MOLECULAR GENETIC ANALYSIS OF MEASLES VIRUSES CIRCULATING IN BULGARIA DURING THE YEAR 2024

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ABSTRACT

Background: Measles is a well-known fever-rash viral disease and remains a significant public health challenge, as cases have rapidly increased in the past few years. From 1 November 2023 to 31 October 2024, 30 EU/EEA Member States reported 18 044 measles cases, of which 13 863 (76.8%) were laboratory-confirmed.

Aim: The study aimed to monitor the circulation of wild-type measles viruses (MeV) in Bulgaria in 2024.

Materials and Methods: During the MeV outbreak in the country in 2024, 126 clinical samples (63 nasal swabs and 63 urine) from 63 patients with a possible measles infection were investigated. Viral RNA was extracted using real-time PCR and conventional one-step RT-PCR assays, and the nucleoprotein (N) gene from the viral genome was detected. The MeV-positive samples were sequenced by direct Sanger sequencing. Phylogenetic analysis was performed using the software program MEGA v. 11.

Results: Acute measles infection was proved in 26 out of 63 tested individuals (41%). The affected were mostly children aged 1-4 (12/26, 46%) and 5-9 years (6/26, 23%). Our studies have established nasal swabs as a more biologically applicable material for rapid PCR diagnostics of MeV, and in all patients confirmed clinically and epidemiologically, the virus was detected in their nasopharynx. The affected regions of the country were Varna (20/26), Sofia city (3/26), and single cases in Sofia district (1/26), Stara Zagora (1/26) and Burgas (1/26). In 2024, phylogenetic analysis of MeV sequences showed a predominant circulation of the D8 and B3 genotypes with imports of the viral strains from the United Kingdom, Germany, Romania, and Austria.

Conclusion: Despite the wide availability of effective vaccines, measles outbreaks continue to occur due to imported cases and transmission of the virus among unvaccinated children in communities.

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Keywords: measles, real-time PCR, sequencing

INTRODUCTION

Measles (MeV) is an RNA virus within the *Paramyxoviridae* family [1]. Dating back to very early in the history of mankind, the social and economic impact of the virus is immense. Three rare but serious measles complications exist, pneumonia being the most common one; it is the cause of most deaths associated with the virus [2]. Acute disseminated encephalomyelitis (ADEM) and subacute sclerosing panencephalitis (SSPE) [3] are rare but affect severely the central nervous system and can be fatal [2, 4]. From 1 November 2023 to 31 October 2024, 30 EU/EEA Member States reported a total of 18 044 measles cases, of which 13 863 (76.8%) were laboratory-confirmed [5].

As a part of the European Regional Commission for Measles and Rubella Eradication Control (RVC), case monitoring in Bulgaria is conducted regarding the progress towards complete eradication of the disease [6]. This aim has been achievable since the introduction of MMR vaccines with high efficacy and immunogenicity. Those provide lifelong immunity. In addition, MeV is relatively stable genetically, with no

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rapid mutagenicity [7].

Analyzis of the sequence variability within the 450 nucleotides encoding the last 150 residues of the C-terminal region of the nucleocapsid protein (N-450), a highly variable segment of the viral genome, is an essential part of the standard genotyping protocol [8]. Of the 24 MeV genotypes recognized by the World Health Organization (WHO) over the years, 18 are currently considered inactive as they have not been detected for at least 10 years [9]. Only five of the 6 genotypes currently identified as active, (D8, B3, D9, H1, and D4) were reported in the WHO Global Measles Nucleotide Sequence Database (MeaNS) [10].

The sequencing and phylogenetic analysis of the N (450) fragment permits to trace transmission pathways during outbreaks; in addition, it helps to confirm, dismiss, or identify connections between cases [11]. The genotype of clinical samples can distinguish the vaccine strain from wild-type viruses and indicate whether an individual has a wild-type MeV infection or a post-immunization rash [12].

