

LABORATORY COMPARATIVE ANALYSIS OF SEROLOGICAL AND MOLECULAR BIOLOGICAL METHODS FOR DETECTION OF MEASLES VIRUS IN BULGARIA

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ABSTRACT

This study **aimed** to perform a comparative analysis between the frequency of detection of the measles virus in Bulgarian patients by using two types of laboratory methods - serological and molecular.

Materials and Methods: Two types of clinical material (serum samples and nasal swabs) A from a total of 202 patients with were tested. The specimens were collected during the measles outbreak in Bulgaria in 2019. The serological - indirect EIA test for detection of specific IgM antibodies and molecular methods - extraction and detection of viral RNA were used.

Results: In the present study, tested Bulgarian patients were divided into 11 age groups. The majority of patients were under 9 years of age (126/202, 62%), including children under 1 year of age (31/202, 15%). Acute measles infection was confirmed by ELISA-IgM in 136/202 (67%) and by RT-PCR in 138/202 (68%) of cases. The positive patients detected only by PCR methods were mainly among the younger patients. In 123/202 of the patients (60,89%) measles infection was confirmed by a combined serological and molecular-biological approach. The rate of coinciding results obtained

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was 87%, including double positive (n=123) and double negative (n=52) tests. No significant differences in the results in terms of gender and age were found.

Conclusion: The combined laboratory approach (immunoenzymatic and molecular assay of each suspected case) is a requisite for measles detection, especially before the onset of symptoms when specific IgM antibodies could not be detected. Molecular biological techniques are basic and preferred approach in the field of modern biomedical sciences. They play an important role in the early and accurate etiological diagnosis and monitoring of viral infections, in particular the measles virus.

Keywords: *measles, RT-PCR, ELISA assay, IgM antibodies*

INTRODUCTION

The study of viral and bacterial infectious agents can be a difficult challenge. This is due either to nonspecific manifestation or atypical clinical course after the implementation of specific prophylaxis, including active vaccination. Therefore, a comprehensive differential diagnosis should be generated, taking into account the diversity of the clinical manifestation, the attendant complications, and the epidemiological parameters of the patient. The accurate diagnosis and proper etiologic treatment often can be difficult.

Serological (immuno-enzyme) methods are based on the high specificity of the antigen-antibody reaction. This type of diagnosis supposes the identification of the infectious agent structural proteins and/or the detection of specific antibodies of classes Ig M, Ig G, Ig A, which are an important diagnostic marker for current (acute) or past infection (immune status) (1, 2).

Development and establishment of different molecular genetic techniques are important for improvement of diagnostic methods. The development of molecular diagnostic procedures for detecting nucleic acid, especially immunogenic regions of the viral or bacterial genome, are current methods for detection of infectious agents. Molecular diagnostics is a tendency in para clinical practice that uses laboratory techniques to detect a large number of pathogens (3, 4).

Measles virus is a single-stranded, negative-sense RNA virus and a member of the Morbillivirus genus in the family of Paramyxoviridae. Measles virus is an antigenically monotypic virus. Point mutations have been accumulated in different parts of the virus genome, mainly in regard to hemagglutinin and nucleocapsid. However,

only a small number of these accumulations induce amino acid substitutions enabling the appearance of new biological characteristics. That determines the measles virus stability (5). Measles is transmitted from person to person by the airborne route. It is characterized by a high contagious index (over 95%), which determines the rapid spread of infection among unprotected population groups (6, 7, 8, 9, 10).

According to World Health Organization (WHO) criteria, measles infection is confirmed by clinical and laboratory diagnostics (11).

Clinically, measles is diagnosed in the presence of one or more typical symptoms: appearance of generalized maculopapular rash lasting 3-4 days, combined with fever (38.5°C or higher), and at least one of cough, coryza or conjunctivitis (11, 12).

Laboratory diagnosis of measles is based on one of the following indicators:

- isolation of measles virus from clinical specimens (throat swab, nasopharyngeal aspirate, conjunctival swab, urine);
- detection of measles RNA;
- detection in serum or oral fluid specimens of specific antibody response against measles virus typical for acute infection;
- detection in clinical specimens of measles virus antigen by Direct Fluorescent Assay (DFA) using measles virus-specific monoclonal antibodies.

