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GENOTYPIC DIVERSITY OF SARS-COV-2 DURING THE PANDEMIC AND EARLY POST-PANDEMIC PERIOD

313.02 –MEDICAL MICROBIOLOGY AND VIROLOGY

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INTRODUCTION

Research relevance. The emergence and rapid spread of SARS-CoV-2 in late 2019 triggered one of the most serious global public health crises, with major consequences for healthcare systems, economies, and society [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**].

On 5 May 2023, the World Health Organization declared the end of the public health emergency of international concern; nevertheless, the virus continues to circulate globally, keeping research in this field relevant [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**]. The WHO statement also noted that SARS-CoV-2 would continue to circulate and evolve.

The genetic mutability of SARS-CoV-2 drives the continuous emergence of variants and subvariants with altered biological properties, including increased transmissibility and immune escape. These changes may affect the performance of diagnostic tests, antiviral therapies, and vaccines, making continuous viral genome monitoring necessary [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**].

Genomic sequencing is a core tool of modern epidemiological surveillance, providing detailed information on viral evolution and transmission dynamics [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**]. Combining genomic, clinical, and epidemiological data supports rapid outbreak detection and evidence-based public health measures [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**]. In the Republic of Moldova, the implementation of modern molecular biology techniques and genomic sequencing has enabled national monitoring of SARS-CoV-2 circulation [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**].

Wastewater-based genomic epidemiology also enables early detection of SARS-CoV-2 in both symptomatic and asymptomatic individuals [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**]. This approach is an effective tool for monitoring viral circulation and assessing transmission dynamics at the community level [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**].

Therefore, the relevance of this research lies in the need to maintain integrated SARS-CoV-2 surveillance through genomic sequencing and wastewater-based epidemiology, enabling rapid detection of emerging variants and more effective public health interventions [**Eroare! Fără sursă de referință.**,**Eroare! Fără sursă de referință.**].

The aim of the study is to identify SARS-CoV-2 variants circulating in the Republic of Moldova and perform phylogenetic analysis of the detected isolates, in order to improve COVID-19 surveillance and morbidity-control measures.

Research objectives:

1. To characterize phylogenetically the SARS-CoV-2 isolates detected in the Republic of Moldova.
2. To study the evolution of SARS-CoV-2 by identifying substitutions, insertions, and deletions, and comparing them with global viral genome diversity.

3. To analyze the dynamics of SARS-CoV-2 circulation and the emergence of new genetic variants, and to investigate the relationship between viral genotypes and epidemiological data.
4. To monitor wastewater as a method for predicting epidemiological trends in SARS-CoV-2 infection.
5. To assess the impact of different SARS-CoV-2 viral genotypes and substantiate proposals for the laboratory diagnosis of COVID-19.

Research hypothesis. Continuous monitoring of SARS-CoV-2 circulation and identification of circulating variants in clinical and environmental samples from the Republic of Moldova will strengthen epidemiological surveillance of viral respiratory infections, improve epidemic forecasting and preparedness, and increase the effectiveness of the response to a potential future pandemic.

Scientific novelty and originality of the research findings. For the first time in the Republic of Moldova, original data were obtained on the genotypic and phenotypic characteristics of SARS-CoV-2 circulating within the country. The use of sequencing techniques to study SARS-CoV-2 strains made it possible to determine their position within global phylogenetic trees, which is essential for understanding viral evolution.

The study has scientific and practical relevance not only for public health, but also for the healthcare system, local public administration, and the academic community, by supporting and optimizing COVID-19 control and response measures.

As a result of this research, SARS-CoV-2 genovariants circulating in the Republic of Moldova were identified in both clinical and wastewater samples.

SARS-CoV-2 sequencing data were used as prognostic indicators for the dynamics of the epidemic process. The results will be used in the development of documents such as national clinical protocols and guidelines, and in the training of specialists, students, and resident physicians at "Nicolae Testemițanu" State University of Medicine and Pharmacy.

Applied scientific value of the study. This study characterized the evolution of SARS-CoV-2 in the Republic of Moldova during the pandemic and early post-pandemic periods by identifying and evaluating the genotypic and phenotypic diversity of circulating strains. It demonstrated the impact of newly emerging strains on COVID-19 morbidity and mortality in the country. The study also showed the high performance of Freyja for the analysis and interpretation of wastewater sequencing data.

Implementation of the research results. Based on the findings of this research, the good practice guide *"Metagenomic Sequencing Using Illumina Technology"* was developed. The study also led to the preparation of the information booklet *"Good Practices in Genomic Sequencing"* for students, resident physicians, researchers, and medical specialists. Another information booklet, *"Fundamentals of a Responsible Biosafety and Biosecurity Culture",* was developed for the same professional groups. The study findings were also used to prepare the monograph *"Nucleic Acid Sequencing: An Overview of Evolution and Technological Innovation".* In collaboration with the team involved in developing the national clinical protocol, the National Clinical Protocol *"Novel Coronavirus Infection (COVID-19)",* NCP-371, Chișinău, 2025, was updated.

Approval and validation of the results. The study methodology and design underwent a three-stage approval process: review by the Scientific Council of the National Agency for Public Health on 18 January 2023, favorable approval by the Research Ethics Committee of "Nicolae

Testemițanu" State University of Medicine and Pharmacy on 20 September 2023, and validation by the specialized scientific seminar on 20 December 2023. This process confirmed the research topic, the scientific supervisor, and the advisory committee. The research was carried out in the Virology Laboratory of the Public Health Laboratory Diagnostics Directorate of the National Agency for Public Health and in the National Reference Laboratory for Genomic Sequencing of the same institution. The thesis was reviewed and approved by the Scientific Council, minutes no. 7 of 18 November 2025; by the joint meeting of the doctoral supervisor, advisory committee, and primary research unit, minutes; and by the specialized scientific seminar in 313 Immunology, Microbiology, Virology and 321 General Medicine / 313.02 Medical Microbiology and Virology / 321.09 Infectious, Tropical, and Parasitic Diseases, minutes of 27 May 2026. The Scientific Council of the Consortium recommended the thesis for public defense by decision no. 4 of 1 July 2026.

Dissemination of research findings. The research findings resulted in 15 scientific works, viz. 6 articles published in scientific journals, including one journal article, 3 articles in peer-reviewed international journals, and two articles in national category B journals; 4 first-author articles; 9 abstracts published in the proceedings of national and international scientific conferences; one monograph; 13 active presentations, including oral and poster presentations at national and international scientific conferences and congresses; 2 innovator certificates; 2 copyrighted scientific works; one national clinical protocol; and 7 implementation acts documenting the practical application of the research results.

Thesis structure and volume. The thesis comprises 94 pages of main text, including the following sections: title page, table of contents, lists of annexes, abbreviations, figures and tables, introduction, four chapters, general conclusions, recommendations, 189 references, and 12 annexes. The illustrative material includes 8 tables and 42 figures.

Keywords: COVID-19; SARS-CoV-2; sequencing; NGS; viral genome; genetic monitoring; mutation variants; wastewater.

THESIS CONTENT

1. MOLECULAR CHARACTERISTICS OF EMERGING SARS-COV-2 VARIANTS AND LABORATORY DIAGNOSIS OF ASSOCIATED INFECTIONS

This chapter reviews current evidence on the biology, genetic evolution, and molecular characteristics of SARS-CoV-2, with emphasis on the mechanisms underlying mutation, recombination, and viral adaptation. It describes the viral genome and protein structure, the replication cycle, and the role of structural proteins in infection and host-cell interaction.

The chapter also discusses SARS-CoV-2 variant classification and nomenclature according to the main international systems, including WHO, PANGO, GISAID, and Nextstrain. Particular attention is given to variants of concern and their effects on transmissibility, virulence, and immune escape. The molecular epidemiology of COVID-19 is also addressed, including transmission mechanisms, risk factors, and key clinical and epidemiological features.

A separate section focuses on modern diagnostic approaches, including RT-PCR, next-generation sequencing, and bioinformatic analysis, highlighting their importance for variant identification and epidemiological surveillance. The main sequencing platforms are presented, together with their advantages for genomic research.

Furthermore, wastewater monitoring of SARS-CoV-2 is also discussed as an innovative approach to epidemiological surveillance. The chapter highlights its value for early detection of viral circulation, assessment of transmission dynamics, and identification of emerging variants. It also addresses the advantages of this approach, its technical limitations, and the need for standardized methods and appropriate bioinformatic tools. This terminology is consistent with current literature on wastewater genomic surveillance, which describes wastewater sequencing as a tool for early detection of SARS-CoV-2 variants and community-level monitoring.

2. RESEARCH MATERIALS AND METHODS

The study was initiated in 2022 at the Virology Laboratory of the National Agency for Public Health. It was designed as a comprehensive investigation conducted during the implementation of whole-genome sequencing for SARS-CoV-2 in the Republic of Moldova, covering the period 2021–2025.

The analysis used SARS-CoV-2 sequences obtained from nasopharyngeal and oropharyngeal swabs, as well as from wastewater samples. SARS-CoV-2 infection was confirmed by RT-PCR using different commercially available reagent kits. Positive samples were selected for sequencing according to predefined inclusion and exclusion criteria.

The study included a representative set of biological samples from different regions of the country, collected from patients with varying demographic characteristics and disease severity, together with wastewater samples. The sample size was calculated for a 95% confidence level and a 5% margin of error, resulting in a minimum of 240 samples according to the formula $n = P(1–P)(Z\alpha/d)^2$.

Thus, the study analyzed 733 biological samples collected between November 2021 - November 2024 for SARS-CoV-2 monitoring by genomic sequencing and phylogenetic analysis. As PCR testing declined, wastewater surveillance was introduced, and 31 wastewater samples collected during 2024–2025 were sequenced, allowing circulating strains to be identified.

The study was carried out in three stages. *First*, the literature was reviewed to summarize current evidence on the genetic characteristics and evolution of SARS-CoV-2, newly emerging variants with epidemiological significance, and their public health impact.

Second, RT-PCR-positive samples meeting the inclusion and exclusion criteria were selected for sequencing. Whole-genome sequencing was performed on the Ion Torrent Genexus and Illumina MiSeq platforms, and the data were analyzed using international databases and bioinformatic tools, including Nextclade, Nextstrain, GISAID, Pangolin, Freyja, and Kallisto.

In the *third stage*, the findings were disseminated through scientific publications, including articles in national and international journals, conference abstracts, oral and poster presentations at national and international scientific meetings, and the publication of guidelines, booklets, and a monograph.

3. GENETIC CHARACTERISTICS OF SARS-COV-2 STRAINS IDENTIFIED IN CLINICAL SAMPLES

3.1. Genetic Analysis of SARS-CoV-2 Isolate Variability in the Republic of Moldova, 2021–2024

A total of 733 SARS-CoV-2 whole-genome sequences were obtained during the study period. Of these, 49 sequences, 6.7%, were assigned to Delta, B.1.617.2; 680 sequences, 92.77%, to Omicron, B.1.1.529; and 4 sequences, 0.6%, to recombinant variants.

The sequenced samples were collected from patients aged 3–94 years, with a mean age of 57.5 ± 21.3 years, 95% CI: 56–59. Men accounted for 44.3%, 95% CI: 41–48%, and women for 55.7%, 95% CI: 52–59%.

Most patients were from urban areas, 79.1%, 95% CI: 76–82%, while 20.9%, 95% CI: 18–24%, were from rural areas.

Phylogenetic analysis against the Wuhan-Hu-1 reference strain showed variable amino acid polymorphism across the sequenced genomes. SARS-CoV-2 genetic diversity is driven by the frequent recombination of genomic RNA, which contributes to the emergence of new viral variants with altered properties and the potential to trigger new pandemic waves, Figure 1.