The current study **aims** to describe the circulation of wild MeV in Bulgaria in 2024, identify the pathways of virus import to the country, and define the main risk groups among the population.

MATERIALS AND METHODS

Study design

All measles-positive cases from twelve Bulgarian regions in 2024 were analyzed to trace MeV genotypes circulation and to identify imported strains (Figure 1). The genetic characterization provides all the information for detecting the pathways of viral transmission and the possible sites of disease contraction.

Patients and clinical materials

To monitor the measles genotype circulation in the country, we examined 126 clinical specimens from 63 patients who provided nasopharyngeal swabs and urine samples and were diagnosed with possible “measles infection” during an outbreak in the country.

- *Throat, nasopharyngeal swabs, and nasal aspirates* were collected by swabbing the mucous membranes of the nasopharynx with Viral CULTURETTE® or a sterile swab placed in sterile viral transport medium (VTM), within the first 3 days after the onset of clinical symptoms of infection, when the virus is

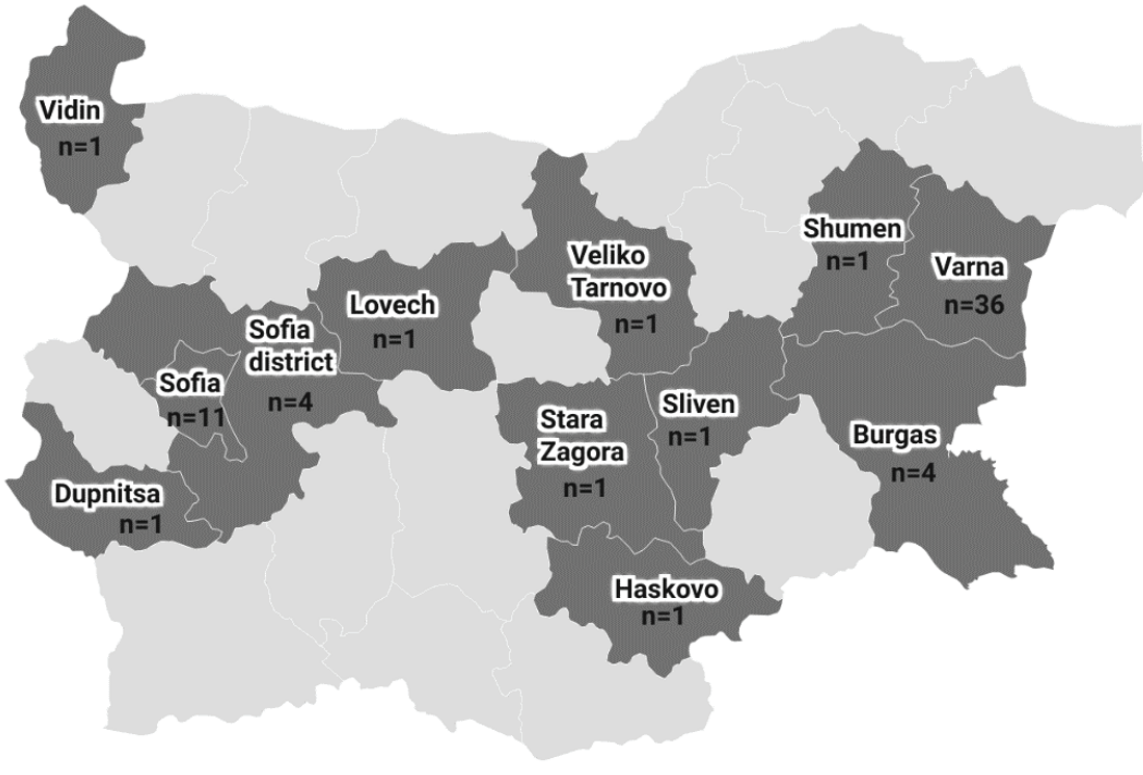

Figure 1. Regional distribution of the studied "possible measles cases” (n=63**)**

