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PROBLEMS
of Infectious and Parasitic Diseases

NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 48, NUMBER 2/2020

ISSN 0204-9155

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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 48, NUMBER 2/2020

Preprinting and Printing by Boris Zl. Pavlov & Alliance print

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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 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*

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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^{0}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 %).

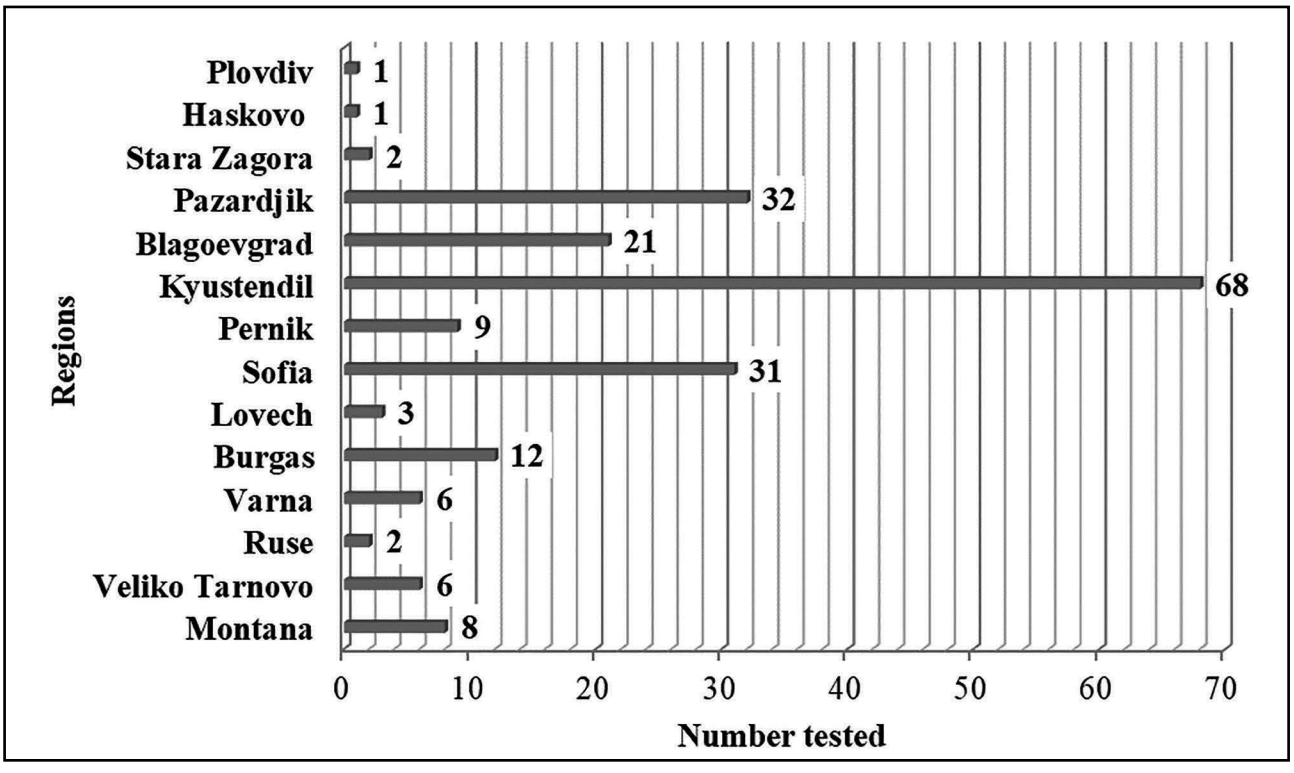

Figure 1. Distribution of the studied patients (n = 202) by country region

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, occured 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.

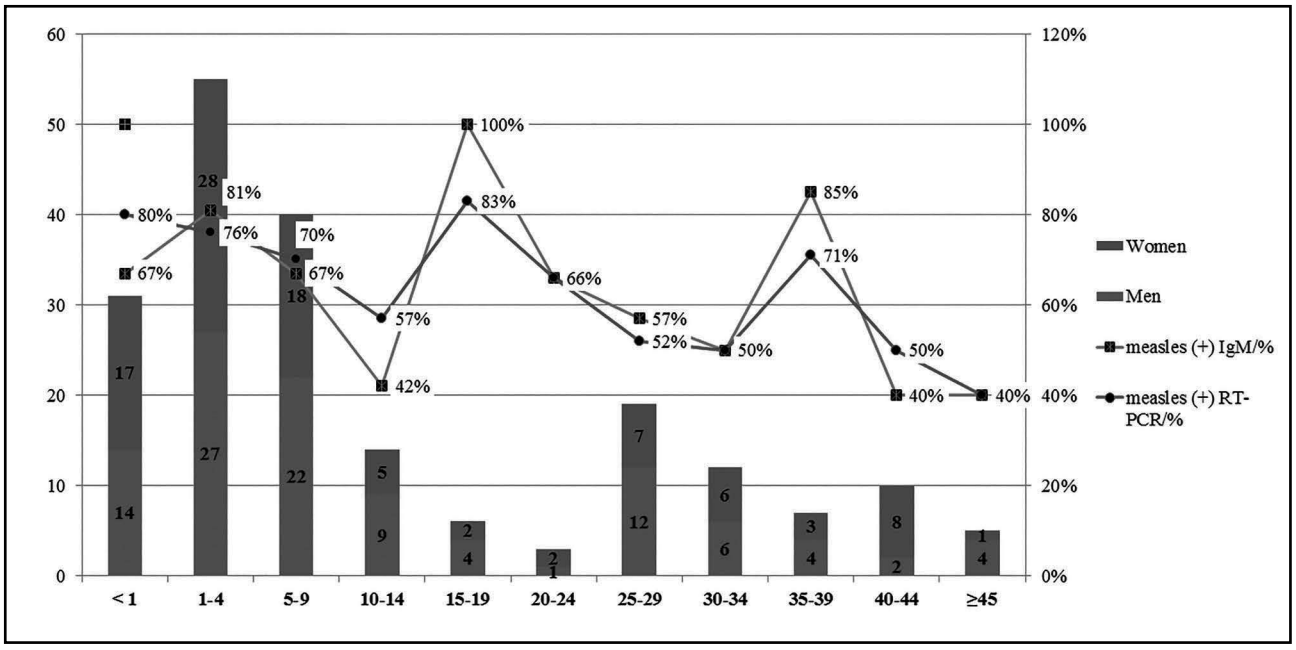

Figure 2. Distribution of confirmed positive patient samples (serum samples and nasal swabs) for measles with a combination of diagnostic markers in percentages by age and sex (n = 202)

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.

Acknowledgements

This work was funded by the European Fund for regional development through Operational Program Science and Education for Smart Growth 2014 - 2020, **Grant BG05M2OP001-1.002-0001-C04 "Fundamental Translational and Clinical Investigations on Infections and Immunity**".

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IMMUNE RESPONSE TO COVID-19 COMPARED TO THE IMMUNE RESPONSE TO SARS, MERS AND INFLUENZA

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ABSTRACT

The course, form and outcome of an acute respiratory illness, as well as its patho-histological features largely depend on the level of inflammatory cytokines. The most important proinflammatory cytokines and chemokines are: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α and GM-CSF. There are many similarities in the human immune response to influenza, SARS and MERS-CoV. Available studies of COVID-19 show a completely different immune response, i.e. immunological indifference or suppression.

Influenza is a disease we have known for a long time. WHO has been successfully following the antigenic drift of influenza virus ever since 1952 (WHO's Global Influenza Surveillance and Response System (GISRS). This is necessary to monitor epidemiological characteristics of influenza as well as for the components of the seasonal vaccine which contains the antigenic characteristics of the subtypes and variants of influenza A virus that circulated in the previous season in the southern hemisphere. Throughout this period, many viruses and bacteria caused respiratory infections, sometimes in increasing epidemic numbers, but it was only the flu that caused serious problems. The epidemics were accompanied by high morbidity and significant mortality. Beta-corona viruses caused a serious warning in 2002 when SARS Cov-1 and MERS in 2012 appeared, followed by high mortality. Alpha corona viruses have been present all this time, but have caused mild upper respiratory infections and rhinitis, without serious consequences. Depending on the season and the region, corona viruses have been present in 10 to 35% of respiratory infections with the immune response to any infectious agent, may be mild, moderate and consequently heal, or severe when due to the high level of cytokines many barriers and membranes can be damaged and cause death. In influenza, the immune response is adequate. Only in a small percentage of cases, an overactive immune response is observed that causes damage and even death. SARS and MERS-CoV have been also shown to elicit a strong immune response.

COVID-19 has been present for only a few months, and despite the efforts of many scientists, the epidemiological characteristics and pathogenesis of the disease are still not completely clear. Although COVID-19 belongs to beta corona viruses along with SARS and MERS-CoV, there are differences in the immune response. Whether COVID-19 weakens the immune system, or the immune system does not recognize it as a serious threat, there is a weak immune response during this infection. Such a significant discrepancy in the immune response can help understand the pathogenesis of COVID 19 and the causes of primary viral pneumonia and ARDS followed by high mortality.

Keywords: *COVID-19, immune response, SARS, MERS, Influenza*

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INTRODUCTION

The purpose of the present paper was to analyze the differences in the immune response between influenza, SARS and MERS-CoV on the one hand and COVID-19 - on the other, and to explain the possible causes of mortality in COVID-19. Papers from highly rated scientific journals, monographs and textbooks were reviewed to analyze the immune response to influenza during epidemics and pandemics, and to compare it to the immune response observed during corona infections.

Flu is caused by influenza types A and B, while influenza type C causes mild upper respiratory infections. Until now, pandemics have only been caused by influenza type A, with subtypes H1, H2 and H3. During a flu epidemic, caused either by influenza virus type A or B, a clinical picture with severe symptoms of general infectious syndrome always develops. In infectious diseases, viral or bacterial, the symptoms of the general infectious syndrome appear due to the cytokine response. The severity of these symptoms, such as fever, malaise, headache, bone and muscle pain, drowsiness, sore throat, etc. depend on the levels of cytokines and other elements of the immune system that modulate the immune response. During influenza infection, there is secretion of the interferon-α (IFN-α), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) α and β, interleukin-6 (IL-6), interleukin-8 (IL-8) and

monocyte-attracting chemokines (2, 7,12, 13, 14, 15, 18, 22). A higher level of released cytokines has been accompanied by more pronounced symptoms of the general infectious syndrome. The human immune system senses the influenza virus as a serious threat and is activated immediately after the virus enters the respiratory epithelium cells. As a result of the rising cytokine levels preceeding the death of the respiratory epithelium cells the clinical picture of flu is observed, whereby after an incubation of 1-2 days the symptoms of general infectious syndrome appear. Symptoms due to the death of a large number of upper respiratory tract epithelial cells occur two to three days after the onset of the disease, such as sneezing, coughing, runny nose, and lacrimation. The latter are present for a long time after the symptoms of the general infectious syndrome have subsided. The temperature normalizes 4-5 days after the onset of the disease, as well as the other symptoms of the general infectious syndrome, as a result of the normalization of the cytokine values and other elements of the inflammatory response of the humoral and cellular immunity. The symptoms caused by the death of the respiratory epithelium cells (sneezing, coughing, runny nose) remain present until those cells are restored (2, 14, 15, 16, 22). But in a small number of people (0.01–0.02% of patients), the immune system reacts too strongly, secreting very high doses of cytokines, leading to a violent immune response that damages the barriers and membranes in the body. The most severe condition is encephalopathy, which is associated with increased levels of numerous cytokines, but in particular, with an increase in IL-6 levels. When the blood concentration of IL-6 is between 80 to 150 pg / ml there is no risk of brain dysfunction. Above 150 pg / ml a mild brain dysfunction occurs. When the level of IL-6 exceeds 6000 pg / mL, the hematoencephalic barrier is damaged, and blood elements such as bilirubin, urea, and creatinine that are toxic to neurons come into contact with CNS cells. Due to the death of CNS cells, he clinical picture of encephalopathy develops. Encephalopathies are severe conditions ending up in 30 to 50% mortality. Encephalopathies with IL6 values above 15 000 pg / mL always end in death. In addition, the impaired hematoencephalic barrier during influenza viral infection, is associated with significantly increased rates of purulent meningitis (7,16,19,20) .