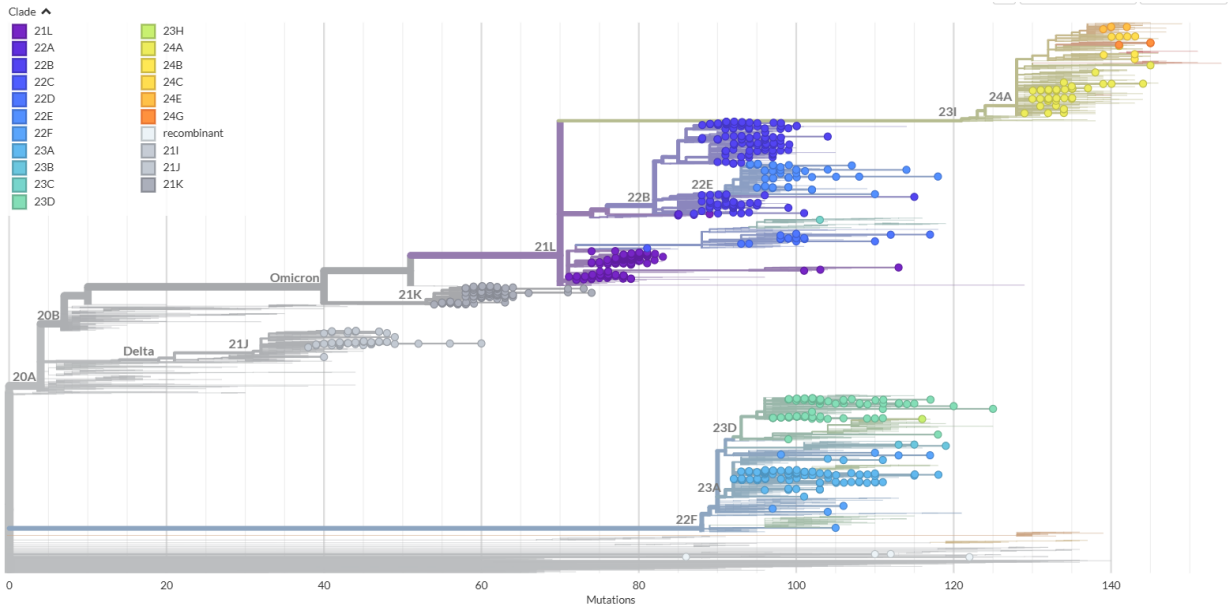

Figure 1. Phylogenetic relationships among the SARS-cov-2 isolates analyzed in this study

The *SARS-CoV-2 genome sequences* identified in the Republic of Moldova were distributed across a broad phylogenetic range, from clade 20A to clade 24A, and clustered with variants circulating globally during the study period. Whole-genome analysis identified 21 distinct clades: 21L, 21I, 21J, 21K, 22A, 22B, 22C, 22D, 22E, 22F, 23A, 23B, 23C, 23D, 23H, 24A, 24B, 24C, 24E, and 24G, together with one recombinant group. Of these, six clades, 21L, 22B, 22E, 23A, 23D, and 24A, represented major evolutionary branches, as they included most of the sampled genomes.

The analysis also revealed unexpected findings. Four recombinant sequences, shown as white points, were identified. These may indicate either local viral evolution or simultaneous infection with two sublineages that subsequently gave rise to recombinant lineages. Recombination and co-infection are recognized mechanisms in SARS-CoV-2 evolution and have been described in phylogenomic studies.

Omicron showed rapid evolutionary change, progressing from clade 21K to 21L with approximately 60 mutations. Clades 22A, 22B, 22C, 22D, 22E, and 22F showed further divergence, reaching 90–120 mutations. Divergence in clades 23A, 23B, 23C, 23D, 23H, 24A, 24B, 24C, 24E, and 24G increased to 120–140 mutations. Notably, Omicron descendants reached approximately 140–150 mutations, about four times more than Delta, clade 21J, which had up to 40 mutations in its genome. The high divergence of Omicron compared with pre-Omicron variants was previously associated with changes in pathogenicity and a tendency toward milder clinical manifestations.

Clades 24K, 21L, and 22B represent earlier evolutionary groups with fewer mutations, usually around 80, and include strains that circulated predominantly during 2021–2022. Clades 22E, 22F, 23A, and 23D, which were widespread during 2022–2023, accumulated up to 100 mutations per genome, whereas the more recent major clades, 23I and 24A, reached approximately 120–140 mutations.

3.2. Molecular Characteristics of Substitutions, Deletions, and Insertions in SARS-CoV-2 Evolution

Comparative analysis of SARS-CoV-2 genomic variants showed that viral evolution and genomic change were driven mainly by substitutions, with a mean of 72.6 ± 21.1 substitutions per genome, 95% CI: 71–74, and deletions, with a mean of 48.6 ± 20.1 per genome, 95% CI: 47–50. Insertions and DCC were detected much less frequently, with mean values of 4.5 ± 8.3, 95% CI: 3.9–5.1, and 1.7 ± 2.2, 95% CI: 1.5–1.8 per genome, respectively.

Figure 2 presents boxplots showing the distribution of nucleotide substitutions across the Nextstrain clades, together with the distribution of amino acid substitutions across the different clades. These graphical representations clearly show that, over time, from 2021 to 2024, the numbers of both nucleotide (a) and amino acid substitutions (b) increased substantially. Thus, the plots also show that Omicron subvariants of SARS-CoV-2 had higher average numbers of mutations than the Delta variant.

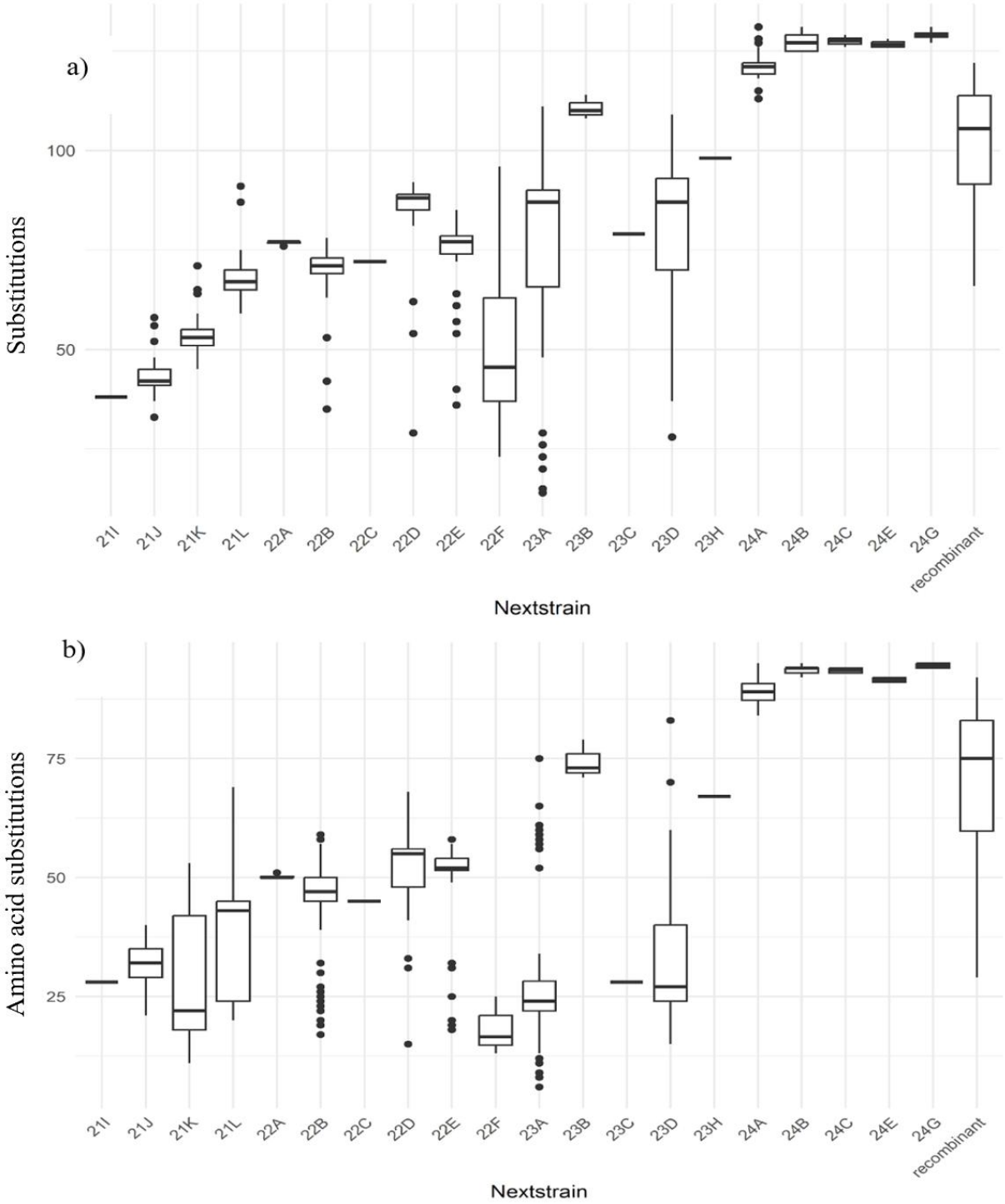

Figure 2. Boxplot distribution of the number of nucleotide and amino acid substitutions according to SARS-CoV-2 clades.

Mutation analysis of the sequenced viral genomes was performed at two levels: the nucleotide level and the amino acid level. As shown in Figure 2, the total number of substitutions was clearly clade-dependent. Both nucleotide and amino acid substitutions increased with viral evolution and the emergence of new clades. The greatest genetic diversity was observed in the Omicron clades, with mean values reaching around 120 nucleotide substitutions and more than 80 amino acid substitutions. Recombinant strains also showed high mean mutation counts, at approximately 110 nucleotide substitutions and 75 amino acid substitutions.

The distribution of deletions in the SARS-CoV-2 genome according to the Nextstrain clade group is shown in Figure 3.