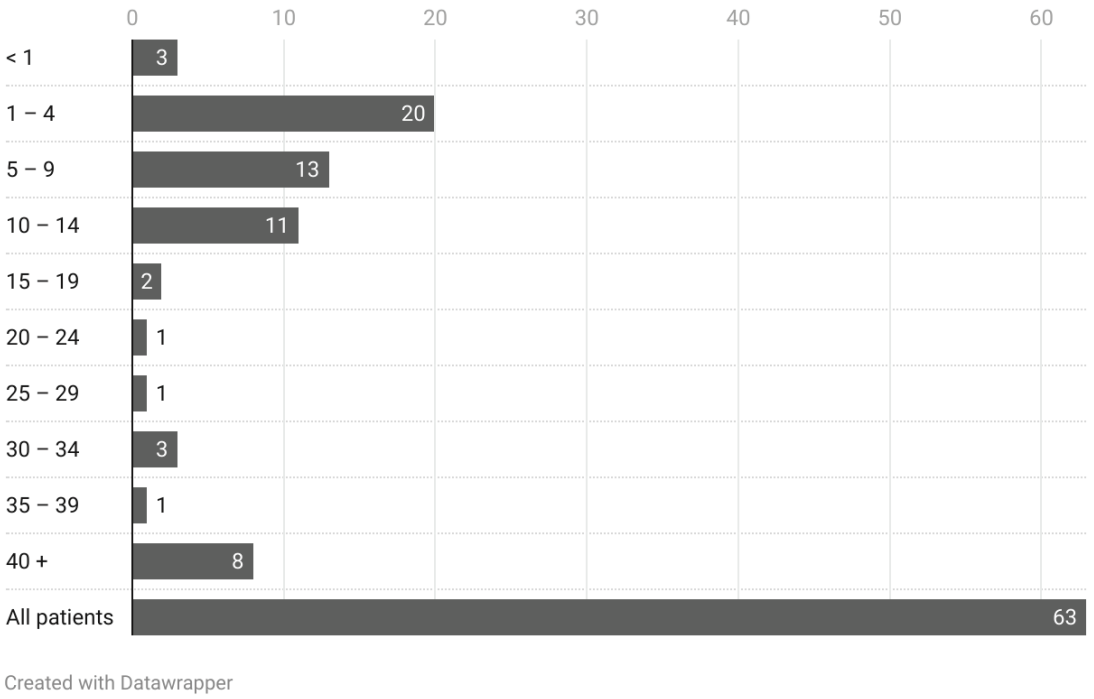

Figure 2. Age distribution of atients examined and confirmed measles cases in 2024.

present in the highest concentration. Storage was carried out while maintaining a cold chain of 6-8°C, and the samples were promptly transported to the laboratory.

- *Sterile urine* - 10 - 50 ml of morning urine was collected in sterile 50 ml containers within 5 days of the onset of the rash, stored in a cold chain (4-8°C), and transported to the laboratory.

The materials were supplied by the Biological Bank of the National Reference Laboratory for Measles, Mumps, and Rubella, Department of Virology, National Center for Infectious and Parasitic Diseases (NCIPD), Sofia. The laboratory collaborated with medical institutions, which provided patient data.

METHODS

The first step of clinical samples processing was column-based extraction with Invitrogen kit (PureLink® Genomic DNA Mini Kit, UK).

Extracted RNA was analyzed by real-time Transcription Polymerase Chain Reaction (PCR) using System Gentier 96E, Tianlong Technology Co. and TaqPath-tm 1-Step Multiplex Master Mix, Applied Biosystems, Thermo Fisher Scientific. Primers and probe used were:

Forward Primer (MVN1139F): 5` TGG CAT CTG AAC TCG GTA TCA C 3`

Reverse Primer (MVN1213R): 5` TGT CCT CAG TAG TAT GCA TTG CAA 3`

Probe (MVNP1163P): 5` FAM CG AGG ATG CAA GGC TTG TTT CAG A BHQ1 3`

After that, amplification of the oligonucleotide consensus primer pairs MeV216 (5’ TGG AGC TAT GCC ATG GGA GT 3’) and MeV214 (5’ TAA CAA TGA TGG AGG GTA GG 3’) for detection of the N gene of the virus via the One Step Reverse Transcription PCR (RT-PCR) method was used. Further on , the samples underwent capillary Sanger sequencing and phylogenetic analysis. The sequencing was performed with the "GenomeLab GeXP" genetic analysis system.