Numerous studies have analyzed episodes of beta corona virus infections and the occurrence of SARS-CoV in 2002, and the 2012 Mers-CoV Middle East Respiratory Syndrome. (1, 6, 18, 25, 26, 27) In both cases, a strong immune response and a cytokine storm with an increase in IL-8, IL-1β, IL-1b and IL-6 were observed. IL-8 appears to be the major cytokine responsible for the inflammatory response in the lungs accompanied by infiltration of neutrophils, monocytes, and NK cells. Experiments in mice in which IFN- α, CCL2, IL-6, TNF- α, and IFN-γ had been induced, demonstrated inflammatory infiltrates with numerous neutrophils, monocytes, and NK cells. IL-1β was associated with tissue damage, neutrophil infiltration, acute inflammatory reactions, and severe respiratory viral infection.

From the numerous papers on COVID-19 available until now, it is evident that there is no strong immune response to this infection **(Table 1).** Leukopenia or normal leukocyte counts are present in most of the cases. The frequently observed lymphopenia is somewhat surprising. Procalcitonin level is normal. CRP increase is detected in a small percentage of cases. (1, 3, 4, 5, 12, 17,18, 22-24, 26, 27). Chuan Chin (9) analyzed the immune characteristics in a group with 452 patients with COVID-19, of which 286 were with severe infection, and 168 - with a mild clinical form. There was a slight increase in IL-6 values only to 13.3 pg / ml in patients with a mild clinical picture, as opposed to 25.2 pg / ml in severe cases (normal values 0.0–7.0 pg / mL). The other tested cytokines, IL -2R, IL-8, IL-10, and TNF-α as well as serum IgM, IgG, and IgA were within the normal ranges in both groups. The absolute numbers of helper (CD3 + CD4 +) and suppressor T cells (CD3 + CD8 +) in patients with COVID-19 were below the normal values. The decrease of helper T cells in the group with severe infection was more pronounced. The function of CD4+, CD8+ T cells, and NK cells (in terms of IFN-γ expression) were in normal ranges and no significant difference was established between severe and non-severe cases. Lymphocyte subsets were analyzed in 44 patients with COVID-19 on admission. The total number of lymphocytes: B cells, T cells, and NK cells was significantly lower in patients with COVID-19 (mean values of 852.9 cells/μL, and even lower) in severe cases, (743.6 vs. 1020) in the non-severe group. Similar results were also obtained by Yishan (22) who analyzed 125 patients, 103 of whom had COVID-19 and 22 non-COVID-19 pneumonia. Wei-jie Guan's analysis of 1,099 patients concluded that there was a difference in the clinical picture and immune response between COVID-19 and other acute respiratory infections (influenza, SARS CoV-1, MERS Cov (19). Other authors (4, 6, 18, 26) also found that the values of interleukins and other cytokines in COVID-19 were either unchanged or slightly elevated. Leukopenia and lymphopenia were present. The fact that there

is no strong immune response during COVID-19 upper respiratory tract infection is in favour of the mild clinical picture whereby the symptoms of the general infectious syndrome are either absent or of low intensity. By the second week of April, more than 2,350,000 SARS-CoV-2 positive patients have been registered worldwide, over 162,000 of whom have died. So far, not a single case of COVID-19 who has died from encephalopathy has been described. This fact is also in favour of the absence of strong immune response, or cytokine storm in SARS-CoV-2 infection.

Table 1. Cytokine levels registered in hospitalized patients during COVID-19 pandemic

author	Reference N	Number of Patients tested	TNF-α (pg/mL)	IFN-γ (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-10 (pg/mL)
Chuan Qin	6	452	8.6 (6.9–10.9)		21 (6.1–47.2)	16.7 (10.2–27.0)	5.4 (5.0–9.7)
Fei Zhou	27	191			7.4 (5.3–10.8)		
Nanshan Chen	4	99			7.9 (6.1–10.6)		
Huan Han	10	102	3-64	1-8	4-32 64-250 (6 patients)	4-16 16 - 64 (7 patients)	

Cytokine storm is the term for a dramatic increase in cytokine concentrations. Cytokine storm occurs at concentrations greater than 4.000 pg/ml for NF-α, IFN-γ, IL 1, and IL 8, greater than 3.000 pg/ml for IL2 and IL 6, more than 2.000 pg/ml for IL 4, more than 1.500 pg/ml for IL 10 and more than 400 pg/ml for IL-12 (9, 10)

Based on previous experience with respiratory infections, the absence of strong immune response may be due to several reasons. The first possible option is that the respective causal agent is part of the normal microflora of the upper respiratory tract and the immune system does not recognize it as a foreign antigen and a danger to the body. The second option is the existence of specific secretory IgA. After an acute respiratory infection, in addition to IgM and IgG antibodies, the level of IgA secretory antibodies increases. These virus-specific IgA antibodies, in the case of COVID-19, neutralize the virus before it enters the cells of the respiratory epithelium, preventing the infection, so that viral antigens do not come into contact with the human immune system at all. It is possible that we currently have a second wave of COVID-19. IgG antibodies to COVID-19 are found in about ten percent of the population. A third option is the existence of cross-immunity so that antibodies to alpha corona viruses (causing seasonal infections in humans) neutralize SARS CoV-2 as well. The fourth possible explanation for the lack of a strong reaction (and absence of general infectious syndrome) is the nature of the virus itself, which manages to avoid the initial strong immune response, , as is the case with HIV infection for example. The virus itself does not cause much damage when entering the body, so there is no proper immune response. HIV enters the body without much resistance, after which it multiplies in the cells of the immune system (CD4 T lymphocytes). Indeed, in the case of SARS-CoV-2, it is paradoxical to reach a state of leukopenia lymphopenia, a decrease in T lymphocytes, for a short period of several days, in an acute infection with short incubation of about 4 (2 -14) days).

Regarding the occurrence of primary viral pneumonia, there is a large difference in the clinical manifestations of epidemic vs. pandemic influenza. During epidemics lower respiratory infections and primary influenza pneumonia are not typically observed. In general, pneumonia during influenza epidemics is due to a secondary bacterial infection. Outbreaks in inter-pandemic period are caused by small drifts in the antigenic structure of viral hemagglutinin. When the new variant appears, memory B lymphocytes recognize a similar antigen, followed by a rapid rise in antibody titters from the corresponding clone. At the same time, there is a complete immune response to the new antigenic influenza virus variant. But partial cross-action is enough to slow the spread of the influenza virus to the lower respiratory tract. When a large antigenic shift occurs and a new subtype of influenza A virus appears in the circulation, there is a lack of cross-immunity help, permitting the new subtype to descend in the lower respiratory system and cause primary viral pneumonia. In addition, during the first encounter with the new subtype of influenza A virus, a significant number of people have a very strong immune response and secretion of large amounts of

cytokines. These primary influenza pneumonias are the cause of high mortality during pandemics. (16, 19, 20) Unlike pandemic influenza viruses, COVID-19 in a large percentage of patients (about 20%) causes primary viral pneumonia. It is also very unusual that a pathogen that is not extremely virulent (has not yet been shown to have a direct cytopathogenic effect) is able to cause massive bilateral pneumonia. The second paradox is that patients with uncompromised immune system have no problem recovering from this massive viral pneumonia. (4, 5, 8, 27) This is contrary to previous experience in science, where primary viral pneumonias are accompanied by high mortality. On the average, COVID-19 pneumonia occurs 18 days after the infection, when the virus is no longer present in the body. in contrast to primary viral pneumonia presenting during the acute phase of pandemic influenza There is a rich finding on X-rays, but no enlarged lymph nodes in the mediastinum (4.8). Although there is evidence of pneumonia, very few inflammatory cells are detected in the autopsy. All this data require consideration and analysis of another possible condition or co-infection in the settings of COVID-19.

Conclusions

The host's immune response to influenza virus is strong, sometimes even excessive.

The host's immune response during SARS and MERS is strong.

Although it is a similar upper respiratory infection, the immune response in COVID-19 is weak, and in some patients it is even suppressed.

Although SARS-CoV-2 does not stimulate the immune response, it does manage to cause primary viral pneumonia in 20% of those infected.

Patients with well-functioning immune system are (paradoxically) easily cured from massive bilateral COVID-19 pneumonia.

Co-infection with another infectious agent or other condition might be the cause of COVID-19 pneumonia.

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SKIN REACTIONS TO ALLERGENS FROM PROCESSIONARY CATERPILLARS (GENUS THAUMETOPOEA)

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ABSTRACT

Background: Moths of the genus *Thaumetopoea* are widespread pests in the coniferous and deciduous forests in Bulgaria. Contact with the caterpillars, larval form of different *Thaumetopoea* species, causes a series of complaints in humans: mainly contact dermatitis (erucism), but also IgE-mediated allergic reactions.

The aim of the present pilot study is to investigate the skin reaction after prick tests with allergens from different *Thaumetopoea* species in a group of people who have frequent contacts with the processionary caterpillars.

Material and methods: A group of 42 subjects was surveyed comprising 37 men and 5 women between the ages of 18 and 87. Specific sensitization to caterpillars of three *Thaumetopoea* species: *Thaumetopoea pityocampa* (pine processionary); *Thaumetopoea processionea* (oak processionary) and *Thaumetopoea solitaria* (pistachio processionary) was assessed by allergy skin prick tests (SPT) with specially designed caterpillar allergens.

Results: Positive allergy skin tests to one or more caterpillar's allergens were measured in 18 (43%) participants. A simultaneous test with the three allergens from the different *Thaumetopoea* species showed that in 6 (33%) of the cases, skin hypersensitivity only to *T. pityocampa* allergens was present. Monosensitization to *T. processionea* was observed in 2 (11%) cases. The rest 10 (56%) participants with positive skin test showed different profiles of polysensitization to the studied *Thaumetopoea* allergens.

Conclusions: The SPT evaluation of skin reactivity to different caterpillar's allergens outlined the important role of processionary allergens, especially those from *T. pityocampa,* in the development of IgE-mediated allergic complaints in different groups of forestry professionals. In view of these results, it seems that IgE-mediated hypersensitivity allergic reactions to *Thaumetopoea* caterpillars are at least as important as those with no allergic mechanism.