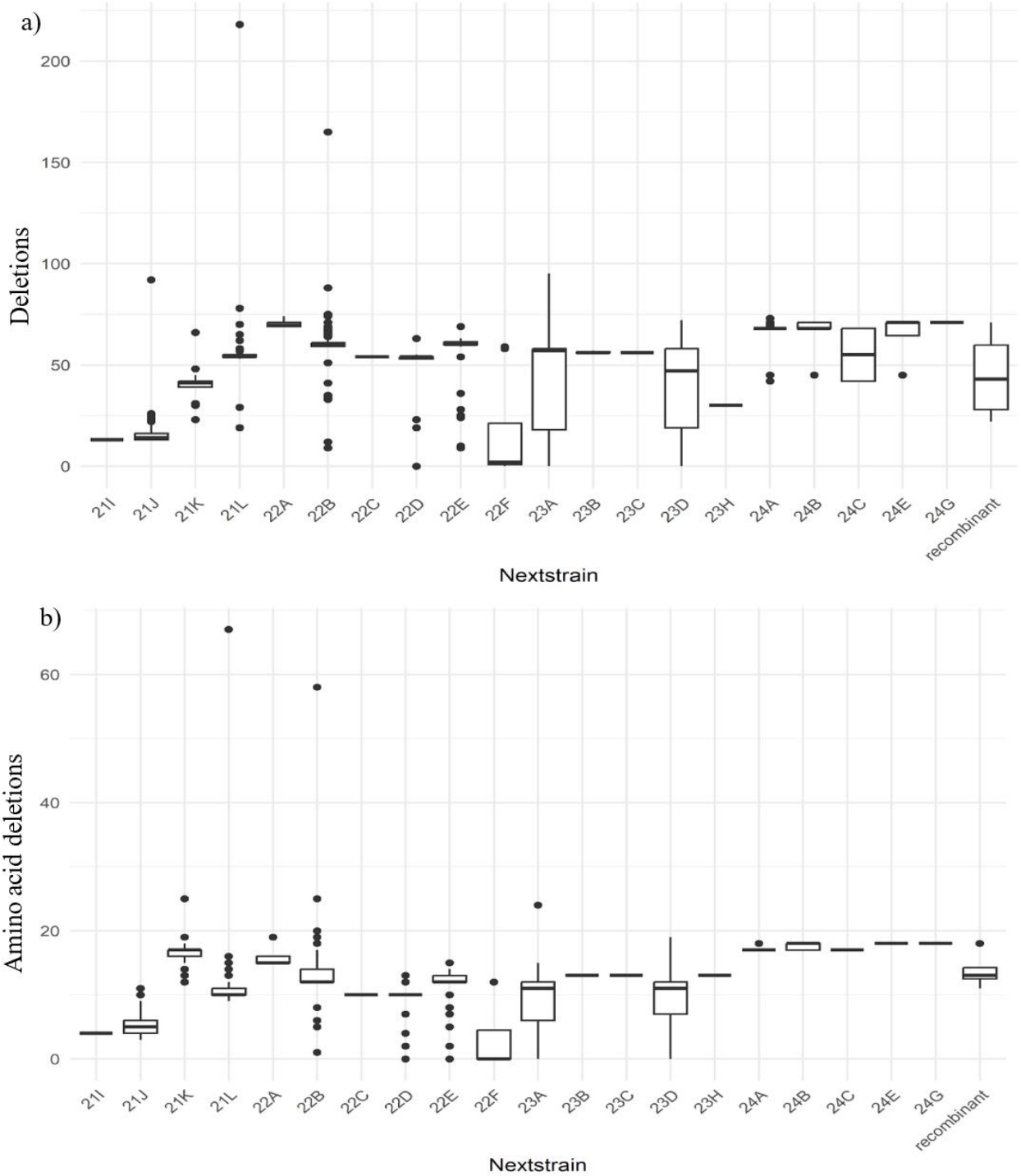

Figure 3. Distribution of the number of nucleotide and amino acid deletions across SARS-CoV-2 clades

A notable finding is the clear increase in the number of deletions when the Delta clades (21I, 21J), collected at the end of 2021, are compared with the Omicron clades. In contrast, the mean total number of deletions across the Omicron clades (21K, 21L, 22A, 22B, 22C, 22D, 22E, 22F, 23A, 23B, 23C, 23D, 23H, 24A, 24B, 24C, 24E, 24G) and the recombinant variant showed a more stable pattern. In some clades, however, the number of deletions increased, particularly in 21K, 22B, 23A, and 23D, likely reflecting differences among isolates within these groups and the acquisition of additional genomic mutations.

Analysis of SARS-CoV-2 evolution in relation to the occurrence of insertions in the genome showed that, in most cases, insertions were either very few or absent. As shown in Figure 4,

nucleotide (a) and amino acid insertions (b) were observed mainly in clades 22F, 23A, and 23D, where the median values were clearly higher than in the other clades, suggesting greater variability among isolates in these groups. A higher number of insertions was also observed in recombinant strains compared with the other groups.

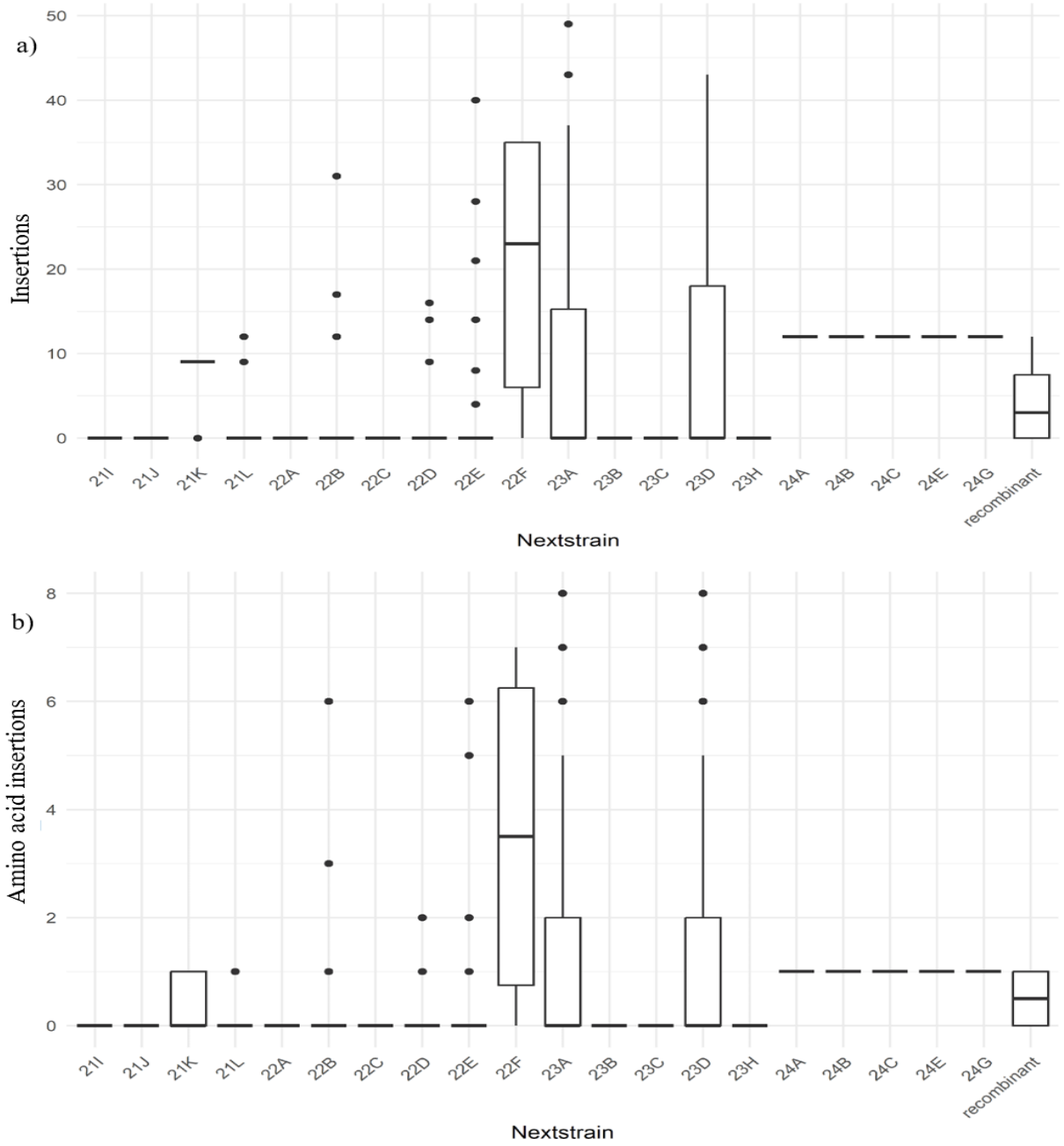

Figure 4. Distribution of nucleotide and amino acid insertions across SARS-CoV-2 clades

Short insertions, like deletions, tend to occur in regions containing identical nucleotides or tandem di- and trinucleotide repeats. These insertions may result from slippage of the RNA-dependent RNA polymerase (RdRp). Another possible explanation is that most, if not all, frameshift insertions are sequencing artefacts; however, some insertions in other genes may represent true events, reflecting the non-essential role of these genes in viral replication.

3.3. Synonymous and Nonsynonymous Mutations in SARS-CoV-2 Genomic Dynamics

Synonymous mutations do not change the encoded amino acid but may still influence cellular processes. In contrast, nonsynonymous mutations alter protein structure and may affect key viral properties, including transmissibility and immune escape.

A total of 13,672 synonymous mutations and 54,080 nonsynonymous mutations were identified, indicating selective pressure toward protein-altering changes. The Spike gene carried the highest number of nonsynonymous mutations, consistent with its central role in viral adaptation and interaction with the ACE2 receptor. A substantial number of mutations was also detected in the N and ORF1ab genes, suggesting their involvement in viral replication and genome stability.

The uneven distribution of mutations across genes highlights their different functional roles and their contribution to the ongoing evolution and adaptation of SARS-CoV-2.

Nearly all nonsynonymous nucleotide changes corresponded to amino acid substitutions in viral proteins. The ten most frequent amino acid mutations identified in each coding gene (Figure 5) illustrate key variability hotspots in the viral proteome. This analysis complements the assessment of synonymous and nonsynonymous mutations across the genome and links genetic variability to potential functional effects on viral proteins, with implications for the biology and epidemiology of SARS-CoV-2.

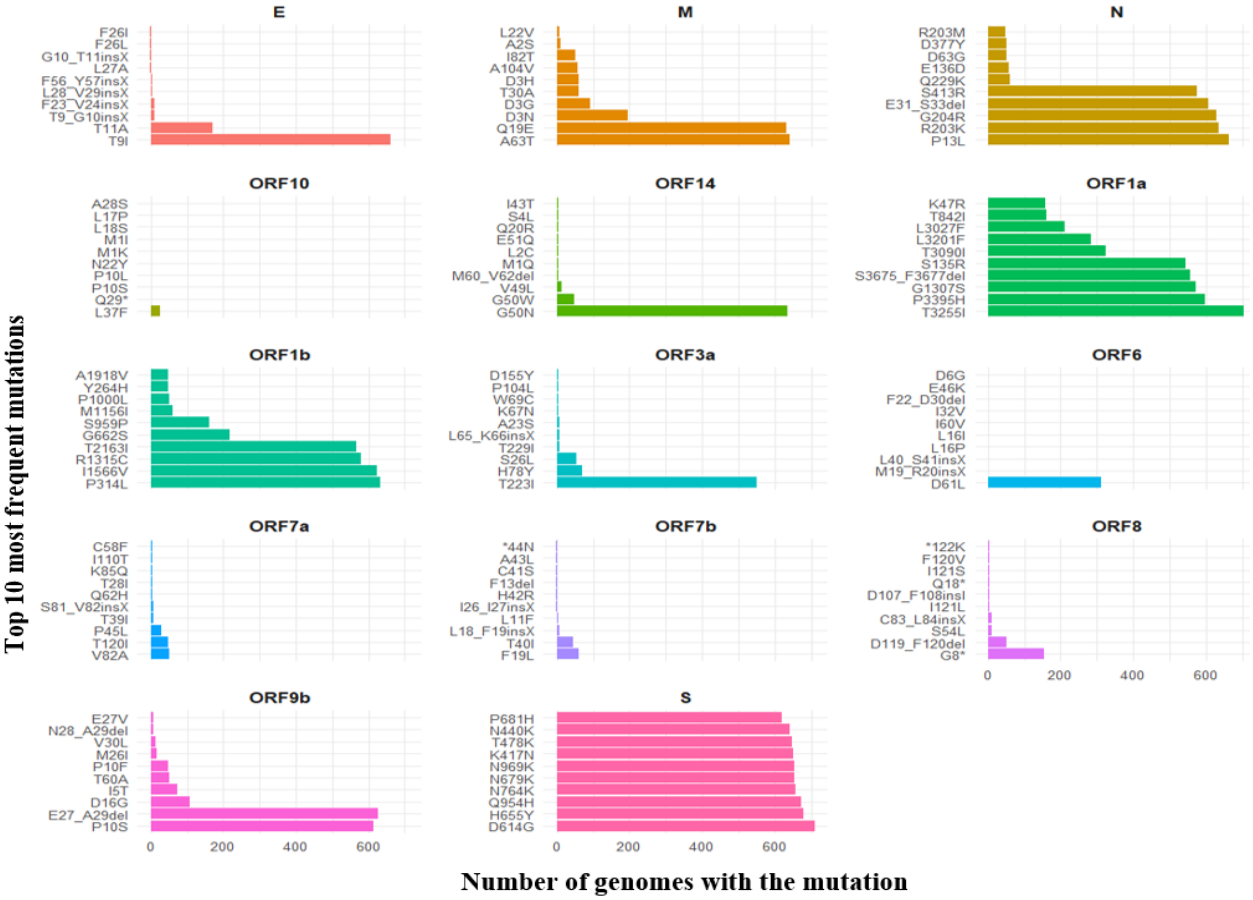

Figure 5. Ten most frequent amino acid mutations in the SARS-CoV-2 genome

Major proteins involved in immune recognition and host-cell interaction, particularly Spike and nucleocapsid, showed substantial accumulation of recurrent amino acid mutations with high absolute frequencies. This pattern suggests important functional changes in these proteins during the rapid spread of specific viral variants.