Epidemiological analysis

Information about patients' immunization status as well as the incidence in different age groups wasretrieved from the web-based data collection and dissemination system for epidemiological surveillance of measles, mumps, and rubella in Bulgaria [13].

Phylogenetic analysis

MEGA software, version 11., and the BLAST (Basic Local Alignment Search Tool) server were implemented for phylogenetic analysis [14].

Phylogenetic trees were constructed using MEGA 11.0 software, the neighbor-joining algorithm, and

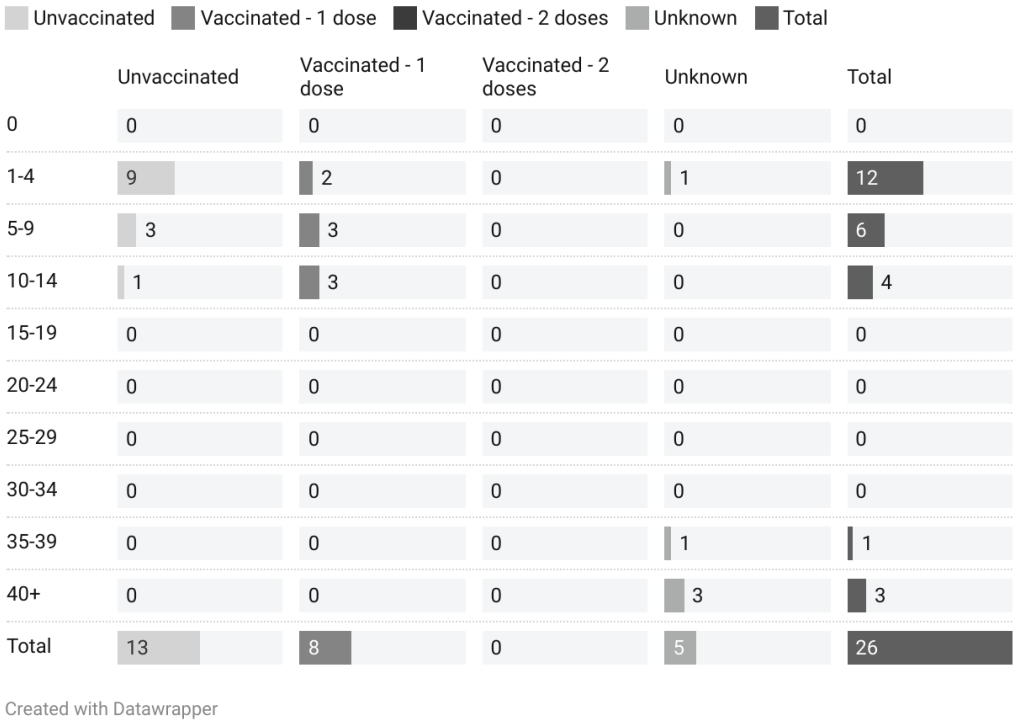

Figure 3. Distribution of confirmed measles cases in 2024 by immunization status and age groups.

bootstrap analysis with 1000 replicates. Alignment and sequence processing were previously performed using the Muscle program built into the MEGA 11.0 software. The best nucleotide substitution model was selected using MEGA 11.0. The genetic distance was determined using the Kimura two-parameter method. Genetic lineages and sub-lineages of DNA isolates were formulated as a cluster of sequences having bootstrap probability ≥70% at the branching point [15].

