases:**

1. **Ulinici, M.,** Covantev, S., Wingfield-Digby, J., Beloukas, A., Mathioudakis, A.G., Corlateanu, A. Screening, Diagnostic and Prognostic Tests for COVID-19: A Comprehensive Review. In: *Life.* 2021;11(6), p. 561. ISSN: 2075-1729. https://doi.org/10.3390/life11060561, (**IF: 3,817**).

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- **Abstracts/theses submitted at national or international scientific conferences**

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✓ **international**

16. **Ulinici, M.,** Strengthening epidemiological surveillance capacity to address COVID-19 and other epidemics - Presentation of project's results and impact. *Workshop "Strengthening epidemiological surveillance capacity to address COVID-19 and other epidemics*", Online event 21-22-23 SEPT 2021, A Republic of Moldova-Italy cooperation.

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- **Participation in media programmes on science and education, innovation and technology transfer**

27. **Ulinici M.,** Interviu ICGEB: https://www.youtube.com/watch?v=Ve_6DXFZ8sM

28. **Ulinici M.,** "Informații despre genomul complet al SARS-CoV-2 izolat de la pacientii din RM" Participare la emisiune radio 23 09.2020. ora 10.15. http://trm.md/ro/spatiul-public/spatiul-

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31. **Ulinici M.,** Participare la Emisiunea „Concret" despre vaccinurile anti-COVID și maratonul vaccinării", TV Moldova1, 24 mai la 17:15 . https://www.facebook.com/tvmoldova1/videos/230641248427050

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- **Invention patents, patents, registration certificates, materials at invention salons**

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- **International research traineeships**

45. THE ARTURO FALASCHI ICGEB Short-term FELLOWSHIP at a PhD level (F/MDA20-01). Perioada: 04.06.21-30.08.21.

46. Visiting researcher în cardul proiectului: Capacity building in Virus Surveillance to tackle COVID-19 and beyond. Parteneri: ICGEB, Trieste Italia. (ICGEB, grant nr. CUP:D87D20000020009), Laboratorul de virusologie moleculară, ICGEB, Trieste, Italia: 15.10.21- 19.12.21