Laboratory testing depends on important factors such as collecting an appropriate clinical specimen for virus detection, its storage, transport and interpretation of the results in a certified laboratory. Genotype data should be used in conjunction with epidemiological information to track transmission pathways and identify sources of infection. On the other hand, sequencing analysis is the only way to distinguish whether a person has wild-type measles virus infection, or a rash caused by a recent measles vaccination (10).

This study **aimed** to perform a comparative analysis between the frequency of detection of measles virus in Bulgarian patients by using two types of laboratory methods - serological and molecular.

MATERIALS AND METHODS

Materials

A total of 404 clinical specimens, collected from 202 patients with diagnosis "probable measles infection", were tested by applying a combined differential diagnostic approach (serological and molecular-biological). Cases were reported during the 2019 measles outbreak in Bulgaria. Sera samples (n=202) and nasal swabs (n=202) were provided from each patient.

Materials were provided by the biological bank of the National Reference Laboratory "Measles, Mumps, Rubella", Department "Virology" at the National Center of Infectious and Parasitic Diseases (NCIPD), Sofia.

Methods

• Serological analysis

All serum specimens were tested for presence of anti-Measles IgM with a commercial indirect enzyme-linked immunosorbent assay (Anti-Measles IgM/IgG ELISA, Euroimmun, Germany). The tests were carried out according to manufacturer's instructions. The absorbance values of tested samples were divided by the mean absorbance values of cut-off calibrator and the results were interpreted qualitatively as positive, negative or equivocal.

• Molecular analysis

- Extraction of viral RNA from starting specimen (nasal swabs) with commercial test Qiagen Viral RNA Mini kit, Germany was performed.
- Detection on nucleoprotein (N) gene and determining the frequency of its proof as a diagnostic marker by commercial kit Qiagen One-Step RT-PCR. CDC consensus primers MeV216 and MeV214 (concentration 20 µM) were used for the detection of MeV.
- Electrophoresis in 2% agarose gel stained with ethidium bromide for visualization of measles PCR products.

• Statistical Analysis

For the statistical processing of the results obtained we used relative percentages (%), graphical and table analysis.

RESULTS AND DISCUSSION

Demographic data

A total of 202 patients with "probable measles infection" (Obs. Morbilli) were investigated. Samples were collected over a twelve month period during outbreak in 2019 in Bulgaria. Two types of clinical material were provided from all investigated patients to perform accurate measles diagnosis. According to WHO recommendation, combined serological and molecular approach for viral detection was used.