RESULTS

Clinical cases

The studied cases ranged in age from 1 to 51 years (median 14.5) and were infected during 2024 (twelve months from January 2024 to December 2024 were monitored). Patients were divided into ten age groupsthe highest percentages of tested were in the groups 1 to 4 years (20/63, 32%, 95%CI 20,48÷43,52) and 5 to 9 years (13/63, 21%, 95% CI 10,94÷31,06) (Figure 2). MeV was detected in 26 of them (26/63, 41%, 95%CI 28,85÷53,15).

For the purposes of study and in order to support accurate laboratory diagnosis, two types of clinical material suitable for molecular biological studies were provided by each patient. (63 nasal swabs and 63 urine samples) MeV RNA was detected by Real-time PCR in 47 samples, including 26 nasal (26/63, 41%, 95%CI 28.86÷ 53.14) and 21 urine samples (21/63, 33%, 95%CI 21.39÷44.61).

Next, a conventional one-step RT-PCR analysis was conducted on the available 126 clinical samples. The extracted RNA examined in the first Real-time PCR procedure was used as initial material.

MeV RNA was detected by conventional PCR in 32 probes, including 22 nasal swabs (22/63, 35%, 95%CI 23.22÷46.78) and 10 urine samples (10/63, 16%, 95%CI 11.38÷20.62). Again, the nasal swabs stood out as a better clinical material for laboratory diagnosis and isolation of MeV. Using onventional PCR, MeV RNA was detected in only 38% (10/26) of urine samples of patients already confirmed for measles infection, as compared with 85% (22/26) of nasal swabs. Thus, in twelve patients, MeV RNA was detectable by conventional PCR only in nasal swab, but not in urine samples.

According to the collected epidemiological data on theimmunization status, 13 cases were unvaccinated (13/26, 50%), 8 were with only one dose of measles vaccine (8/26, 31%), and 5 - with unknown immunization status (5/26, 19%) (Figure 3).