ORF1a and ORF1b also contained several frequent mutations, some of which have been associated in the literature with changes in replication efficiency in host cells. Other genes, generally shorter and less central to the replication cycle, showed high-frequency mutations at specific positions. These changes may provide selective advantages related to immune escape or to the interaction between viral proteins and host antiviral mechanisms. Some mutations were associated with deletions, insertions, or premature stop codons, indicating protein-truncating

events that may alter or abolish accessory functions, a phenomenon reported in SARS-CoV-2 epidemiology.

The marked differences between genes in mutation distribution and frequency suggest that the evolutionary landscape of SARS-CoV-2 is strongly shaped by the functional role of each protein, the degree of immune pressure, and the structural constraints required to preserve protein-complex integrity.

Significant mutations. Functionally relevant mutations may alter transmissibility, immune escape, and response to therapy, and are often associated with variants of concern. The most relevant mutations identified in this study, including D614G, N501Y, L452R, and E484A/K, were located mainly in the Spike protein and are associated with increased infectivity and reduced effectiveness of the immune response. Some mutations enhance binding to the ACE2 receptor, whereas others facilitate escape from antibody-mediated neutralization. Mutations in the N protein and ORF1ab region are also involved in increased viral replication and broader viral adaptation. Overall, the distribution of mutations confirms the central role of the Spike protein in SARS-CoV-2 evolution and the continuing adaptive capacity of the virus.

Private mutations specific to genomes from the Republic of Moldova. These mutations were characteristic of viral strains circulating in the Republic of Moldova and were absent or detected only very rarely in other countries. Because such mutations may result from genome assembly errors, only private mutations that occurred repeatedly in the analyzed sequences were included in this study. Statistical analysis of private mutations by WHO SARS-CoV-2 variant identified significant differences for ten private mutations, with p-values < 0.05; the most frequent mutations were further interpreted.

The private mutations A23048G and T22686C were detected exclusively in Omicron, with frequencies of 10.9% and 13.2%, respectively, and showed statistically significant differences, $p < 0.05$. A10447G and A10449C were found predominantly in Omicron, but also in recombinant variants, while T44C was observed in both Omicron and recombinant forms.

The most frequent mutation was T9344C, located in ORF1ab, which was detected exclusively in Omicron at a frequency of 38.2% and showed high statistical significance, $p < 0.001$.

These findings highlight the genetic diversity and local adaptation of SARS-CoV-2 and support the need for continued monitoring to assess potential effects on vaccine and treatment effectiveness.

3.4. Evolutionary Dynamics of SARS-CoV-2 and Implications for the RBD Immune Escape Score

The continued evolution of SARS-CoV-2 may reduce antibody-mediated recognition of the virus in humans. To assess how amino acid substitutions affect binding between the SARS-CoV-2 RBD and the host ACE2 receptor, molecular dynamics analysis was performed on the viral RBD–human ACE2 complex, and the effect of different mutations on complex stability was evaluated. The SARS-CoV-2 phylogenetic tree was also analyzed to determine how RBD amino acid mutations contribute to escape from antibody binding.

Analysis of the SARS-CoV-2 phylogenetic tree, Figure 6, constructed according to the ACE2 affinity score, showed an increasing trend in strains with the highest number of mutations. The ACE2 affinity score increased in samples carrying 60–80 mutations, with a peak in the range of 120–130 mutations. The graph shows a sharp increase in ACE2 affinity for Omicron compared with Delta, 21I and 21J, and a clear upward trend across Omicron sublineages, consistent with increased viral transmissibility. Among Omicron clades, low ACE2 affinity scores were observed

for 24B and 24C, moderately low scores for 21L, 22B, 24E, and 24G, and moderately increased scores for 23A and 23D.

The results indicate that ACE2 binding is a highly evolvable trait. In almost all cases in which the RBD binds to a given ACE2 receptor, amino acid mutations can increase this binding several-fold. This capacity for mutation-driven enhancement of ACE2 binding was observed with the emergence of different Omicron sublineages, which are characterized by a high number of genomic mutations.

A key aspect of monitoring SARS-CoV-2 evolution is the detection of genetic recombination events, as these may generate new viral strains with altered virulence and biological properties. Recombinant viral RNA was identified in four of the analyzed samples; however, no change in the ACE2 affinity score was observed, suggesting no evident epidemiological consequence.

Analysis of the RBD immune escape score in relation to SARS-CoV-2 evolutionary dynamics (Figure 7) showed a clear association between viral evolution, the emergence of new variants, and increasing immune escape potential.

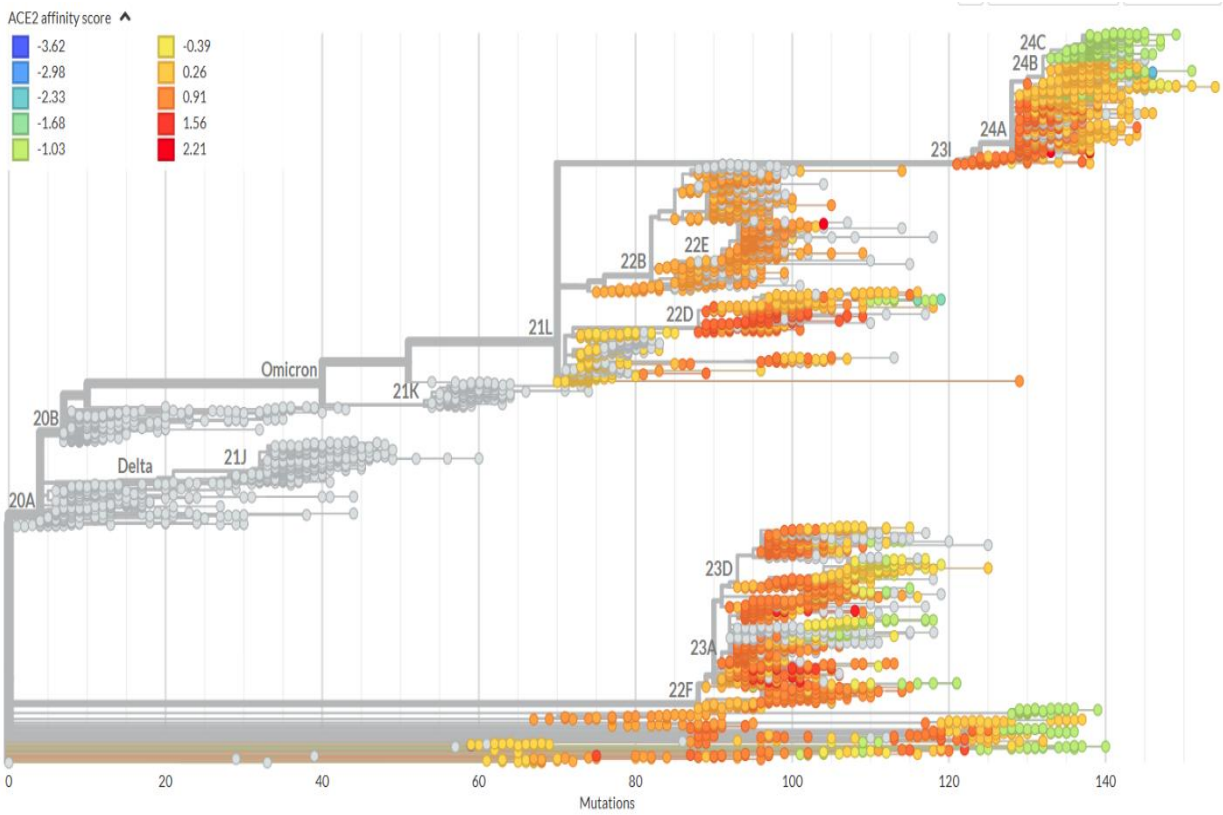

Figure 6. Phylogenetic tree of SARS-CoV-2 isolates according to ACE2 affinity score

In clades 21I, 22A, 22B, 22C, 22D, and 22E, the immune escape score ranged from 0 to 1.0. The mutation-driven immune escape profile changed substantially over time. In particular, the RBD immune escape score began to increase in variants from clades 22F, 23A, 23B, 23C, 23D, 23E, 23F, 23G, and 23H, reaching values between 1.0 and 1.4. In variants from clades 23I, 24A, 24B, 24C, 24E, and 24G, as well as in the recombinant variant, the RBD immune escape score ranged from 1.4 to 1.962.

Analysis of the RBD immune escape score in relation to SARS-CoV-2 evolutionary dynamics showed that both immune escape and ACE2 binding affinity correlated independently with mutation frequency. The highest immune escape coefficients, shown by the red-colored points in Figure 7, were observed in clades 24A, 24B, 24C, 24E, and 24G, which include the

lineages JN.1, JN.1.11.1, KP.2.3, KP.3, and KP.3.1.1. This terminology is consistent with current literature describing recent Omicron lineages, including JN.1, KP.2, and KP.3, as variants with increased RBD mutations and enhanced immune evasion.

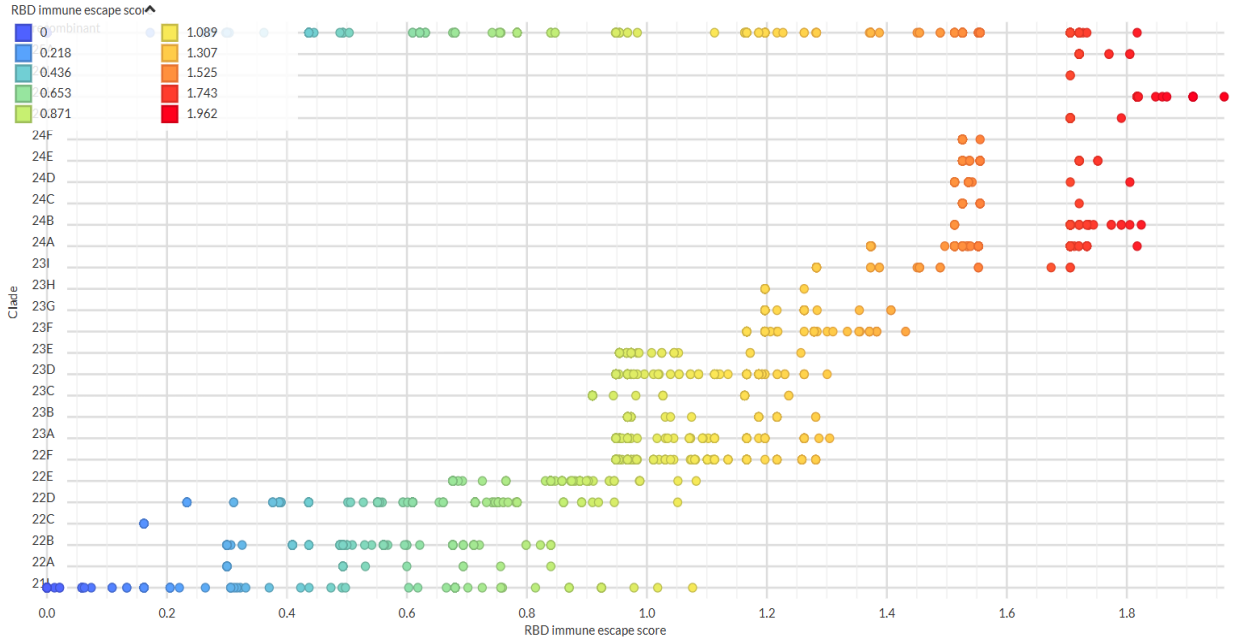

Figure 7. RBD immune escape score in relation to SARS-CoV-2 evolutionary dynamics

These lineages circulated in the Republic of Moldova in 2024 and support the observation that recent Omicron subvariants showed a higher rate of antibody escape than earlier SARS-CoV-2 variants.