Keywords: *Thaumetopoea pityocampa, Thaumetopoea processionea, Thaumetopoea solitaria, IgE-mediated allergy, skin prick tests.*

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INTRODUCTION

Moths of the genus *Thaumetopoea* are widespread pests in the coniferous and deciduous forests in Bulgaria. They are mainly represented by the following species: *Thaumetopoea pityocampa* (pine processionary); *Thaumetopoea processionea* (oak processionary) and *Thaumetopoea solitaria* (pistachio processionary) (1).

There is a plenty of literature and clinical evidences that contact with caterpillars, the larval form of different *Thaumetopoea* species, causes a series of complaints in humans dominated by contact dermatitis (erucism), and more rarely - conjunctivitis or keratitis, developed through a toxic-irritant mechanism (2,3). Toxic reactions are caused by the urticogenic hairs covering the caterpillar body. Upon contact with human skin the hairs break down and secrete toxic proteins causing irritation. One of the proteins, called thaumetopoein, has a histamine-releasing effect (4).

In recent years, in addition to the toxo-irritant reactions, IgE-mediated allergic reactions were also defined. These reactions are caused mostly by direct contact with caterpillars or inhalation of their airborne urticogenic hairs. Allergic reactions affect mainly forest workers in areas with excessive development of the caterpillars causing allergic rhinitis or asthmatic attacks and even anaphylactic shock (5,6). Therefore, the larval form of *Thaumetopoea* moths should be considered not only as a source of occupational contact allergy, but also as an aeroallergen causing inhalative and ocular allergic symptoms, which may affect a wide range of people found in such an environment (7).

Due to the climate changes in recent years, different moths of the genus *Thaumetopoea* have expanded their habitat in Bulgaria and represent a serious health threat for the communities of people working and residing within the forests (8).

In this regard, the aim of the present pilot studywas to investigate the skin reactions after prick tests

with allergens from different *Thaumetopoea* species in a group of people having frequent contacts with the processionary caterpillars in order to prove their sensitization.

MATERIAL AND METHODS

Surveyed persons

The skin sensitivity to most spread *Thaumetopoea* species in Bulgaria was studied in a group of people working daily in the forests and having frequent contact with processionary caterpillars. A total of 42 subjects were surveyed, 37 men and 5 women between the ages of 18 and 87.

After giving their informed consent, the project participants completed a specially designed questionnaire. Each participant was then subjected to a detailed examination to determine the presence of allergic complaints while working in the forest.

Skin prick tests with allergens from different *Thaumetopoea* species

Specific sensitization to caterpillars of three *Thaumetopoea* species: *Thaumetopoea pityocampa* (pine processionary); *Thaumetopoea processionea* (oak processionary) and *Thaumetopoea solitaria* (pistachio processionary) was assessed by allergy skin prick tests (SPT).

For this purpose in the Laboratory for Allergenic preparations at Bul Bio NCIPD, Sofia, Bulgaria, special diagnostic allergens from the above mentioned *Thaumetopoea* species were prepared. As a raw material for the production of allergenic extracts, caterpillars in L4/L5 stage were collected and provided by scientists from the Forest Research Institute at Bulgarian Academy of Sciences. The allergens were prepared by an original methodology consistent with the procedures for production and standardization of allergens for SPT diagnostics and complying with the requirements of Good Manufacturing Practice (GMP).

To prove the specificity of the allergens from studied *Thaumetopoea* species a control group of 21 volunteers (11 healthy, non-allergic individuals; 5 patients, sensitized to grass pollen and 5 – sensitized to house dust mites), was tested with the above mentioned experimental extracts by SPT and showed no positive skin reactions.

The diagnostic allergy skin tests were performed simultaneously with the allergens from the three *Thaumetopoea* caterpillars in a volume of 0.05 ml on the volar side of the forearm of each participant. Negative (Coca I solution) and positive (histamine 1 mg/ml) controls were applied in parallel. The reactions obtained were read in 20 minutes according to size of wheal and flare. Wheal and flare with a mean diameter > 3mm were considered positive.

To determine |The degree of skin reaction to the allergen was determined using the following grading scale based on the size of the wheal (Table 1.)

Table 1. Degree of skin reaction to the allergens from caterpillars of three *Thaumetopoea* species

Wheal size (mm)	Interpretation of skin reaction
< 3	Negative
3-5	Slight positive
5-10	Moderate positive
10-15	Strong positive
>15	Very strong positive

Statistics

All analyses were performed using the software package GraphPad Prism 6.0 (GraphPad Software, Inc.). Descriptive analysis of the wheal areas in SPT as well as Comparisons of means and ratios were performed by Mann-Whitney test for unpaired data. Probability values of $p < 0.05$ were considered statistically significant.

Results

During the examination for allergy complaints 27 (64%) participants declared no health problems. On the other hand, 15 (36%) persons reported allergic symptoms during their daily professional activities in the forest (**Fig.1.**).

The complaints were mainly from the skin: itching in 7 and rash - in 8 participants. In 4 forestry workers skin complaints were combined with symptoms from the upper respiratory tract (runny nose and sneezing).

Determination of the specific sensitization to different species of *Thaumetopoea* caterpillars was performed by SPT using specially designed diagnostic allergens.

Positive allergy skin tests to one or more caterpillar's allergens were measured in 18 (43%) participants. According to the obtained data, 15 (36%) of the forest workers were with positive skin reactions

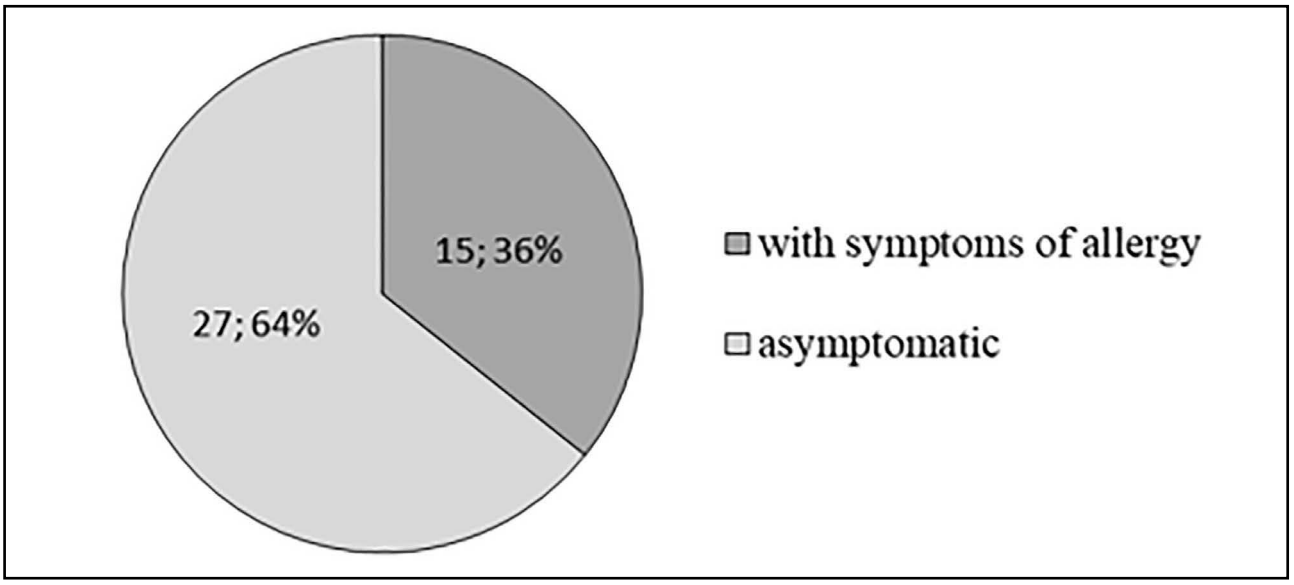

Fig. 1. Distribution of participants after the examination for allergy

to the allergen from *Thaumetopoea pityocampa* (*T. Pit.)*; 12 (29%) - to allergen from *Thaumetopoea processionea (T. proc.)* and only 7 (17%) to the allergen from *Thaumetopoea solitaria (T. solit.).* There were no significant differences between the three extracts regarding the wheal areas in SPT (*p >0,05 for all comparisons*).

The distribution of the degree of positive skin reactions after testing with the studied allergens from different Thaumetopoea species is shown in **Fig. 2.**

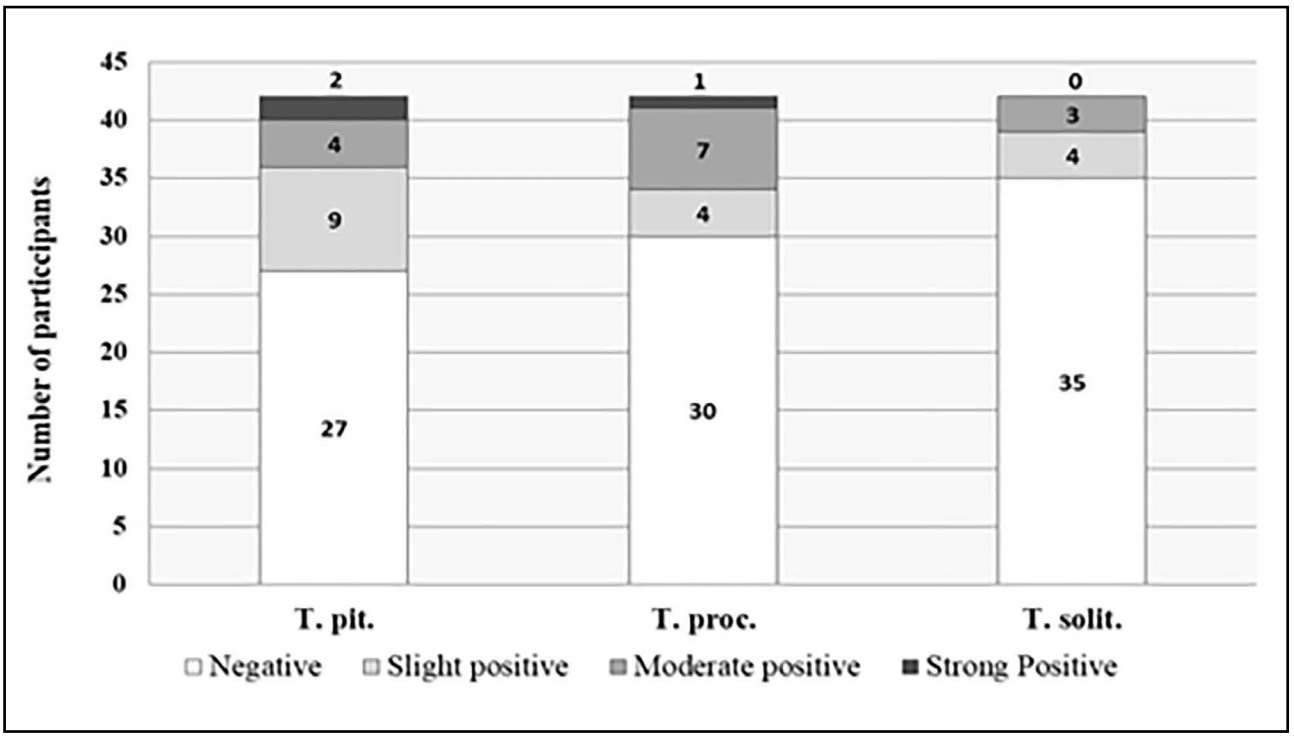

Fig. 2. Degree of positive skin reactions from the studied allergens from different *Thaumetopoea* species

After a SPT with *T. pit*. allergen 9 participants (60%) showed slight positive; 4 (27%) - moderate positive and 2 (13%) - strong positive skin reactions. The skin reactions to the allergen from *T. proc*. were as follows: - 4 (34%) slight positive; 7 (58%) moderate positive and 1 (8%) strong positive . In response to allergens from *T. solit.* No strong positive skin reactions were observed, Slight positive reactions were observed in 4 (57%) and moderate positive reactions - in 3 (43%) of the tested.