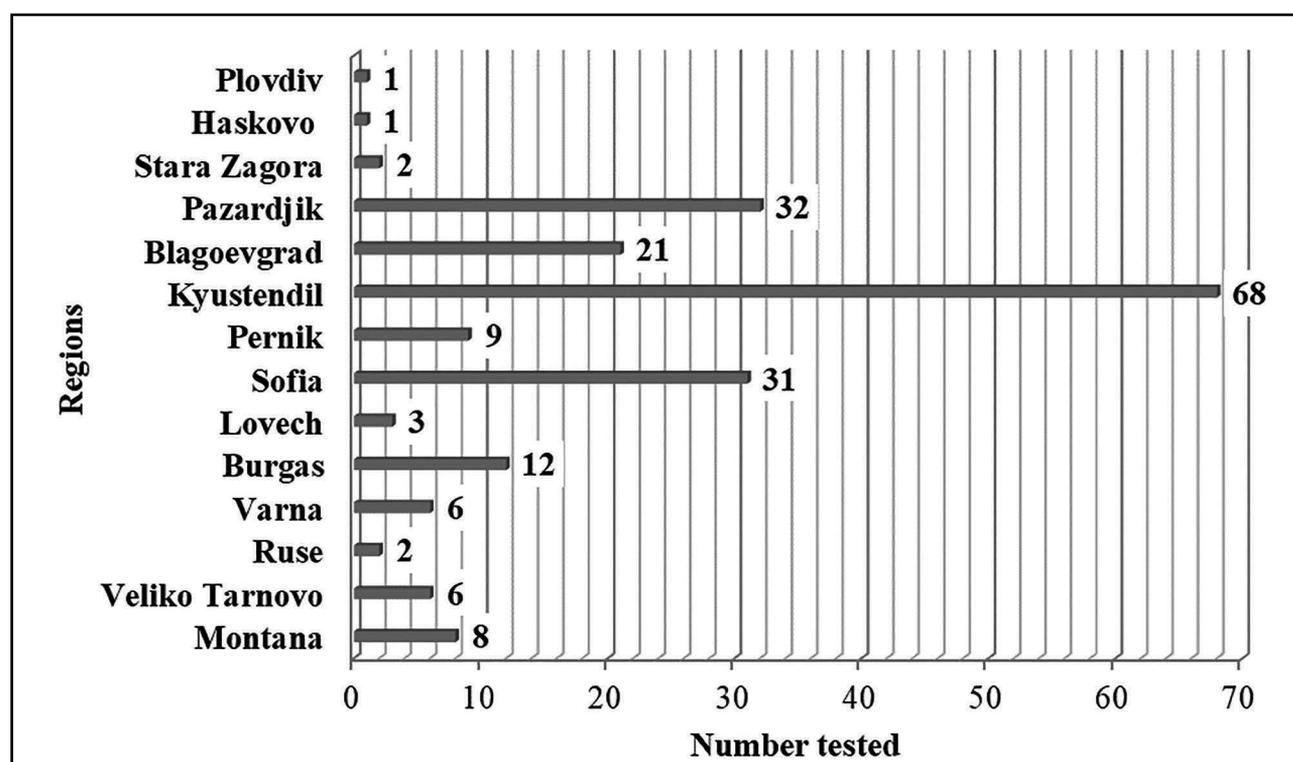
The tested patients were divided into 11 age groups. Most of the infected were children from the groups: less than 1 year, 1-4 years and 5-9 years old. The majority of patients were under 9 years of age (126/202, 62%), including those under one year of age (31/202, 15%), who according to the health policy of the country, had not yet received the measles vaccine and were potentially at risk (Table 1).

Table 1. Distribution of the tested patients (n = 202) by age and gender, in numbers and percentage

Age (years)	Tested patients		Gender	
	Number	Percent (%)	Male (n/%)	Female (n/%)
<1	31	15%	14 (45%)	17 (54%)
1-4	55	27%	27 (49%)	28 (50%)
5-9	40	19%	22 (55%)	18 (45%)
10-14	14	6%	9 (64%)	5 (35%)
15-19	6	2%	4 (66%)	2 (33%)
20-24	3	1%	1 (33%)	2 (66%)
25-29	19	9%	12 (63%)	7 (36%)
30-34	12	5%	6 (50%)	6 (50%)
35-39	7	3%	4 (57%)	3 (42%)
40-44	10	4%	2 (20%)	8 (80%)
≥ 45	5	2%	4 (80%)	1 (20%)
Total	202	100%	105 (52%)	97 (48%)

The current study included 14 of the 28 regions of the country. The samples are from hospitalized persons in the local hospitals (Figure 1). 2019 is characterized by increased measles morbidity among the population in the country and registration of over 1000 infected. Despite the measures taken by the Ministry of Health, the number of patients continued to increase in 2020. The measles outbreak started in the first half of February 2019 in Blagoevgrad

district. The number of tested samples in present study from there is 21/202, 10.40%. The genetic and epidemiological analysis proves the import nature of the disease from the Republic of Western Macedonia. Subsequently, Sofia district (31/202, 15.35%), Kyustendil district (68/202, 33.66%) and Pazardjik (32/202, 15.84%) were covered and tested, isolated cases were investigated in other 9 areas (50/202, 24.75 %).