4. GENETIC MONITORING OF SARS-COV-2 IN WASTEWATER VIA WHOLE-GENOME SEQUENCING

4.1. Wastewater Genomic Surveillance of SARS-CoV-2 as an Alternative Approach for Monitoring Viral Evolution in the Republic of Moldova

Wastewater genomic surveillance of SARS-CoV-2 was implemented for the first time in the Republic of Moldova and provides an alternative approach for monitoring viral circulation in the population and for supporting further research on SARS-CoV-2 spread within the country. This approach is particularly valuable in a context where individual COVID-19 testing has become sporadic and inconsistent after the pandemic, while pandemic fatigue and declining public interest in public health measures have become increasingly evident.

Although the quality of SARS-CoV-2 genomic sequencing data obtained from wastewater samples was lower because of high dilution and mechanical degradation of viral material in water, the data were sufficient for Freyja analysis and for the identification of viral lineages.

Analysis of the wastewater samples sequenced in 2024 identified 32 genetic lineages corresponding to the Pango lineage classification system, which is used globally to monitor SARS-CoV-2 evolution. The detected lineages included: A.12, AZ.4, AZ.5, B.1.1.243, B.1.1.305, B.1.1.357, B.1.1.363, B.1.1.374, B.1.104, B.1.160.32, B.1.177.25, B.1.177.66, B.1.289, B.1.292, B.1.411, B.1.420, B.1.431, B.1.539, B.1.579, B.10, B.6, B.6.1, B.6.3, B.6.4, B.6.5, B.6.6, B.6.8, XT, JN.1.1.1, AE.4, B.1.1.45, and B.11.

The identified variants and their changes in abundance over time are shown in Figure 8. In this graph, the Y-axis represents the prevalence, or relative proportion, of each SARS-CoV-2 mutation variant among all sequences analyzed for the corresponding sampling month, with values

ranging from 0 to 1. The X-axis shows the month of sample collection: September, October, and November. During these months, viral concentrations in wastewater were sufficiently high, with RT-PCR Ct values up to 35, allowing sequencing to be performed. Changes in prevalence and mutation patterns reflect the temporal dynamics of the genetic lineages identified in the analyzed samples. Green indicates wastewater samples, while pink indicates the human sample used as a positive control.

Temporal analysis of viral lineage abundance made it possible to identify lineages that persisted throughout the three-month period of increased viral concentrations in wastewater, such as B.1.177.66, as well as lineages that became dominant only during specific periods, including B.1.1.243, B.1.160.32, and B.1.1.305.

According to published data, some identified lineages, such as B.11, are no longer circulating globally. Their detection may therefore indicate either local endemic persistence or isolated reintroduction. Both scenarios support the need for continued genomic surveillance to better understand the re-emergence of SARS-CoV-2 variants that appear to have disappeared from global circulation.

Each plot in Figure 8 shows the dynamics of a lineage circulating in Chișinău in autumn 2024. The X-axis shows the wastewater sampling months, September, October, and November 2024, and the Y-axis shows the abundance of each variant in the analyzed samples, from 0 to 1. The most abundant lineages included B.1.177.66, B.1.1.243, B.1.160.32, and B.1.1.305. B.1.177.66 was repeatedly predominant across several samples, suggesting intensive circulation of this lineage in the capital of the Republic of Moldova, whereas the other lineages reached high abundance only during specific time intervals.

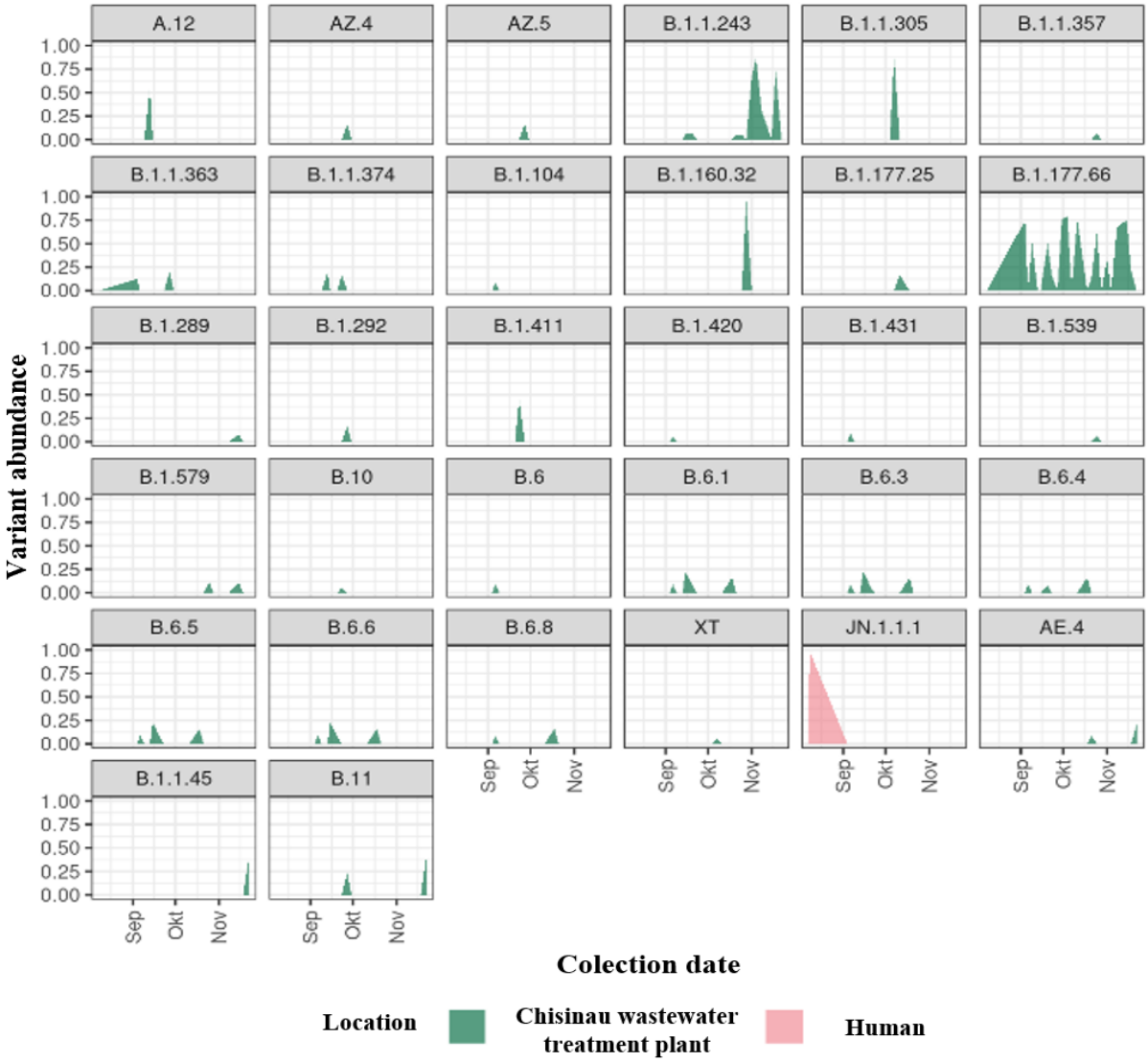

Figure 8. Abundance and temporal dynamics of SARS-CoV-2 lineages in wastewater samples from Chișinău, September–November 2024

The highest relative abundance at the specified time point, approximately 80%, was observed for B.11 and B.1.1.45 (Figure 9). According to outbreak.info, B.11 has been detected in at least 21 countries, mainly in Western Europe, India, and North America, but has been reported less frequently in Eastern Europe. Its detection is noteworthy because this lineage circulated during earlier stages of the pandemic. Its presence in wastewater from Chișinău in October and November 2024 may reflect either isolated cases or low-level local endemic circulation. This finding highlights the importance of continued SARS-CoV-2 genomic monitoring, including the detection of possible reintroductions of variants that appear to have disappeared from global circulation.

The 2025 genomic dataset, which included nine wastewater samples (S5–S14) and six human clinical samples (S15–S20) used as reference controls, also showed low viral abundance, making bioinformatic analysis more difficult. Despite these limitations, circulating sublineages were identified and SARS-CoV-2 variants were quantified.

According to the data obtained, the increase in SARS-CoV-2 RNA concentration in wastewater correlated with the rise in the number of infection cases in Chișinău municipality. This made it possible to sequence samples collected during the warm season, when the number of COVID-19 cases began to increase in the Republic of Moldova.

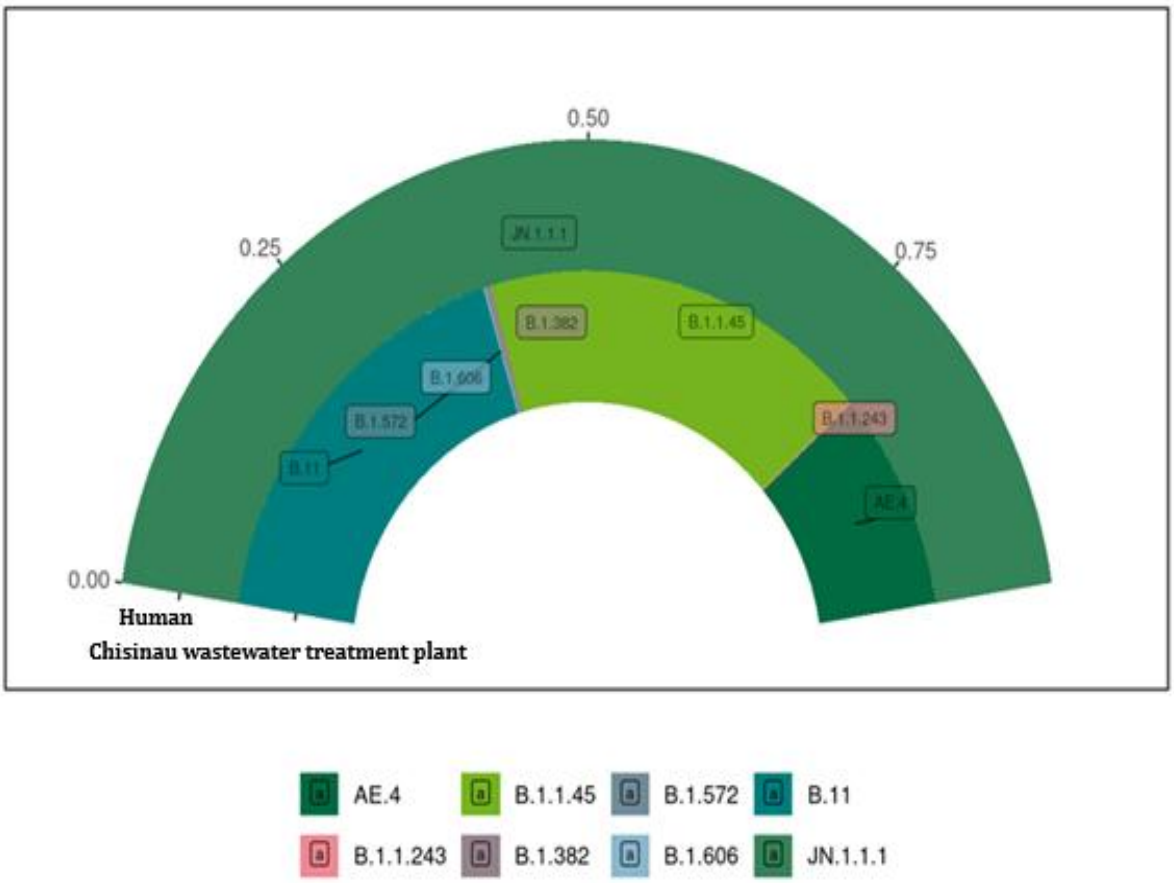

Figure 9. Recent SARS-CoV-2 lineages detected in wastewater compared with the human sample

As shown in Figure 10, during June–August 2025, the predominant SARS-CoV-2 lineages were AY.57, BA.3, BF.22, NS.3, NT.1, XFG.12, XP, XFG.5, and XFG.4, which is consistent with WHO data for the same period. WHO designated XFG as a variant under monitoring in June 2025, noting its increasing global proportions.