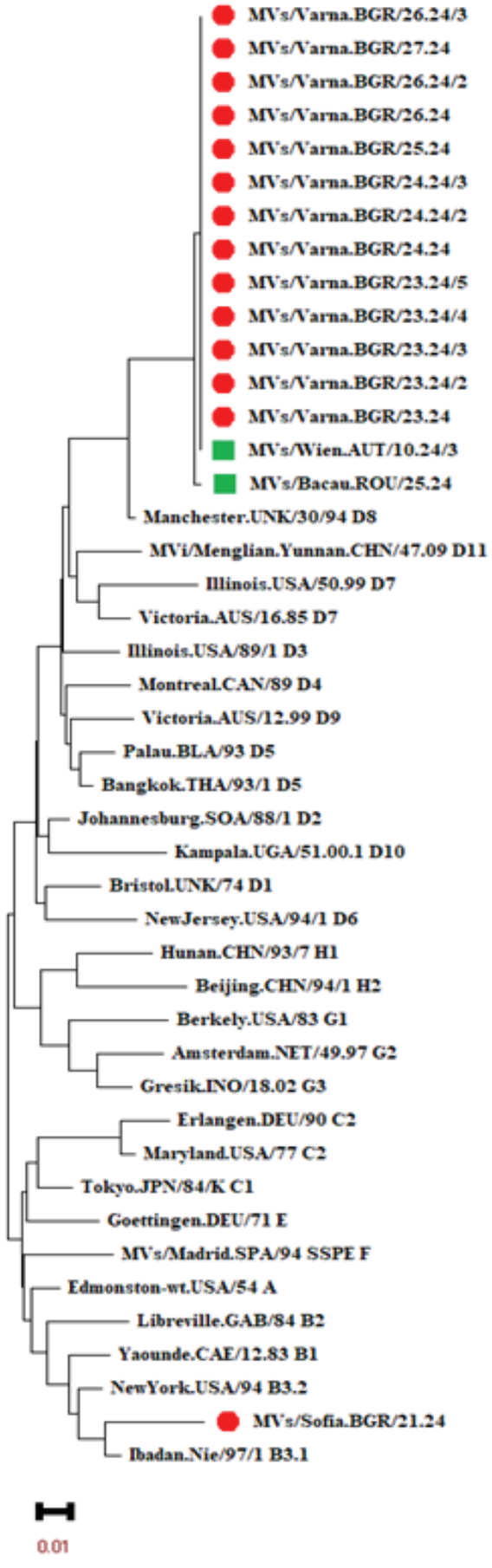

Figure 4. The phylogenetic tree included 44 nucleotide sequences of which 14 Bulgarian isolates indicated by a circle and 30 sequences of reference viruses, representatives of the known MeV genotypes.

Sequencing and phylogenic analysis

Applicable data for genotyping was obtained for 14 of the 26 patients confirmed positive for MeV. The sequence analysis aimed to determine the genetic similarity of Bulgarian sequences with the reference sequences as well as with the MeV viruses isolated in recent years, including those circulating in the European region. Sequence comparison is the most sensitive method for locating and mapping transmission chains. The phylogenetic analysis was based on the N-450 region (450 nt) amplified by conventional PCR. The constructed phylogenetic tree displayed two MeV genotypes, with 13 of the sequences belonging to the D8 genotype, evidently clustered in the Varna region, and only one viral strain (MVs/Sofia. BGR/21.24) was member of B3.1 from Sofia city. Additional analyses in BLAST-NCBI showed 100% similarity of Bulgarian isolates from 2024 with those from Austria (Vienna) and Romania, downloaded from GenBank These findings suggest the potential for import/export of similar MeV strains between these countries (Figure 4).

Another important step in the confirmatory patient analysis is their geographic localization and the definition of the main affected areas in the country. The measles outbreak reported in the past year affected five regions, with the highest number of infections in Varna (n=20) (in the villages of Sindel and Beloslav), followed by Sofia city (n=3), Sofia region (n=1), Burgas (n=1), and Stara Zagora (n=1). It was confirmed that the virus was imported from Austria, Germany, Romania, and the United Kingdom asthe first verified caseshad traveled abroad up to two weeks before the onset of clinical symptoms. Considering the sequence analysis, among measles isolates from Varna of dominated genotype D8, whereas isolates from Sofia City were mainly of B3.1. a

DISCUSSION

Measles is considered s one of the most highly contagious viral infections in recent times. In Bulgaria, laboratory control of measles is carried out by the National Reference Laboratory, where protocols recommended by the WHO and reliable methods for molecular genetic analysis are applied. One of the most accurate methods is Real-time PCR followed by

sequencing of the clinical cases [16, 17]. All countries must provide information on the strains detected or tested to the Global Measles and Rubella Laboratory Network (GMRLN), from where the possible transmission can be sampled or compared [18].

The molecular biological analysis in our study confirmed acute measles infection in 26 out of the 63 tested patients (26/63, 41%). The first registered and laboratory-confirmed cases in the country for 2024 were reported during epidemiological week 14, eV cases were confirmed and observed up to epidemiological week 41, with a peak of cases in July. The affected were mostly children in the age groups of 1-4 (12/26, 46%) and 5-9 (6/26, 2%) years. Our study proved that most of the affected had not completed the first immunization course or had not received a measles vaccine at all. In Those groups, Immunization practices in those groups should be strictly monitored since they are the most vulnerable, and the infection is commonly transmitted among them. A total of 50% (13/26) of measles-confirmed patients had not received immunization. The general trend of infected people who were unimmunized while subject to immunization, or already vaccinated is alarming. Similar data is reported by ECDC, according to which over 80% of confirmed cases in Europe in the last 12 months are among unimmunized individuals [19]. This raises questions about postponed immunization, on one hand and about unsuccessful or incorrectly administered vaccine, on the other. In 2023 alone, 500,000 children in the European Region did not receive the first dose of the measles vaccination (MCV1) as programmed byroutine vaccination programs [20]. The main reason for the increase in cases is the COVID-19 pandemic, which prevented millions of children from following their immunizations and therefore led to a 43% increase of anticipated measles fatalities and an 18% increase of expected measles infections in 2022 as compared to 2021 [20]. Thirty-seven nations reported large or disruptive outbreaks. The European Region accounted for one-third of all global measles cases in 2024 and Romania, which is a neighboring country, accounted for the highest number of cases in the region in 2024 (30,692 cases) [21]. Measles among young age groups is associated with the highest risk of life-threatening complications [21, 22].