A simultaneous test with the three allergens from the different *Thaumetopoea* species showed that in 6 (33%) of the cases, skin hypersensitivity only to allergens from *T. pit.* was present. Monosensitization

to *T. proc.* was observed in 2 (11%) participants. The rest 10 (56%) participants with positive skin test showed different profiles of polysensitization to *Thaumetopoea* allergens under study (Table 2).

Table 2. Profiles of sensitization to the different species of *Thaumetopoea* caterpillars among the participants with positive allergy skin pricks tests.

Positive SPT	Number	%
T. pit.+	**6**	**33**
T. proc.+	**2**	**11**
T. pit.+ T. proc.+	**3**	**17**
T. proc.+T. solit.+	**1**	**6**
T. pit.+ T. proc.+ T. solit.+	**6**	**33**

When comparing the data from the history and the SPT, it was found that in 8 (53%) of the surveyed 15 forest workers with complaints after contact with caterpillars, the symptoms developed without specific sensitization. In the remaining 7 (47%) the clinical history was supported by data for specific sensitization, while in 11 (41%) the detected sensitization to the caterpillars was asymptomatic (**Fig. 3**).

DISCUSSION

Our findings from a first of its kind pilot study unequivocally prove that representatives of the genus *Thaumetopoea* in Bulgaria cause health problems in people who have daily contact with their larvae, most often workers and scientists performing activities in the forests.

According to the results from a detailed allergic examination 15 (36%) persons reported allergic symptoms after a contact with *Thaumetopoe*a caterpillars. Skin reactions were the most common clinical manifestation. 47% of the participants with symptoms reported severe itching, sometimes with no visible skin lesions. In 53% there was a rash, mainly of two different types: rapid hives (contact urticaria) or late-onset papular itchy rash, which persists for several days. In most of the studied participants, contact urticaria was IgE-mediated.

The pathogenic effects of the *Thaumetopoe*a caterpillars are not limited to the skin. In 27% of participants with skin symptoms, upper respiratory tract involvement in the form of seasonal runny nose, coughing, swallowing disorders and difficulty breathing was also observed.

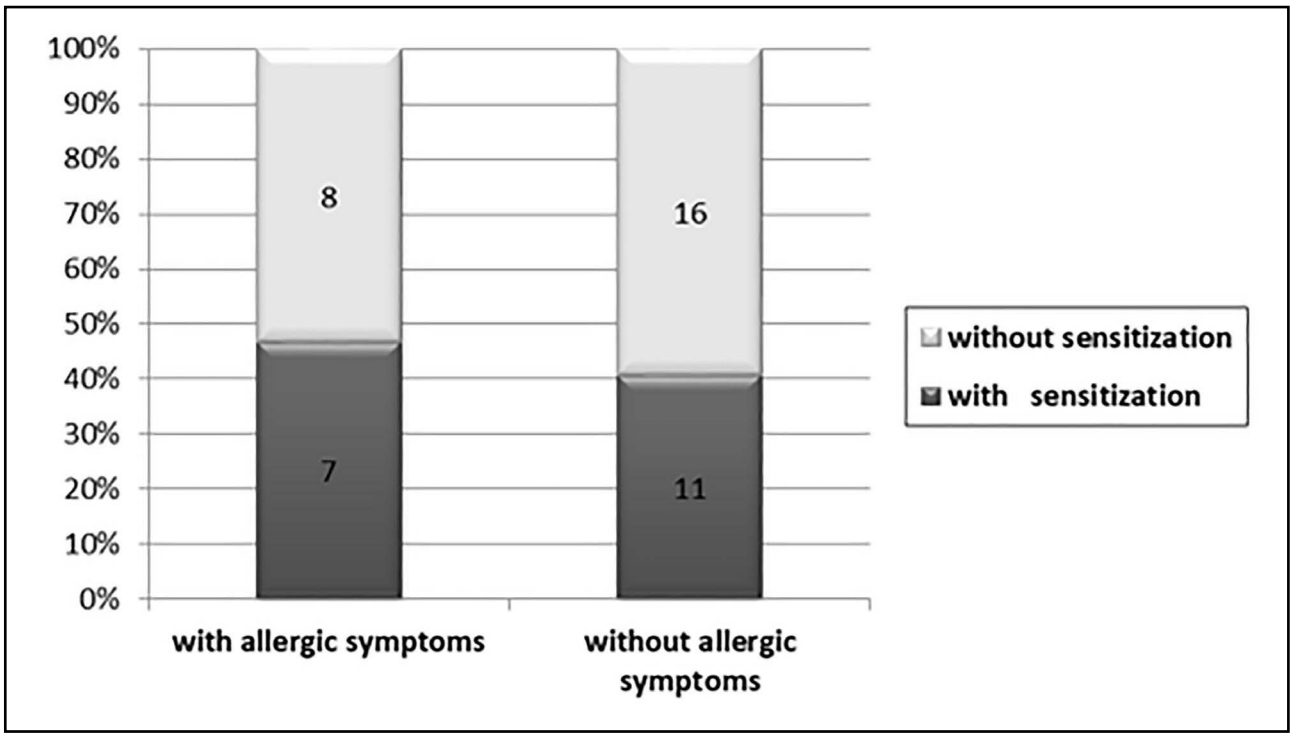

Fig. 3. Comparison between data from clinical history and presence of specific sensitization to processionary caterpillars

There are several possible explanations in the literature for the pathogenetic mechanism of the inflammatory response that develops after contact with the urticogenic hairs of different *Thaumetopoe*a species. Several studies have described a specific protein (thaumetopoein)

contained in hairs, which has a direct effect on the mast cells and leads to their degranulation and release of histamine (4, 9).

The fact that some individuals present with more intense, immediate reactions, while others show minimal or even no clinical manifestations after similar exposure has prompted that an IgE-mediated allergic mechanism might be involved. According to up-to-date studies of whole body allergenic extract from pine processionary it contains a mixture of at least 70 proteins, including 7 allergenic molecules, which penetrate the skin and mucous membranes after exposure to caterpillar hairs (9, 10).

For the purposes of our study, we have prepared whole body diagnostic allergens from different types of *Thaumetopoe*a caterpillars. With their help, specific skin reactions to different caterpllar's allergens were studied for the first time.

The data obtained through the SPT revealed positive skin reactions to different caterpillar's allergens in 18 (43%) of the participants. test., were monosensitized to *Thaumetopoe*a *pityocampa.* Monosensitization to *Thaumetopoea pityocampa* was observed in 6 (33%) of the positive cases and to *Thaumetopoe*a *processionea* - in 2 (11%). The rest 10 (56%) participants showed different profiles of polysensitization to the studied *Thaumetopoea* allergens. A fact that needs to be examined in more detail in our future studies.

Our results clearly show that in the group of subjects having frequent professional contacts with different *Thaumetopoe*a caterpillars, the positive skin reaction to *Thaumetopoe*a *pityocampa* was most common and most pronounced.

Supplementation of SPT data with the initial allergic symptoms survey demonstrated that only 7 from the 18 participants with positive skin tests (39%) reported allergic symptoms on contact with processionary caterpillars. In the remaining 11 (61%) participants, the sensitization to caterpillar allergens was asymptomatic. Indeed, since the allergic immune response is genetically determined, not all sensitized individuals develop allergic symptoms after exposure to caterpillar's allergens.

The results from our pilot study are in line with data from other European studies, which found that in endemic areas, SPT with caterpillar whole body extract yielded positive results in 53% to 58% of individuals with suspected *T. pityocampa* reactions (11). Some epidemiological data in Spain have been published on reactions caused by *T pityocampa* in a cross-sectional, randomized, age-, sex- and habitat-adjusted study of 1224 participants. The prevalence of skin reactions to *T. pityocampa* was 12% in rural areas, 9.6% - in semiurban areas with nearby pine forest, and 4.4% - in urban areas. It was found that the risk of skin reactions to *T. pityocampa* was directly related to the exposure to the caterpillar (12).

Summarizing these data, it can be argued that in significant number of cases the complaints following the contact with different processionary caterpillars have immunological pathogenesis and involve production of allergen-specific IgE. The demonstration of the IgE-mediated mechanism of reactions after contact with *Thaumetopoe*a caterpillars should be performed as early as possible, in order to avoid new contacts with different caterpillar's allergens and progressive increase of sensitization.

CONCLUSION

The present study provides important initial theoretical and practical information on the impact of different *Thaumetopoea* species in Bulgaria on human's health. Forestry workers are most at risk of *Thaumetopoea*-related disease. The evaluation of skin reactivity after SPT with different caterpillar's allergens outlined the important role of processionary allergens, especially this from *T. pityocampa,* in the development of IgE-mediated allergic complaints among forestry professionals. In view of these results, it seems that IgE-mediated hypersensitivity allergic reactions to *Thaumetopoea* caterpillars are at least as important as those with no allergic mechanism.

Acknowledgments: This study is supported by research grant DN 01/17/ 22.12.2016, Bulgarian National Science Fund.

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FIRST SURVEY ON KNOWLEDGE, ATTITUDE AND PRACTICES ABOUT PARASITIC DISEASES AMONG THE POPULATION IN THE CENTER OF MOROCCO: THE CASE OF LEISHMANIASES

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ABSTRACT

Background

The leishmaniases are a group of diseases caused by protozoan parasites from more than 20 Leishmania species. These parasites are transmitted to humans by the bites of the infected female phlebotomine sandfly. It is still a public health problem in several countries, notably in Morocco, where this pathology is widespread and is prevalent in sporadic or endemic forms.

Methods

In order to determine the knowledge of the population in terms of leishmaniases, its vector and means of transmission of the disease, we carried out a survey during the year 2017 with the consulting population at the level of all 45 Health Centers (H/C). The descriptive results are presented as percentages and numbers. The chi-square test was used to test the association between the variables. Values of $p < 0.05$ were considered significant. The calculation of chi^2 (X^2) and the p-value are carried out using the software R.

Results

The grouping of responses shows a lack of knowledge on leishmaniases and phlebotominae by a large majority of the population (97% and 95%, respectively), whereas there is no correlation between the socio-economic level of the population and this lack of knowledge ($X^2 = 2.8$, $p > 0.05$), nor between the socio-economic level and the knowledge of the risk related to insects ($X^2 = 6.4$, $p > 0.05$).