Analysis of the data showed that wastewater sequencing can identify SARS-CoV-2 genotypes circulating in Chișinău municipality, including those not yet detected through clinical sample sequencing because of reduced healthcare-seeking and testing during the post-pandemic period. With more intensive wastewater sampling, this approach may also reveal patterns of viral distribution within communities, helping to clarify transmission and spread during infectious disease outbreaks. Most importantly, the findings indicate that wastewater sequencing can detect newly emerging SARS-CoV-2 genotypes and other pathogenic viruses at the population level.

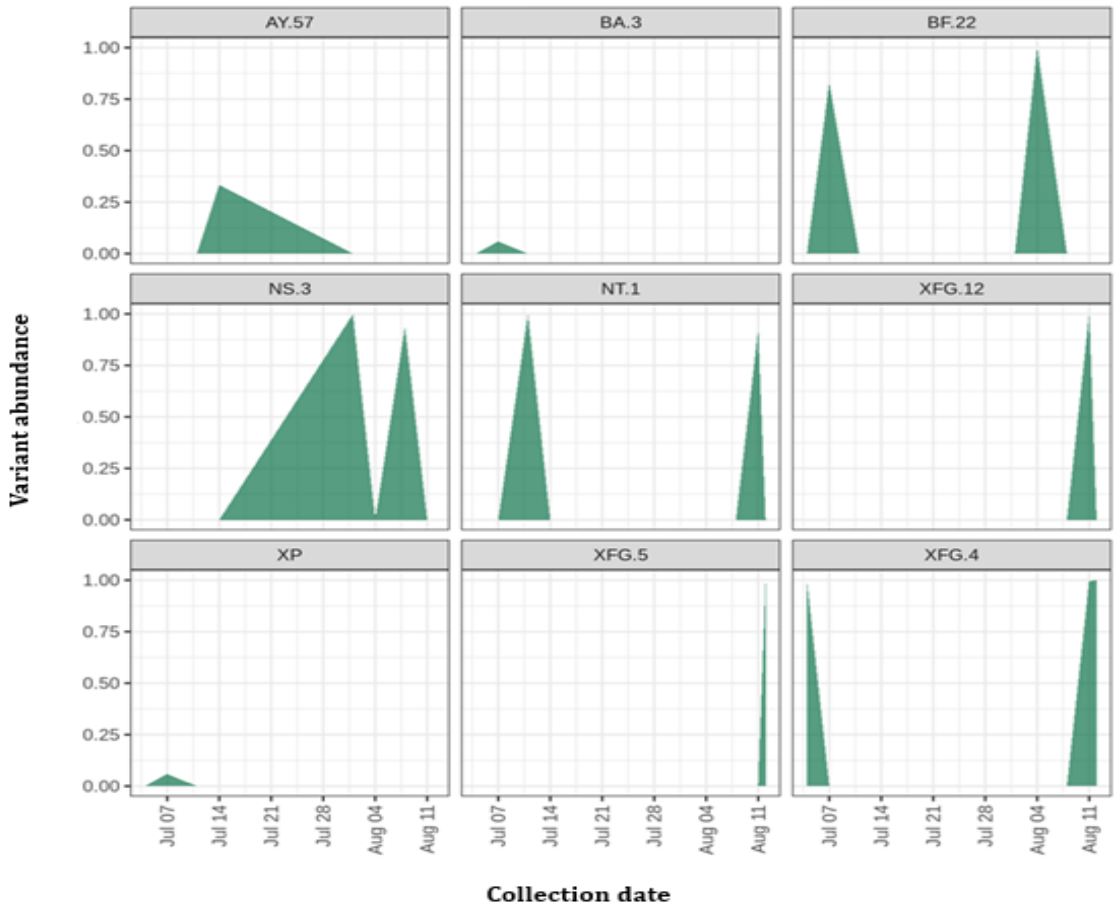

Figure 10. Abundance and temporal dynamics of SARS-CoV-2 lineages identified in wastewater samples from Chișinău, June–August 2025

4.2. Comparative performance of Freyja and Kallisto for estimating SARS-CoV-2 lineage abundance in wastewater monitoring

In wastewater, SARS-CoV-2 is highly diluted and degraded under the effect of various chemical agents, resulting in very low viral concentrations. Therefore, reliable specialized bioinformatic tools are required to analyze sequencing data obtained from wastewater samples.

To evaluate the performance of Freyja and Kallisto as tools for analyzing and interpreting wastewater sequencing data, a comparative assessment was performed. A synthetic dataset comprising 42 wastewater metagenomic sequences with known lineage composition was used. The dataset included 11 SARS-CoV-2 genetic lineages: Alpha, Beta, Delta, Epsilon, Eta, Gamma, Iota, Kappa, Lambda, Theta, and Zeta. Each sample contained 1,092,761 reads, aligned to the SARS-CoV-2 reference genome NC_045512.2 corresponding to the Wuhan-Hu-1 strain.

Each sample mixture was generated artificially to create a lineage-abundance gradient across the dataset. Samples 1–10 contained a dominant lineage, mainly Epsilon, with abundances ranging from 90% to 98%. Samples 11–21 represented transition phases with mixed Alpha and Epsilon lineages. Samples 22–30 contained Alpha and Delta mixtures, reflecting the emergence of Delta, whereas samples 31–42 were dominated by Delta, with abundances exceeding 96%. This design allowed the performance of Freyja and Kallisto to be tested across the full abundance range, from trace levels below 0.1% to dominant lineages above 90%.

Comparative analysis showed strong overall correlations between predicted and true lineage abundances for both tools, but with markedly different accuracy profiles. Freyja showed excellent

performance, with an R^2 of 0.9793, explaining 97.9% of the variation in the true abundance data (Figure 11).

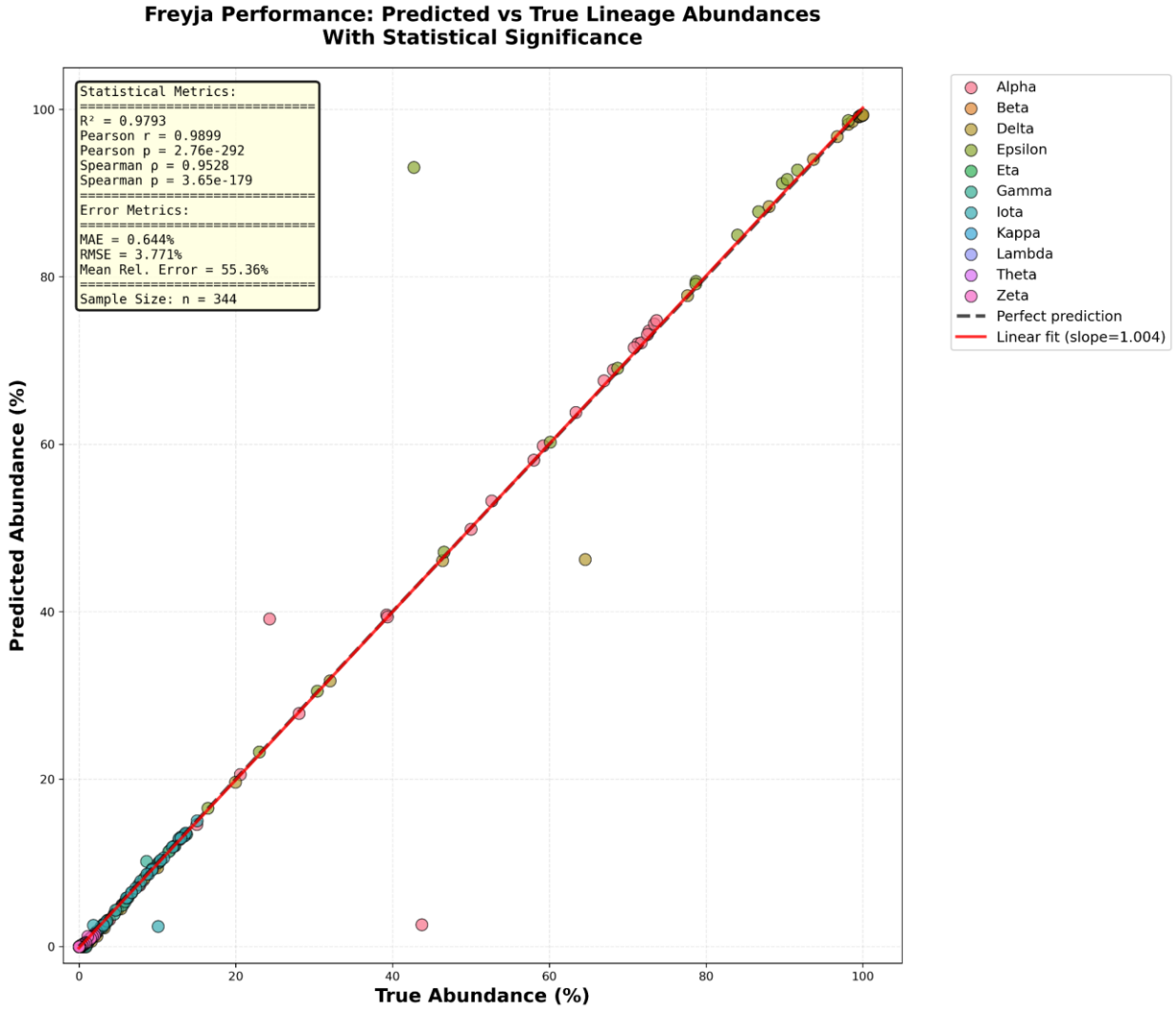

Figure 11. Performance of Freyja in comparing predicted and true SARS-CoV-2 lineage abundances

The Pearson correlation coefficient was r = 0.9899, with $p < 2.76 \times 10^{-292}$, indicating an extremely strong linear relationship. The mean absolute error was only 0.644%, meaning that typical predictions differed from the true values by less than one percentage point. The root mean square error was 3.771%, indicating that occasional larger errors occurred but remained limited. These metrics were calculated across 344 lineage-level comparisons, after excluding measurements in which both true and predicted abundances were below 0.001%.

To assess the quality of the Freyja-based wastewater monitoring results, the prediction-error heatmap was analyzed (Figure 12). In this heatmap, white cells indicate non-significant prediction error, below 1%, compared with the expected value. These results show that Freyja provides the highest accuracy in estimating the relative abundance of SARS-CoV-2 lineages.