Figure 1. Distribution of the studied patients (n = 202) by country region

Serological analysis

Acute measles infection (presence of measles IgM antibody) was confirmed by EIA assay in 136/202 (67%) of tested samples. It is an indicator of an early immune response to the virus (11, 13). In many cases measles IgM marker should be considered in combination with testing for other infectious agents causing fiver rash syndrome (14). In this study, all serum samples were tested by rubella IgM ELISA

assay and proved negative for rubella.

The largest proportion of cases were among children aged 1-4 years (45/202, 22,78%) and 5-9 years (27/202, 13,37%) or 72 /202 (35,64%) in total. These are population groups that must have been subjected to first dose MMR vaccine, but patient`s records showed a delay of the vaccination or gradual decrease in protective immunity. This is the group of patients most affected by measles in Europe and around the world (15).

Table 2. Distribution of tested patients by age and confirmed acute measles infection by measles ELISA IgM and RT-PCR diagnostic markers and age groups (n = 202)

Age (years)	Number of patients (n)	Patients tested by measles IgM ELISA		Patients tested by measles RT-PCR	
		Number (+)	Number (-)	Number (+)	Number (-)
<1	31	21	10	25	6
1-4	55	45	10	42	13
5-9	40	27	13	28	12
10-14	14	6	8	8	6
15-19	6	6	0	5	1
20-24	3	2	1	2	1
25-29	19	11	8	10	9
30-34	12	6	6	6	6
35-39	7	6	1	5	2
40-44	10	4	6	5	5
≥ 45	5	2	3	2	3
Total	202	136	66	138	64

Molecular analysis

Molecular biological methods have been identified as one of the most sensitive assays for determining a viral agent in clinical and tissue samples. Detection of specific target region of viral or bacterial genome with PCR-based technique has been used clinically to improve the diagnostic accuracy. During the first few days after the infection onset, RT-PCR may be diagnostically more useful than serology.

In the current study, MeV-RNA was detected in 138 out of 202 (68%) specimens (nasal swabs) (see Table 2). The majority of cases occurred among children below 9 years old. The results for four patients (younger than 1 year) were positive only by PCR analysis, while their serological test was negative (16).

A comparative analysis between the standard laboratory methods (immunoenzymatic and molecular biological) was carried out, by calculating

the frequency of detection of serological markers - specific IgM antibodies and conservative region of viral genome. Acute viral infection was serologically confirmed in 136/202 (67%) of the cases by serology, and in 138/202 (68%) of the cases by molecular biology. In the majority of the patients tested (123/202, 60.89%), the etiological role of the measles virus was confirmed by a combined serological and molecular-biological approach (Figure 2). Considering that with two types of clinical material taken on the same day of infection were provided from all individuals, the results obtained showed a detection rate of 67% and 68% for the two diagnostic markers - IgM antibodies and viral RNA, respectively. The coincidence rate of results obtained was 87%, including double positive (n=123) and double negative (n=52) ones. Serum-based measles-specific IgM EIAs are the recommended laboratory assays for diagnosis of

acute measles infections and appear to be sufficient for measles control programs. However, serum samples are not ideal for molecular characterization of measles virus (17).

Laboratory confirmation methods are affected by the timing of specimen collection (18, 19). In fact, studies have shown that 30% of serum specimens obtained in the first 72 hours after rash onset reveal negative results, since IgM antibodies are still developing and may be below the detectable levels (20, 21). Taking into account that case investigation and sampling, in our context, occurred

early after rash onset, the number of measles cases reported might slightly underestimated based on IgM serology solely. In similar cases, a number of authors highlighted the advantages of the molecular analysis (16). The added advantage of throat swab samples is the non invasive and easy-to-handle collection as compared to serum samples that require specific transport and conservation conditions to avoid false negatives (22).

Our data show the potential interchangeability of the two approaches - serological and molecular biological in the diagnosis of measles virus.