Conclusion

These data show the extent of the perception problems with parasitic diseases, particularly leishmaniases and also the lack of awareness of the population against this scourge. Indeed, these data can be used to define measures to be taken to limit or even eradicate exposure, and subsequently risk.

Keywords: *leishmaniases , sandflies, knowledge, attitude, practices, population*

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INTRODUCTION

Health and sustainable development are intimately linked. Communities under the pressure of a barrage of endemic diseases face enormous obstacles to improve the quality of life. Leishmaniases are examples of parasitic zoonoses that place these affected communities at a significant risk of morbidity, debility and mortality.

They are parasitic diseases caused by parasites from the genus Leishmania (1,2), and are transmitted from vertebrate to vertebrate following the bite of female sandflies (Diptera: Psychodidae) (3,4). These are emerging diseases and closely related to the state of the environment (5). The leishmanian pathogenic complex (parasite, vector, reservoir), evolves in a geographical area defined by a set of bioclimatic parameters (6). There are three main forms of the disease: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), also known as kala-azar, and mucocutaneous leishmaniasis (MCL) (7, 8). They are endemic in 88 countries, including 72 developing countries. A total of 370 million people are at risk of the disease (9, 10). For the success of any prevention and/ or control strategy for any disease, the participation of the population at risk or affected is an important step in their active involvement in carrying out the activities of the program. Indeed, the understanding of the perception, attitudes and practices of the population towards a disease is a determining factor for the success of control programs.

In Morocco, despite the kingdom's efforts, leishmaniasis in its different forms is endemic in many regions and continues to pose a major public

health problem (11, 12). The number of cases reported in 2015 by the epidemiology department of the Moroccan Ministry of Public Health was 8718 cases of leishmaniases. Several researches (13, 14, 15) have been carried out in order to combat this epidemic. However, no national study has been conducted on the population's perception of leishmaniasis and its vector. In this context, this study aimed to assess the knowledge, attitudes and practices of the population at the level of the prefecture of Meknes in relation to leishmaniases and their vector.

MATERIALS AND METHODS

Study area

The prefecture of Meknes (Figure 1) is located in north-west Morocco in the region of Meknes-Fes, on the plateau of Saïs, 140 km from the administrative border Rabat and 60 km from the spiritual capital Fez.

It is a predominantly urban subdivision: 82.3% of the population lives in urban areas compared to 17.7% in rural areas. The population is estimated according to the general population and housing census of 2014, at 835,695 inhabitants (16).

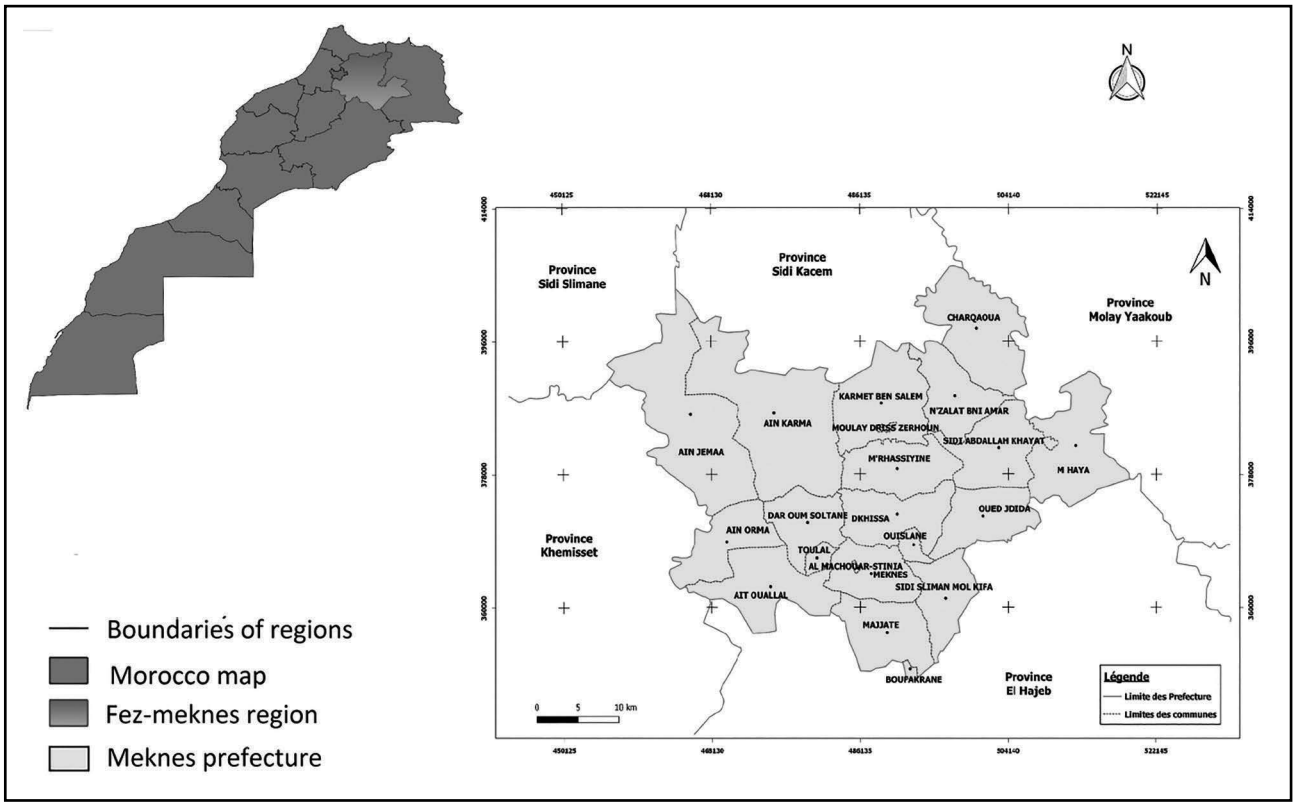

Figure 1. Geographic location of the study area (Meknes Prefecture)

Survey conduction

The purpose of the survey was to draw up a socioeconomic profile of the city of Meknes and the degree of perception of the health risk related to leishmaniases and their vector by the population of the prefecture of Meknes. A descriptive cross-sectional survey was conducted during the months of June and July 2017. The interview was carried out using a questionnaire. People were chosen at random to be interviewed individually.

A total of 383 interviews were conducted throughout the territory studied.

The conduction of this survey required some information concerning the studied phenomenon. Three factors essentially determine the sample size for a population survey:

- The estimated prevalence of the studied variable - leishmaniases in this case (p),
- the desired level of confidence (t),
- the acceptable margin of error (m).

For a survey design based on a random sample, we can calculate the required sample size by applying the following formula (17):

$$n = \frac{\mathbf{t}^2 \text{ x } \mathrm{p}(1-\mathrm{p})}{\mathbf{m}^2}$$

Explication:

- n = required sample size
- t = 95% confidence level (typical value of 1.96)
- p = estimated prevalence of leishmaniases (in case of lack of information, it is recommended to take p = 0.5)

- m = 5% margin of error (typical value of 0.05).

$$n = \frac{1{,}96^2 \times 0{,}5(1-0{,}5)}{0{,}05^2}$$

Statistical processing of results

The descriptive results are presented in the form of percentages and numbers. The Chi-square test was used to test the association between the variables. Values of $p < 0.05$ were considered significant. The calculation of chi^2 (X^2) and of the p-value is carried out using the R software.

RESULTS

The results of this study made it possible to identify the obst acles for the implementation of effective methods for combating and preventing this disease.

1. Socio-demographic characteristics of the studied population (Table 1)

A total of 383 individuals were interviewed, despite certain refusals. The majority of the consultants were women, 253 or 66%, while men represented 34%. represent More than half of the respondents, or a total of 248 Consultants had no profession (housewives, students and the unemployed).

The distribution of consultants according to monthly income showed that almost half of them or 49% had an income between 100 and 300 EUR, followed by 26% with an income between 300 and 800 EUR and 21% with an income below 100 EUR. Only 4% received more than 800 EUR per month.

Analysis of the education level of respondents showed that 32% had a secondary level against 30% illiterate, followed by 25% with a primary level only 7% -with a university level, and finally 6% - with a preschool level.

Table 1: Socio-demographic characteristics of respondents

Socio-demographic characteristics	**Number**	**Percentage**
Sex of the respondents		
Female	253	66%
Male	130	34%
Profession of the respondents		
Without	267	70%
Daily employment	60	16%
Official employment	24	6%
Retirement	19	5%
Other	13	3%
Monthly income of the family		
<1000 DH*	79	21%
1000-3000	190	49%
3000-8000	100	26%
> 8000	14	4%
Educational level of the respondents		
Without	115	30%
Preschool	22	6%
Primary	95	25%
Secondary	122	32%
University	29	7%

** DH, Morrocan dirham = 0.092 Euro*

2. Knowledge of leishmaniases and their vector

Almost all of the participants ignored leishmaniases (97%), while 3% of them had an idea about the disease. Only 10 from the surveyed who knew about leishmaniases confirmed that the disease was transmitted by an insect **(Table 2)**.

Statistical analysis showed that there was no statistically significant relationship between the knowledge of leishmaniases and the socio-economic level of the studied population ($X^2 = 2.8$, $p > 0.05$). Similarly, 19 respondents or 5%, have confirmed in the questionnaire that they knew the sandflies, against 95% who did not. Statistical analysis showed no statistically significant relationship between the knowledge of sandflies and the socio-economic level of the population ($X^2 = 6.4$, $p > 0.05$).

3. Participants' practices and attitudes of related to prevention of leishmaniases

Concerning the probability of an insect to transmit the disease, 358 respondents (i.e. 93%), confirmed the transmission by insects against only 7% who believed the opposite.

The answers concerning the use of some means of insect control showed that the majority of respondents (79%) used such a means to fight against all types of insects while 21% of respondents use no means. Regarding the type of control used, 68% of respondents confirmed using insecticides against 17% who used curtains, are about 11% Basil pest control users , and only 4% who used mosquito nets.

Table 2. Knowledge, attitude and practices related to leishmaniases and their vector

	Number	Percentage
Knowledge of leishmaniases		
Yes	10	3%
No	373	97%
Knowledge of sandflies		
Yes	115	5%
No	22	95%
Probability of disease transmission by insects		
Yes	358	93%
N	25	7%
Use ofsome means against insects		
Yes	303	79%
No	80	21%
Type of Means used		
Secticide	205	68%
Curtains	51	17%
Biological : basil	34	11%
Mosquito nets	13	4%

DISCUSSION

Understanding more about a disease, leishmaniases in particular, means going beyond its clinical and epidemiological characteristics and taking into account the viewpoints of those directly involved in the prevention and control of this epidemic, thus making these actions more effective.

The present study highlighted a number of problems concerning the health risk associated with leishmaniasis. For a young and mostly female population, the participation rate was very satisfactory. This could be explained by the fact that the target population included individuals with health problems, and the collaboration of health professionals certainly contributed to the success of the interviews.