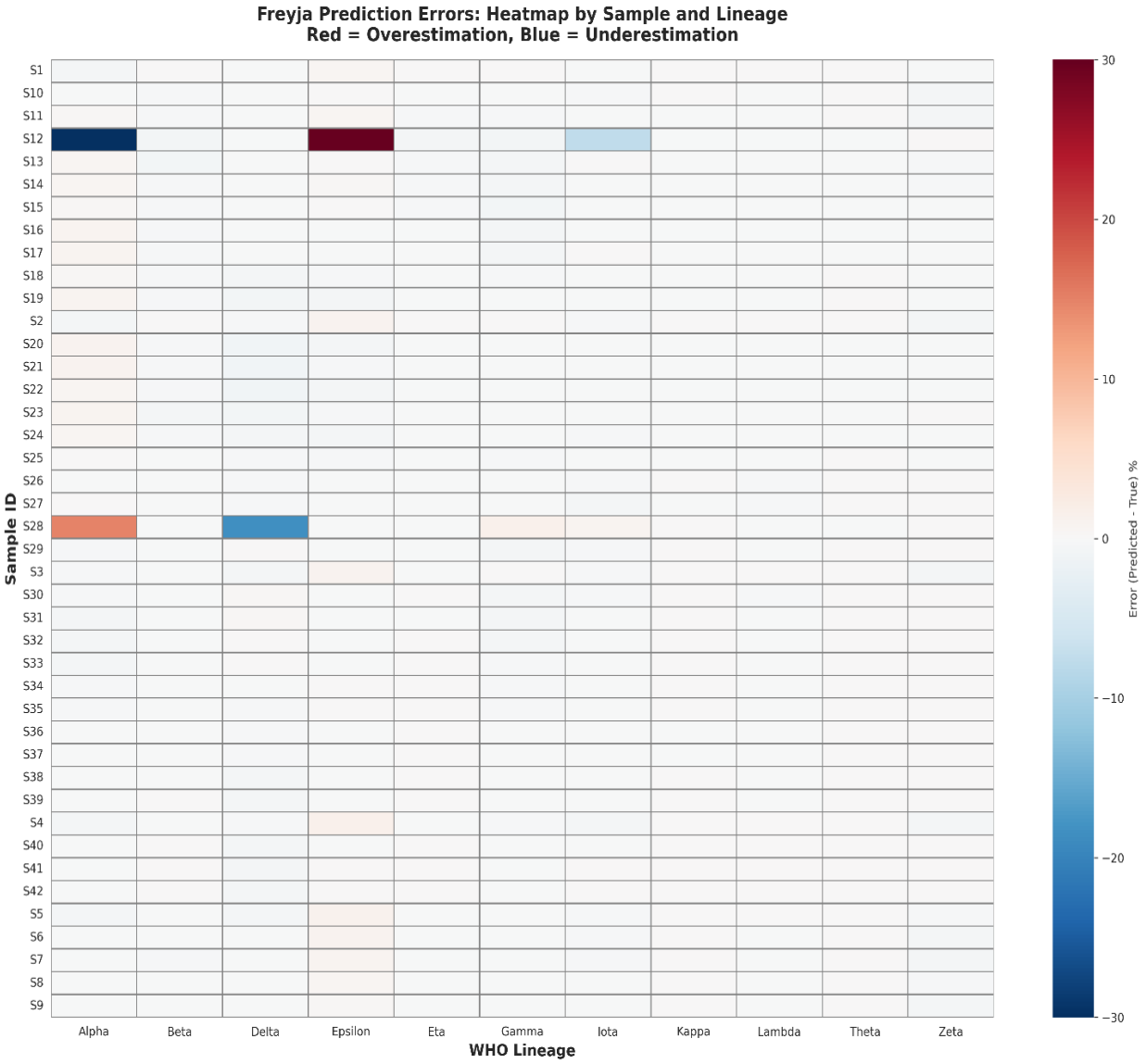

Figure 12. Freyja prediction-error heatmap

Kallisto also showed good performance, although its accuracy was substantially lower than that of Freyja. It achieved an R^2 of 0.853, explaining 85.3% of the variation in true lineage abundances. The Pearson correlation coefficient was $r = 0.950$, with $p < 7.04 \times 10^{-213}$, still indicating a very strong linear relationship.

However, the mean absolute error was 4.48 percentage points, approximately seven times higher than that obtained with Freyja. The root mean square error reached 9.28 percentage points, substantially exceeding the MAE and indicating that occasional large errors had a notable effect on overall performance. These results were based on all 420 comparisons across the 42 samples and 10 WHO-defined SARS-CoV-2 lineages (Figure 13).

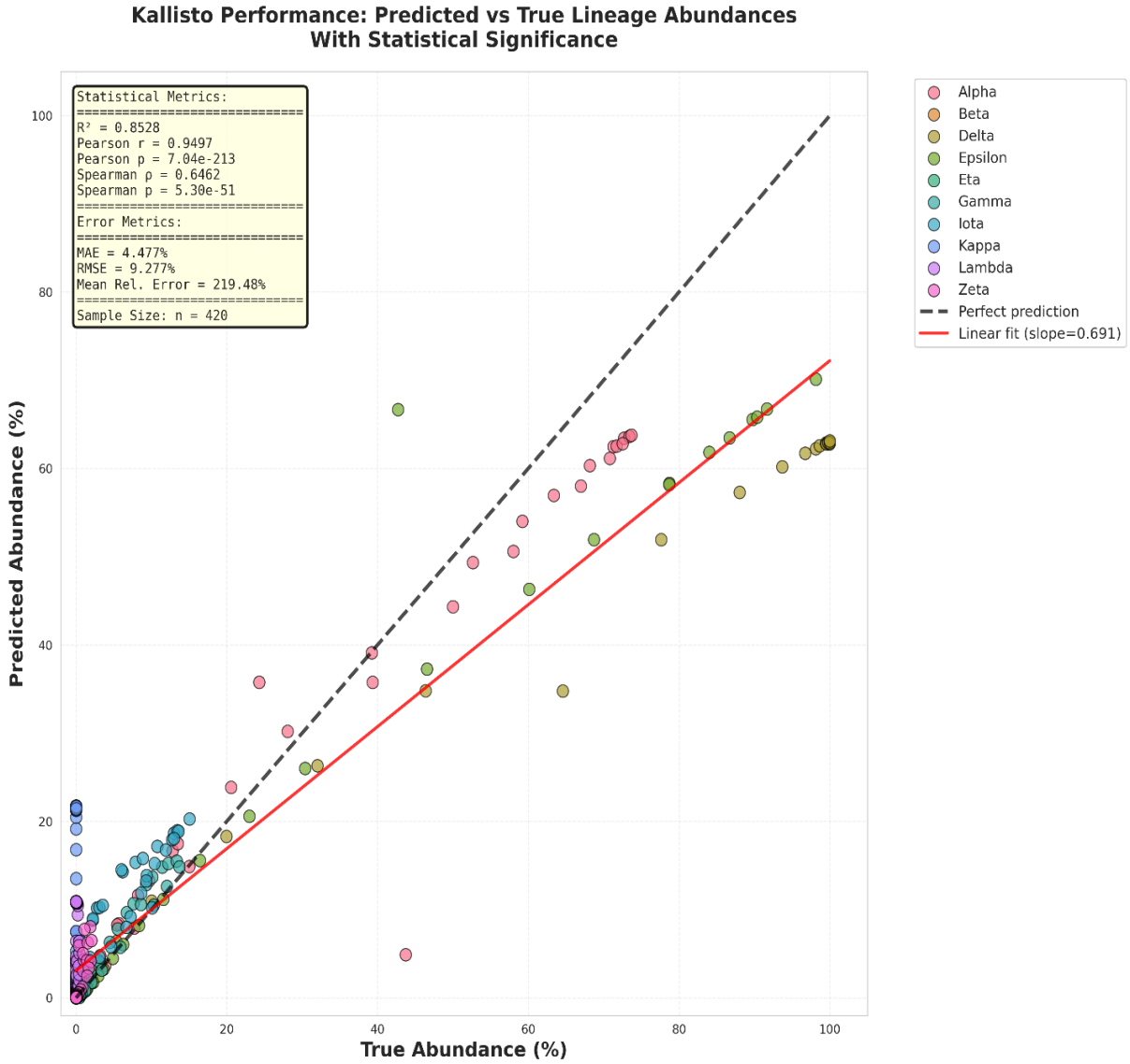

Figure 13. Kallisto performance in comparing predicted and true SARS-CoV-2 lineage abundances by viral variant

Kallisto performance by SARS-CoV-2 variant showed substantial dispersion for many variants. The comparison between predicted and true lineage abundances showed that estimates for Alpha, $R^2 = 0.9230$; Delta, $R^2 = 0.7750$; Epsilon, $R^2 = 0.8882$; and Gamma, $R^2 = 0.8622$, were close to the true relative abundances. In contrast, the remaining variants, Beta, Eta, Iota, Kappa, Lambda, and Zeta, were generally underestimated. The relative abundance estimate was weak for Beta, $R^2 = 0.248$, and negative for Zeta, $R^2 = -10$, see Annex 12. For many variants, the colored points were frequently distant from the prediction line, indicating systematic underestimation or overestimation of lineage abundance. Overall, these findings show that Kallisto performed substantially less well than Freyja

The Kallisto prediction-error heatmap, Figure 14, differed markedly from the Freyja heatmap, Figure 12. These plots show prediction errors for each sample by viral variant; red and blue rectangles indicate prediction errors greater than 15%.