Epidemiological data indicated that MeV was imported in Bulgaria from Germany, Romania, and the United Kingdom, as the first confirmed cases had reported a stay abroad up to two weeks before the onset of clinical symptoms of the disease. The highest number of infected patients were from the region of Varna (20/26, 77%), and single cases were confirmed in Sofia city (3/26, 12%) Sofia region (1/26, 4%), Stara Zagora (1/26, 4%) and Burgas (1/26, 4%). Sequence analysis and genotyping proved the circulation of two measles genotypes: D8 (localized in Varna) and B3.1 (in Sofia city), which have been dominant worldwide in recent years. The number of measles genotypes reported by GMRLN has decreased significantly, from nine in 2013 to just two since 2021. In 2022, of 1470 measles sequences reported, 772 (53%) were of genotype D8 and 698 (47%) were -of genotype B3 [22]. The tendency continues in 2023, when 3,373 MeV sequences from 74 countries have been reported, of which 2,503 (74%) were from genotype D8 and 870 (26%) - from genotype B3 [23]. In 2024 data was the same, and global distribution of D8 and B3 were reported [24]. Studies from Bulgaria in the recent years have shown mainly the transmission of the same two genotypes (D8 and B3) [25].

The present study confirmed that nasal swabs were a more suitable clinical material for timely PCR diagnosis of MeV, as in all clinically and epidemiologically confirmed patients, the virus was detected in their nasal swab samples. In five patients, MeV RNA was detected only in nasal swab samples and not in their urine. This is primarily related to the route of MeV transmission and primary viremia in the regional lymph nodes of the respiratory tract. During the first days of infection, when clinical materials are collected, the virus multiplies mostlyand attains the highest concentration in the upper respiratory tract, with main source of emission –secretions from the nasopharynx [26]. At the same time, urine contains many nucleic acid-destroying compounds, such as urea, which affects the analysis [27, 28].

The comparison of the two amplification methods used (Real-time and conventional PCR), outlined Real-time PCR analysis as the more sensitiveand specific. Four Bulgarian patients with confirmed measles were identified solely by Real-time PCR, while conventional PCR failed to detect the virus. Real-time PCR advantages over traditional PCR techniques s are a

shorter processing time, lower labour requirements, and reduced risk of contamination, as it eliminates the need for post-amplification procedures [29].

The rapid and timely registration of measles cases in the country in 2024, as well as the reference laboratory diagnostics, ensured the containment of the epidemic outbreak in Varna region and the prevention of viral spread in the other regions, where only sporadic cases were reported.

CONCLUSION

Measles surveillance serves as a broader indicator of the immunization system performance. A rise in measles cases frequently indicates weaknesses in healthcare infrastructure and vaccination distribution methods, requiring systemic improvements. Strengthening of surveillance mechanisms, such as laboratory confirmation and real-time reporting, is critical for achieving measles eradication targets and preventing future outbreaks. Listing a lot of potential import sources is difficult, but connecting a sequence to an identified strain in MeaNS submitted to the measles nucleotide surveillance database suggests that the new sequence is a member of a lineage with global spread. Given the recent re-emergence of measles, improvements in surveillance infrastructure and immunization programs are required to sustain progress toward global virus elimination.

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