The results of our study showed that most of the respondents had no idea about leishmaniasis and its vector (97%, and 95% respectively). On the other hand, they were aware of the health risks posed by insects transmitting diseases. These results were very poor as compared to similar studies carried out in Ethiopia, where (87.4%) had heard of leishmaniases and 89.4% of them were well informed (18).

Ten participants who said they knew about leishmaniases also said that the causative agent of the disease was transmitted by the bite of a sandfly. This result is similar to the one found in Sudan (19) where only 6% indicated that the disease was transmitted by the bite of sandflies. However, it is very low compared to the studies conducted in Saudi Arabia where 37.4% of participants could identify sandflies as the vector of leishmaniases (20). Statistical processing of the results showed that there was no correlation between the socio-economic level of respondents and the knowledge of leishmaniasis and its vector ($p > 0.05$) neither between the socio-economic level and the perception of the health risk related insects. Yet, poor housing, and malnutrition are among the risk factors implicated in the prevalence of leishmaniases (21, 22).

The ignorance of leishmaniases and sandfly population as their vector insect could be explained, on the one hand, by the lack of information from the part of the health personnel, and on the other hand, by the small size and the silent flight of vectors, making them difficult to identify. Thus even in endemic areas, the majority of the population may be largely unaware of the presence of sandflies (Diptera: Psychodidade) and their role in the epidemiology of the disease (23).

The adaptation of pathogenic vectors to urban areas and domestic environments (24, 25, 26),

requires that decision makers, organizations and health professionals implement significant actions in collaboration with the population. It is important that both citizens and health professionals take charge of the cycle of this disease so that, together, they could fight against this pathology.

Indeed, awareness programs must be put in place in order to fight against leishmaniases despite the fact that this epidemic is difficult to combat (27). This requires training community health workers in using simplified definitions of the different types of leishmaniasis. Moreover, several studies have recommended strengthening community awareness of leishmaniases and its control through health education (28) while taking into account the perception of the population, its beliefs and altitudes (29) . Several authors (30, 31) have defended the idea that the local population should be involved in the collective resolution of the problems and not only included as the scope of concern, or the source of data or the target of efforts.

These actions are also recommended by WHO experts in the fight against leishmaniases (32), in a technical report emphasizing the role of social mobilization in changing the behavior of the population.

It is also important to note that the use of mosquito nets and curtains as a means to control leishmaniases was low (4%, and 16% respectively). Thees results were much lower than those proved by a study carried out in Nepalwhere 58% used mosquito nets (33). Studies in Iran and Venezuela have shown that the regular use of insecticide-treated mosquito nets and curtains provides some personal protection against the bites of sandflies and the transmission of leishmaniases (34, 35). However, the use of insecticides is more frequent in the majority of the surveys, which explains the low number of cases recorded at the prefecture level (36), and at the same time proving the effectiveness of insecticides in the fight against the disease. These results are consistent with Rioux's research; who in a mission report on the outbreaks of cutaneous leishmaniases observed in the south of Morocco recommends the application of insecticides to fight against this epidemic and also confirms the results of Bettayeba et al., in 2016 (37), who concluded that leishmaniases must be prevented by vector control.

CONCLUSION

In the light of our results, we note that a misunderstanding of leishmaniases and their vector existed among the majority of consultants regardless of their socio-economic level while most of them (93% of consultants) were aware of the risk related to insects, and 79% of the latter used different means to combat these insects.

Indeed, efforts to fight against leishmaniasis are still insufficient, hence the need to set up a preventive strategy to fight against this pathology. Information campaigns improving the awareness of the population could be used to limit or even eradicate the exposure, and subsequently the risk of infection.

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ANALYSIS OF CIRCULATING STRAINS, CAUSING INVASIVE LISTERIOSIS IN BULGARIA FOR TEN YEARS, 2010-2019

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ABSTRACT

Listeriosis is a zoonosis with multiple mechanisms of infection and multiple organ symptoms, severe course and high lethality. An increasing incidence of listeriosis has been reported in several European countries in recent years. A limited range of Listeria strains is responsible for most outbreaks occurring in different countries. The aim of the study is to monitor the spread of the Listeria strains causing invasive listeriosis in Bulgaria for the period 2010-2019 and to analyze the etiological structure of the infection in different hospitals in the country. A total of 56 Listeria strains from 17 hospitals were investigated for confirmation of species and serogroup. The materials were isolated from haemocultures, amniotic fluids, cerebrospinal fluids, anal and throat secretions and two tests were used. Confirmed strains isolated from the clinical samples for the study period belonged to 4 serogroups of Listeria. *Listeria monocytogenes serogroup I* was detected in 28 (50%) of the samples, *Listeria monocytogenes serogroup II* – in 43%, *Listeria innocua* –in 5% and *Listeria welshimeri* – in 2% of the samples. The strains isolated from cerebrospinal fluid predominated (52%) and those isolated from haemocultures were 36%. Nineteen of the 29 isolated strains from cerebrospinal fluid belonged to serotype *4b*. *Listeria monocytogenes serogroup I* was detected in 70% of haemocultures. *Listeria innocua* and *Listeria welshimeri* were detected in cerebrospinal fluid. Serotype *1/2a* was found in six hospitals and serotype *4b* - in five hospitals. The majority of isolated strains were from newborns:12/56 (21.43%). Serotype *1/2a* was detected in 12 hospitals and serotype *4b* in 11 hospitals. The largest variety of strains was found in Plovdiv, UMBAL "Sv. Georgi". The data confirmed a steady trend in the spread of certain listeria serotypes in each hospital over the years. Screening at-risk groups, mainly women of childbearing age, is recommended in order to limit the risk of listeriosis in the future.

Keywords: *Listeria, serogroupes, hospital strains*

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INTRODUCTION

Listeriosis is a zoonosis with multiple mechanisms of infection and multiple organ symptoms, severe course and high lethality (17). The infection is defined as zoonosis of increasing medical, social and economic importance due to its severe course, high mortality and specific diagnosis. *Listeria monocytogenes* is the causative agent of human listeriosis, a potentially fatal food-borne infection. Clinical manifestations range from febrile gastroenteritis to more severe invasive forms, including sepsis, meningitis, thrombencephalitis, perinatal infections and abortions (21). An increasing incidence of listeriosis has been reported in several European countries in recent years. These increases reflect a predominantly higher rate of bactereamic listeriosis in patients ≥65 years of age and are unrelated to geographical location, gender, ethnicity, socioeconomic factors, or infectious serotypes (1).

In Europe, invasive listeriosis is an infection of great concern to public health due to its clinical severity (hospitalization rate > 90%) and high fatality rate (20% to 30%). The infection is characterized with low incidence (0,4 $\%_{ooo}$) as compared to salmonellosis and campylobacterios, food-borne infections with the highest incidence in all European countries (23.7$\%_{ooo}$ and 45.6$\%_{ooo}$ respectively) (6). Statistically significant increasing trends in listeriosis notification rates were noted in Austria, Denmark, Hungary, Italy, Spain and Sweden from 2005 to 2009 (6). One of the deadliest outbreaks from food contaminated with *L. monocytogenes* (melons) was reported in

the United States in 2011. Using *in vitro* and *in vivo* assays, the two isolated strains LS741 and LS743 were shown to differ significantly from the ordinary laboratory strain 10403S. These strains exhibited increased virulence characterized by higher brain colonization (12).

Over the last years, many reports from European countries have shown an increasing rate of listeriosis in older age while the pregnancy-related cases remained stable (13,14). *L. monocytogenes* is a genetically heterogeneous species, with a small number of strains (serotypes 1/2a, 1/2b, 4b) implicated in human listeriosis. However, a limited range of strains is responsible for most blasts occurring in different countries. For example, from May 2015 to March 2016, an outbreak due to *Listeria monocytogenes* serotype 1/2a and clinical pulsotype never previously isolated in Europe occurred in central Italy (4). In the Czech Republic and Poland, an elevated number of listeriosis patients after salmon consumption were recorded, with prevalence of serotype 1/2a (11). Multinational blast from *Listeria monocytogenes* sequence type 6 infections related to ready-to-eat meat products was announced in Italy in 2019 (5).

According to the latest annual ECDC epidemiological report, five individual member States of the European Union reported markedly increasing trends of listeriosis over the period 2013-2017 (Germany, Italy, Netherlands, Poland and Spain) (7). In Bulgaria, confirmed cases of listeriosis for this period range from 0.10 to 0.49 per 100 000.

The aim of the study was to monitor the spread of Listeria strains causing invasive listeriosis in Bulgaria for the period 2010-2019 and to analyze the etiological structure of the infection.

MATERIAL AND METHODS

A total of 56 *Listeria* strains were investigated in the NRL "Vector-borne infections, listeria and leptospires" for confirmation of the species and the serotyping. They were sent from 9 hospitals in the country and 8 hospitals from Sofia town. The resulting strains were isolated from haemocultures, amniotic fluid, cerebrospinal fluid , and anal and throat secretions. Two tests were used for strain confirmation: 1) Himedia Latex test kit (Hilisteria), Germany 2) Api Listeria (Biomerieux), Australia.

RESULTS

Listeriosis is defined as a zoonosis with increasing medical, social and economic importance, due to its severe clinical course, high mortality and specific diagnostics - serological and cultural. The incidence of listeria infection in our country is very low as compared to other infectious diseases. In recent years, it is approaching the average values for Europe(0.47 per 100 000). In Canada approximately 0,4 listeriosis cases were reported per 100 000 , in Greece - 0.3 and in Sweden - 7.5 per 100 000 (8,15). In Bulgaria, the incidence of listeriosis for the period 2010-2019 was wavy (Fig. 1). Four morbidity peaks were observed: in 2012, 2014, 2017 and 2019. The highest incidence was registered in 2019. This was the highest incidence in the country for a period of 20 years (for example, the incidence in 2001 was 0) (25).

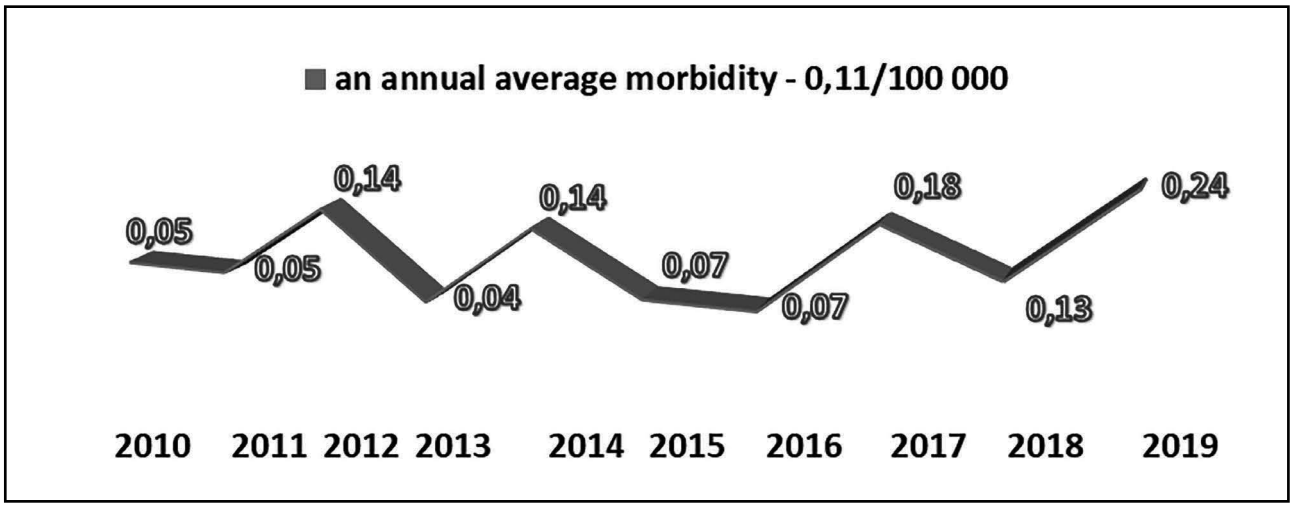

Figure 1. Average morbidity per 100 000 for the period 2010-2019.