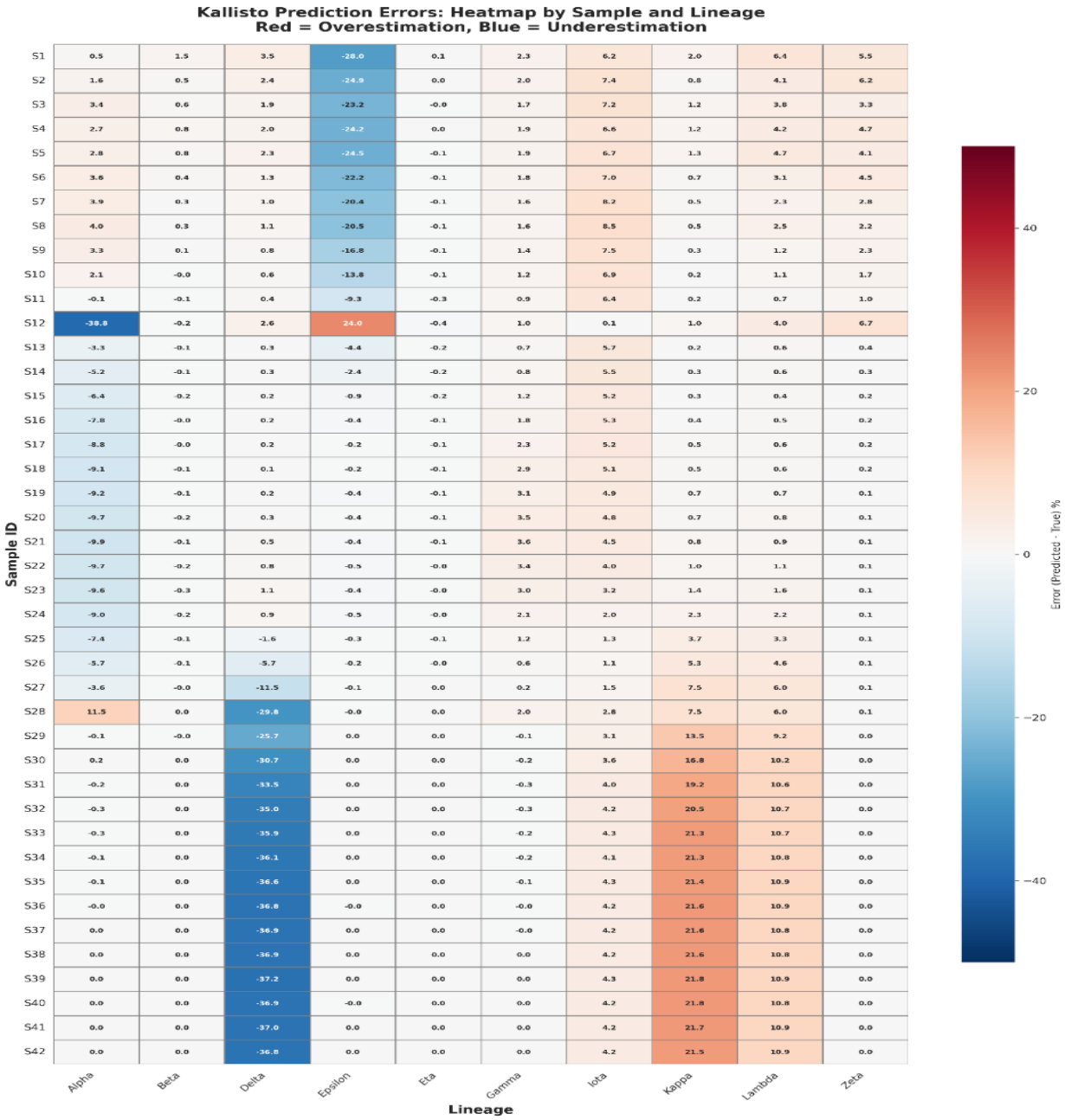

Figure 14. Kallisto prediction-error heatmap

The Kallisto prediction-error heatmap showed a highly heterogeneous pattern, with systematic errors affecting entire columns. For Delta in samples 28–42, the blue signal indicated prediction errors above 25%, consistent with underestimation. Kappa showed increased values in samples 29–42, with systematic prediction errors exceeding 13%. Similarly, Alpha, Epsilon, and Lambda showed prediction errors above 9%, reaching up to 38% in sample 12.

Spearman rank correlation revealed an important difference between Freyja and Kallisto. Freyja achieved a ρ value of 0.953, indicating excellent preservation of rank order, even in non-linear relationships. Kallisto showed a substantially lower Spearman ρ of 0.646, suggesting weaker performance in maintaining the correct relative ranking of genetic lineages, particularly at low abundance levels, where small absolute differences can produce large rank shifts.

Therefore, the comparative analysis of the two bioinformatic tools demonstrated higher accuracy of Freyja for monitoring SARS-CoV-2 genetic variants detected in wastewater and for supporting the prediction of epidemic waves.

GENERAL CONCLUSIONS

1. Phylogenetic analysis of SARS-CoV-2 isolates from the Republic of Moldova showed the sequential circulation of Delta and Omicron, followed by the gradual replacement of Delta by Omicron sublineages. Twenty-one clades were identified, among which 21L, 22B, 22E, 23A, 23D, and 24A represented the main evolutionary groups. These findings show that locally circulating strains were part of the broader global SARS-CoV-2 phylogenetic structure, while also revealing regional patterns of viral circulation.
2. Genomic analysis showed rapid SARS-CoV-2 evolution, reflected by the progressive accumulation of mutations, particularly in spike-associated regions. Omicron displayed markedly greater genomic diversification than Delta, with 140–150 mutations per genome compared with approximately 40 mutations per genome for Delta. This pattern supports the increased genetic adaptability of the virus.
3. Analysis of mutation types showed a predominance of changes with potential functional relevance, especially nonsynonymous mutations, which clearly exceeded synonymous mutations. This pattern is consistent with positive selection and may be linked to viral adaptation, increased transmissibility, and immune escape potential. These findings have direct relevance for molecular diagnostics and the surveillance of emerging variants.
4. Combining genomic and epidemiological data showed that the emergence of new SARS-CoV-2 variants was associated with increased epidemic activity in the Republic of Moldova. The shift from Delta to Omicron corresponded to the rising incidence and the development of epidemic waves, showing that genotypic diversity contributed to the epidemiological course of COVID-19. These results support the need to strengthen genomic surveillance as part of evidence-based public health response.
5. Wastewater monitoring of SARS-CoV-2 in Chișinău municipality was shown to be a sensitive early-warning tool that complements conventional epidemiological surveillance. This approach supported the detection of circulating variants and helped anticipate changes in infection dynamics. It represents an original contribution to integrated environmental and public health surveillance and highlights the need to continuously adapt molecular diagnostic methods to viral genetic variability.
6. Overall, the findings support the implementation of a national integrated SARS-CoV-2 surveillance model combining genomic, epidemiological, and environmental data. This model would improve early detection of emerging variants, support anticipation of epidemic trends, and strengthen a predictive, resilient, and evidence-based public health system.

PRACTICAL RECOMMENDATIONS

1. An integrated SARS-CoV-2 genomic surveillance system should be developed and implemented, based on continuous monitoring of circulating strains and linkage of genomic, clinical, and epidemiological data. Systematic data sharing through international platforms, such as GISAID, should be ensured to support public health strategies, optimize therapeutic and preventive interventions, and improve the performance of the healthcare system.
2. National genomic sequencing capacity should be strengthened through the development and implementation of a national strategy and action plan covering infrastructure, human resources, and the regulatory framework. Innovative approaches, including wastewater

genomic surveillance, should be integrated into routine practice to monitor viral circulation and anticipate epidemiological trends.

3. The research findings should be further used and scientific work continued by expanding genomic surveillance to other pathogens of public health importance and by integrating advanced bioinformatic analysis methods. This would support the development of a predictive, resilient public health system guided by evidence-based decision-making.

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✓ **international:**

20. **Colac S.** SARS-CoV-2 genome sequence analysis: evolution and impact of new Omicron lineages in the Republic of Moldova. *Comunicare orală prezentată la: Conferința științifică internațională Patrimoniul cultural de ieri – implicații în dezvoltarea societății durabile de mâine*; 11-12 febr 2025; Iași–Chișinău–Lviv.
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22. **Colac S.,** Apostol M. Particularitățile circulării virusurilor gripale și non-gripale la copii în perioada pandemiei COVID-19. *Comunicare orală prezentată la: Conferința Actualități în pediatrie și impactul imunizării asupra morbidității și mortalității copiilor*. Chișinău; 22-23 sept 2023.
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26. **Colac S.** Secvențierea genomică – instrument modern în monitorizarea agenților microbieni. *Comunicare orală prezentată la: Conferința Abordarea „O singură sănătate" pentru securitatea sănătății globale*. Chișinău; 20-21 nov 2025.

✓ **national:**

27. **Colac S.** Variabilitatea genetică a virusului SARS-CoV-2, varianta Omicron circulantă pe teritoriul Republicii Moldova. *Comunicare orală prezentată la: Conferința științifică anuală USMF „Nicolae Testemițanu"*. Chișinău; 18-20 oct 2023.

28. **Colac S.,** Ulinici M., Burduniuc O. Analiza diversității genetice a virusului SARS-CoV-2. *Comunicare orală prezentată la: Conferința Sănătatea și fenomenul rezistenței la antimicrobiene în țările cu venituri mici și medii din Europa de Est.* Chișinău; 27 ian 2024.
29. **Colac S.** Secvențierea genomică în supravegherea sănătății publice. *Comunicare orală prezentată la: Conferința ANSP: trecut, prezent și viitor.* Chișinău; 7 oct 2025.

- **Poster presentations at scientific forums:**

✓ **international:**

30. **Colac S.** Semnificația genetică și monitorizarea variantelor circulante ale virusului SARS-CoV-2 în Republica Moldova. *Poster prezentat la: Conferința Abordarea „O singură sănătate – realizări și provocări”*. Chișinău; 23-24 nov 2023. (Premiul mare).
31. **Colac S.** Monitorizarea răspândirii SARS-CoV-2 prin secvențierea genomică a apelor reziduale. *Poster prezentat la: Conferința Abordarea „O singură sănătate pentru securitatea sănătății globale”*. Chișinău; 20-21 nov 2025.

✓ **national:**

32. **Colac S.,** Burduniuc O., Apostol M. Monitorizarea infecției COVID-19 prin secvențierea întregului genom și analiza filogenetică a izolatelor SARS-CoV-2. *Poster prezentat la: Conferința științifică anuală USMF „Nicolae Testemițanu”.* Chișinău; 19-21 oct 2022.

ANNOTATION

to the doctoral thesis in medical sciences by PhD candidate Svetlana Colac: "Genotypic Diversity of SARS-CoV-2 During the Pandemic and Early Post-Pandemic Period." Specialty 313.02 – Medical Microbiology and Virology.

Relevance. SARS-CoV-2 infection remains a major public health challenge because of the continuing evolution of the virus and the emergence of genetic variants that affect transmission and disease control. Genomic monitoring is essential for epidemiological surveillance and for identifying circulating variants.

Aim of the study. To identify SARS-CoV-2 variants circulating in the Republic of Moldova and to perform phylogenetic analysis of the detected isolates, with the aim of improving COVID-19 surveillance and morbidity control measures.

Objectives of the study. To provide a phylogenetic characterization of SARS-CoV-2 isolates detected in the Republic of Moldova; to study SARS-CoV-2 evolution by identifying substitutions, insertions, and deletions and comparing them with global viral genome diversity; to analyze SARS-CoV-2 circulation dynamics and the emergence of new genetic variants, including the relationship between viral genotypes and epidemiological data; to assess wastewater monitoring as a method for predicting epidemiological trends in SARS-CoV-2 infection; and to evaluate the impact of different SARS-CoV-2 genotypes in order to support proposals for improving the laboratory diagnosis of COVID-19.

Scientific novelty and originality. Original data on the genotypic and phenotypic characteristics of SARS-CoV-2 circulating in the Republic of Moldova were obtained for the first time using modern genomic sequencing and bioinformatic analysis. The study identified SARS-CoV-2 genovariants in clinical and wastewater samples, assessed their position within global phylogenetic trees, and analyzed the molecular evolution of the virus. The findings contributed to forecasting epidemic dynamics and optimizing surveillance and control measures for COVID-19.

The obtained study results. The research determined the genetic structure and distribution of SARS-CoV-2 lineages in the Republic of Moldova during 2021–2025. Dominant mutations were identified, and their impact on the biological properties of the virus was assessed. The study also demonstrated the value of wastewater monitoring for early detection of circulating variants, evaluated the performance of bioinformatic tools used for sequencing-data analysis, and developed proposals for improving laboratory diagnosis and epidemiological surveillance.

Theoretical background. The study deepens current understanding of the molecular evolution of SARS-CoV-2 and the mechanisms of viral adaptation, providing a scientific basis for the development of modern genomic surveillance and epidemiological management strategies.

The research practical value. The findings were applied through the development of good-practice guidelines in genomic sequencing, the update of the national clinical protocol, and the integration of the data into the training of students, resident physicians, and specialists in the field.

Implementation of scientific results. The research findings were implemented through the development of a guide on metagenomic sequencing, preparation of a monograph on nucleic acid sequencing, update of the National Clinical Protocol on COVID-19, and use of the results in healthcare institutions, epidemiological surveillance, and university education.

PhD thesis structure. The thesis includes an introduction, four chapters, general conclusions and recommendations, a bibliography of 189 references, 12 annexes, 94 pages of main text, 8 tables, and 42 figures. The results have been published in 32 scientific papers.

Keywords: COVID-19; SARS-CoV-2; sequencing; NGS; viral genome; genetic monitoring; mutation variants; wastewater.