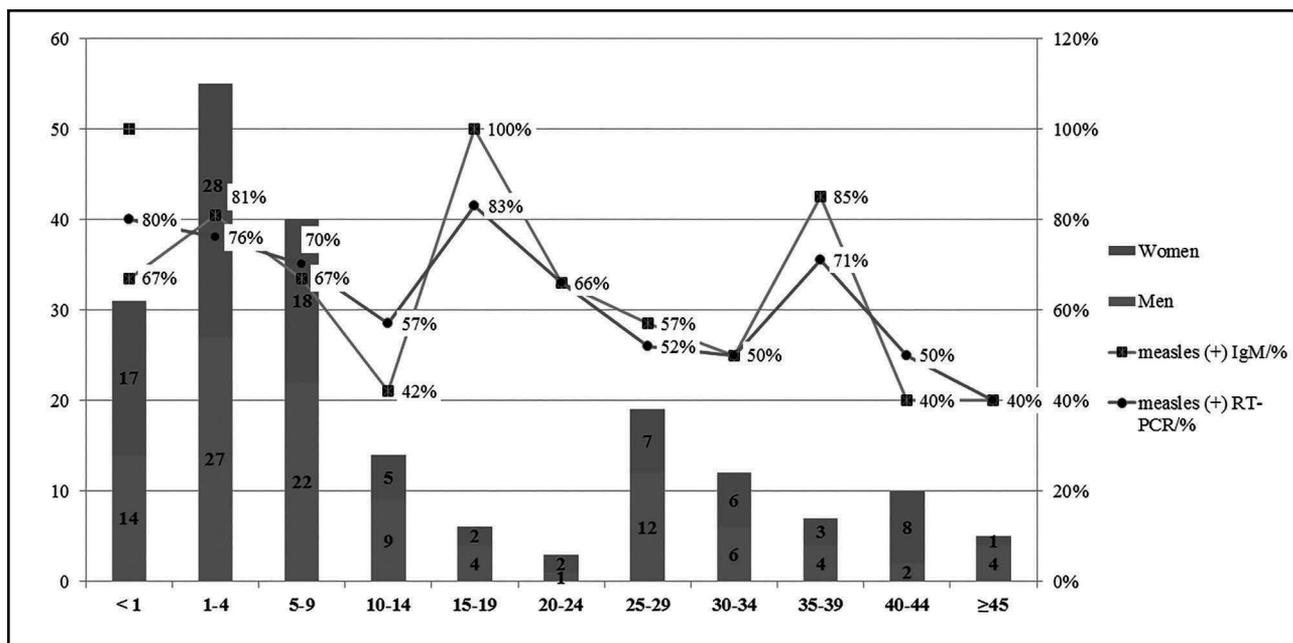


Figure 2. Distribution of confirmed positive patient samples (serum samples and nasal swabs) for measles with a combination of diagnostic markers in percentages by age and sex (n = 202)

The similar results obtained in the diagnosis of acute measles infection show that both laboratory methods have equal sensitivity and specificity. Therefore, the appropriate approach for the diagnosis of measles infection will depend entirely on the specific clinical case, health condition of the patient and doctor's estimation.

Most of the MeV-positive cases were confirmed by both RNA detection and serology. The positive patients detected only by PCR methods were mainly among the younger tested. This could be due either to sample collection during the window period or very early before the onset of symptoms when specific Ig M antibodies cannot be detected, or - eventual reinfection with the virus, in which case the organism may react without acute phase IgM antibodies formation (Figure 1). In these cases, the molecular approach is the only successful choice of diagnostic method (23).

CONCLUSION

Molecular-biological techniques are a basic and preferred approach in the field of modern biomedical sciences. Genomic techniques developed rapidly and became a subject of great interest in the first two decades of the 21st century. They provide an opportunity to study the structure of micro and macro-organisms, the microbiome, and infectious agents that are important for public health. PCR is a particularly appropriate laboratory method for clarification and confirmation of measles virus infection in a variety of circumstances, including atypical or complicated presentations (young children, pregnant women and the elderly, immunocompromised patients) or cases in which serum for antibody testing is not available. PCR facilitates identification of measles virus genotypes and differentiation between vaccine-associated and wild type measles virus infection. Molecular biological methods play an important role

in the early and accurate etiological diagnosis and monitoring of infectious pathogens, in particular the measles virus.

Competing Interest

The authors do not have any competing interest.

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