Morbidity shows a steady trend towards an increase in the number of patients with listeriosis in Bulgaria and correlates with the trends of the epidemic process in the world and Europe in particular.

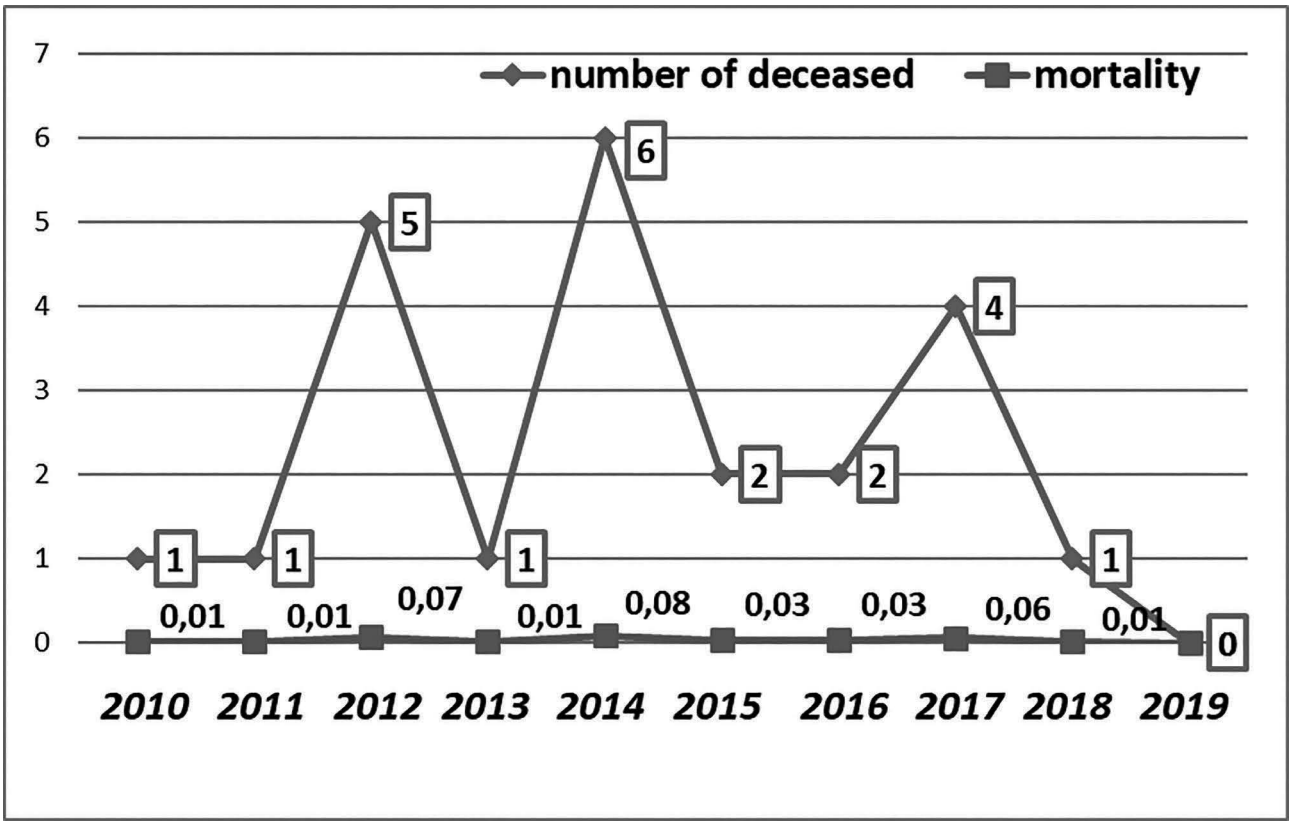

Figure 2. Average mortality per 100 000 for the period 2010-2019.

Fig. 2 presents data of mortality and number of deaths from listeriosis in the study period. Despite of the better detection of listeriosis, the issue of persistently high mortality and letality remains in recent years. The highest mortality rate was registered in 2014, when 6 patients with listeriosis died (0.08 % 000).

Serotyping of the isolated Listeria strains from clinical samples for the study period revealed 4 serotypes (Fig. 3). (28/56) *Listeria monocytogenes I serogroup 1/2a serotype was detected* in the highest percentage of cases, 50%, and - *Listeria monocytogenes II serogroup 4b serotype* - in 43%. *Listeria welshimeri* was detected in only one isolate. *Listeria monocytogenes serotype 1/2a* was isolated in almost all years of the study period. The highest number of isolates of this group were confirmed in 2012 and in 2016, 8 and 6 respectively. The largest variety of Listeria strains was found in 2014 (Fig.4). Interestingly, in 2015 and in 2017 only *4b* serotype was found. *Listeria innocua* was detected in 2014 and comprised 5% of the isolated strains.

The number of isolated Listeria strains is presented in Table 1. Those isolated from cerebrospinal fluid predominated (52%), followed by those isolated from haemoculture (36%) (Fig.5). Only one strain was isolated from anal secretion and one - from abdominal punctat (2%). 19 of the isolated strains from cerebrospinal fluid belonged to *Listeria monocytogenes II serogroup 4b serotype* (19/29) and 7 - to *Listeria monocytogenes I serogroup 1/2a serotype* (Table 1). 70% (14/20) of the isolated strains from haemoculture belonged to *Listeria monocytogenes I serogroup 1/2a serotype*. This serotype was detected from abdominal punctat and anal secretion. Only two strains were isolated from amniotic fluid and they belonged to *Listeria monocytogenes I serogroup 1/2a serotype*.

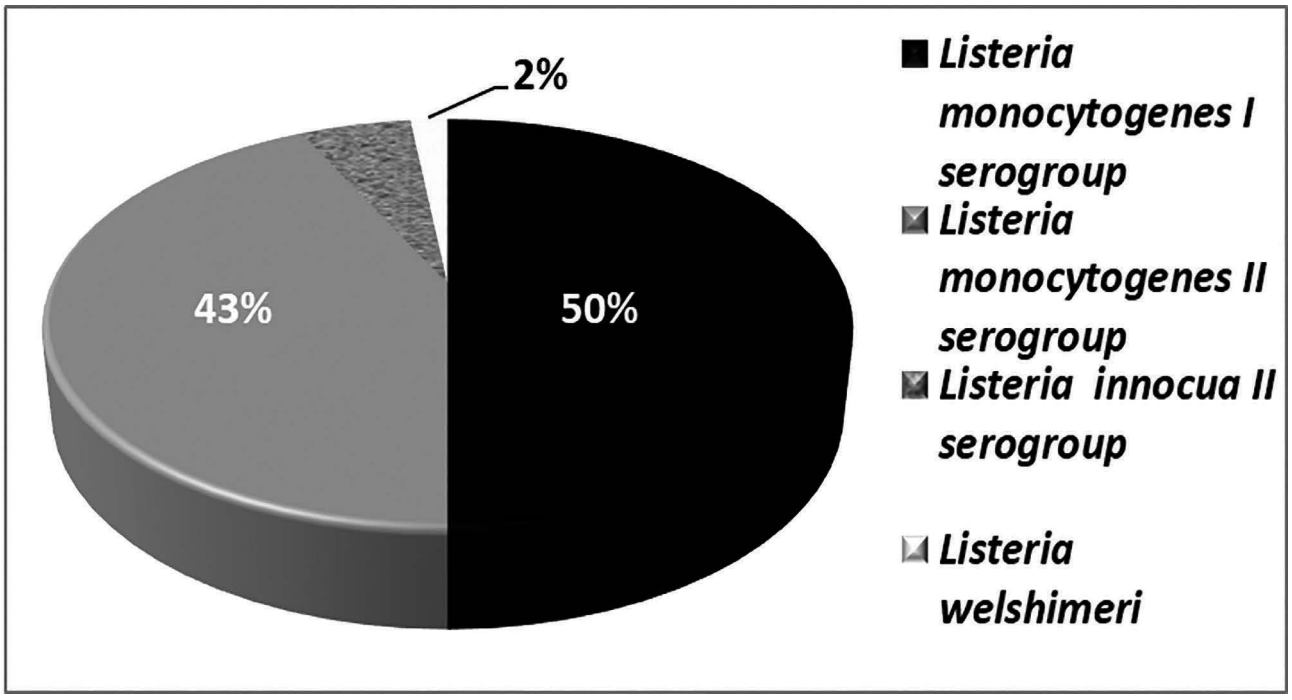

Figure 3. Proportions of Listeria serogroups, 2010-2019.

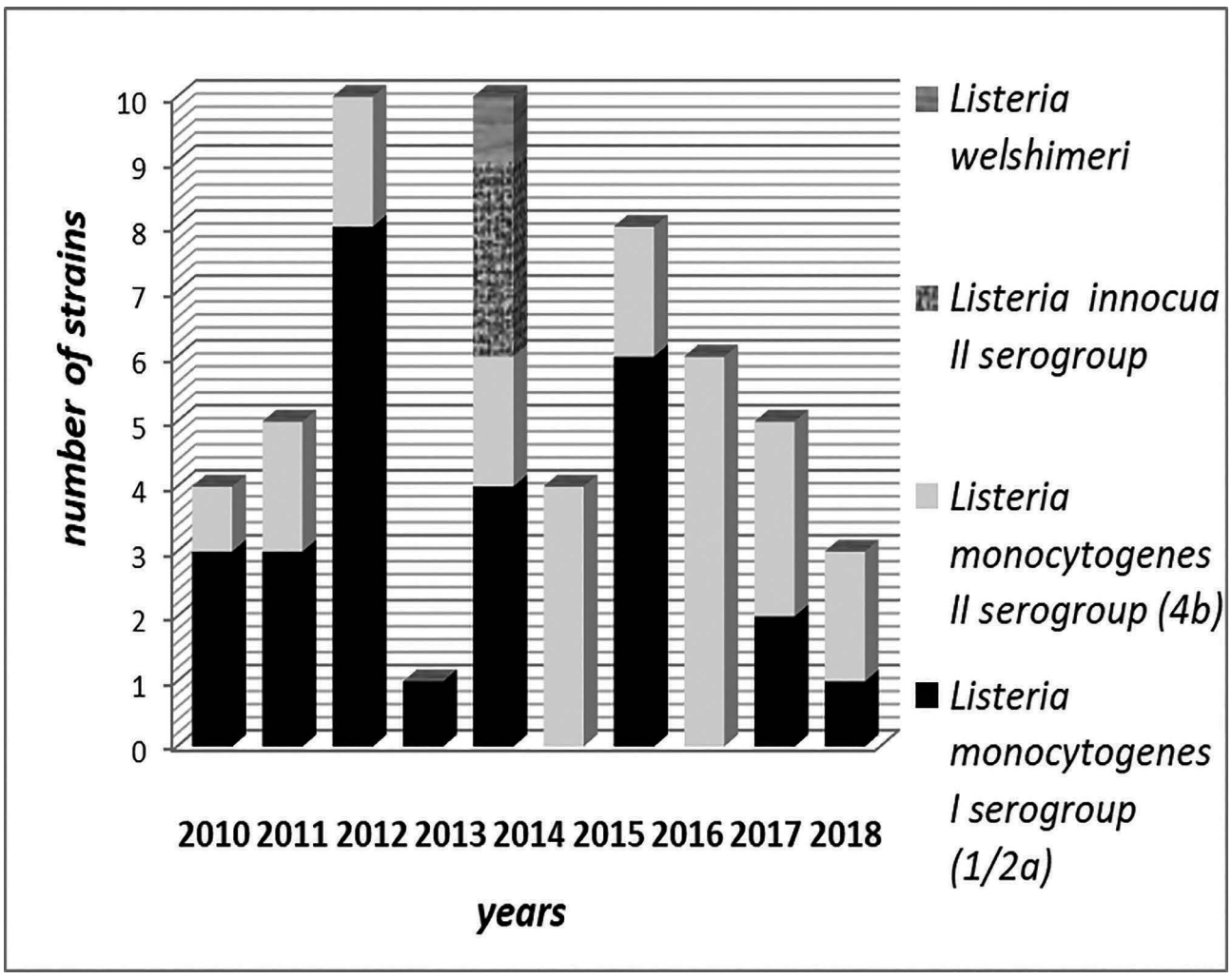

Figure 4. Distribution of isolated strains of Listeria by years.

Table 1. Isolated strains of *Listeria* from the relevant materials for the period 2010-2019

materials / isolated strains	cerebrospinal fluid	haemo culture	amniotic fluid	throat secret	abdominal punctate	anal secretion
Listeria monocytogenes I serogroup	7	14	2	1	1	1
Listeria monocytogenes II serogroup	19	6	1	1	0	0
Listeria innocua II serogroup	2	0	0	0	0	0
Listeria welshimeri	1	0	0	0	0	0
Total	**29**	**20**	**3**	**2**	**1**	**1**

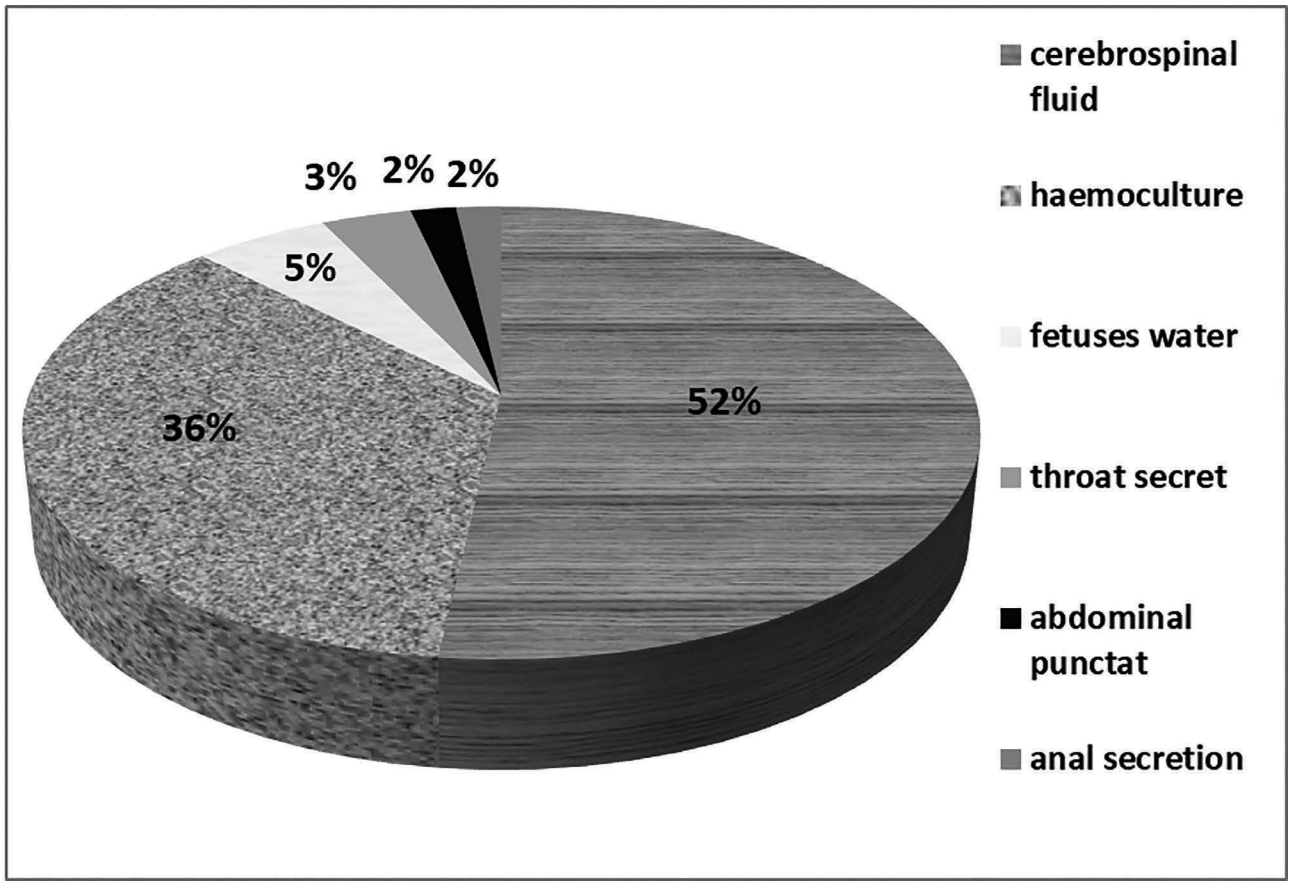

Figure 5. Proportions of clinical samples investigated for Listeria.

The distribution of the studied Listeria strains by hospitals for the studied period is presented in Fig.6. Four serogroups of Listeria were found in "St. George University Hospital", Plovdiv. *Listeria monocytogenes I serogroup* was detected in 6 hospitals and serotype *4b* - in five hospitals. At the same time, both serogroups were found in three hospitals.

The highest percentage of isolated strains was from newborns - 12/56 (21.43%), followed by those isolated from patients aged 60-69 years - 11/56 (19.64%). The fewest strains were isolated from patients in the 10-19 and 40-49 age groups (Fig. 7).

DISCUSSION

It is essential to prepare a spatial epidemiological study of epidemiological features of an infection. In Bulgaria he morbidity of listeriosis during the period 1990-2000was 0.02%$_{000}$ and during the next decade (2001-2010) there were 2 peaks of morbidity (in 2002 - 0.08%000 and in 2007 - 0.14% $_{000}$) (25). During the study period, the incidence of listeriosis was dynamic, with 4 peaks. This is most likely due to more frequent outbreaks as well as the influence of more factors on the epidemic process.

Listeriosis is one of the infectious diseases that often end in death. Despite of the better detection of listeriosis, the persistently high mortality and lethality remain an issue even in recent years. *L. monocytogenes* is the leading cause of high mortality (from 20% to 30%) from foodborne infections in humans (22). In the current study, mortality was higher in three of the years (2012, 2014, 2017). In our country only in 2019 no deaths were reported. Most patients died in 2014 (6 people), when the highest mortality rate was registered - 0.08 % $_{000}$ (25).

Assays (serological and PCR) grouped *Listeria monocytogenes* into 13 serotypes (*1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e* and *7*) (9) with different virulence potential. The studies (18, 19, 23) indicate that most (98%) cases of listeriosis in humans are caused by serotypes *4b, 1/2a, 1/2b* and *1/2c,* and for the most recent sporadic or epidemic cases of listeriosis was responsible serotype *4b*. Different Listeria serotypes are specific to particular geographic areas. Serotype *1/2a* is common in some European countries: Italy, Czech Republic and Poland (4, 11). For our country the predominant serotype is also *1/2a* (50%), followed by serotype *4b*. *Listeria welshimeri* was isolated in only 2% of cerebrospinal fluid strains taken from a 30-year-old man diagnosed with meningitis. This serotype was first isolated in human from a fecal sample in France in 1987. Until then, this serogroup had only been isolated from the environment and from rare animal sources (2). *L. innocua* is almost always non-hemolytic, but a few strains have been found to be hemolytic (24). Biochemically, *L. innocua* is very similar to *L. monocytogenes*. In the present study, it was isolated from cerebrospinal fluid of two patients diagnosed with meningitis. The strains were non-hemolytic.

The predominant Listeria serotype isolated from cerebrospinal fluid was *4b,* while from haemoculture it was *1/2a*. The serotype *1/2a* was also isolated from samples of anal secretions, fetus water, throat secretions and abdominal points. *Listeria monocytogenes II serogroup 4b serotype* was responsible for the latest sporadic or epidemic cases of listeriosis, but it is also more virulent. In some countries, such as Sweden and the United States, there is greater variability in serotypes (20,26), while in Algeria, for example, serotype *4b* is isolated mainly from food products (16).

Analyzing the distribution of Listeria serotypes by hospitals, we found that serotype *1/2a* was detected in 12 of the 17 hospitals included in the study (Fig. 6). Serotype *4b* was detected in 11 of the hospitals, both serotypes simultaneously - in 6 of them. The largest variety of strains was found in Plovdiv, UMBAL "Sv. Georgi" (4 types of serotypes), where the largest number of strains -13/56 (23.21%) were studied. Listeria strains isolated from cerebrospinal fluid 12/13 predominated and only one strain was isolated from haemoculture. Serotype *4b* prevailed for this hospital -5/13. In this hospital, 3 strains belonging to *Listeria innocua* and one to *Listeria welshimeri* were also detected.

The strains belonging to serotype *1/2a* (5/11) predominate in hospital "Maichin dom", Sofia. Of the 11 samples tested, 8 were from haemoculture. Only serotype *4b* was confirmed in five of the studied hospitals: MBAL "Burgas" and MBAL "Kustendil" (samples from cerebrospinal fluid) and UMBAL "St. Anna", MBAL "Shumen" and MBAL "Pazardzhik" (samples from haemoculture).

Only serotype *1/2a* was found in six of the studied hospitals: Varna SBAGAL and Sofia MVR (samples of

cerebrospinal fluid), UMBAL "Aleksandrovska", Vtora MBAL, Sofia and MBAL "Stara Zagora" (samples from haemoculture) and SBALXZ Sofia (sample from abdominal puncture) (Fig. 6). These data confirm a steady trend in the spread of certain listeria serotypes in each hospital over the years. Serotypes' circulation should be considered when analyzing epidemiological data on listeriosis in these regions.

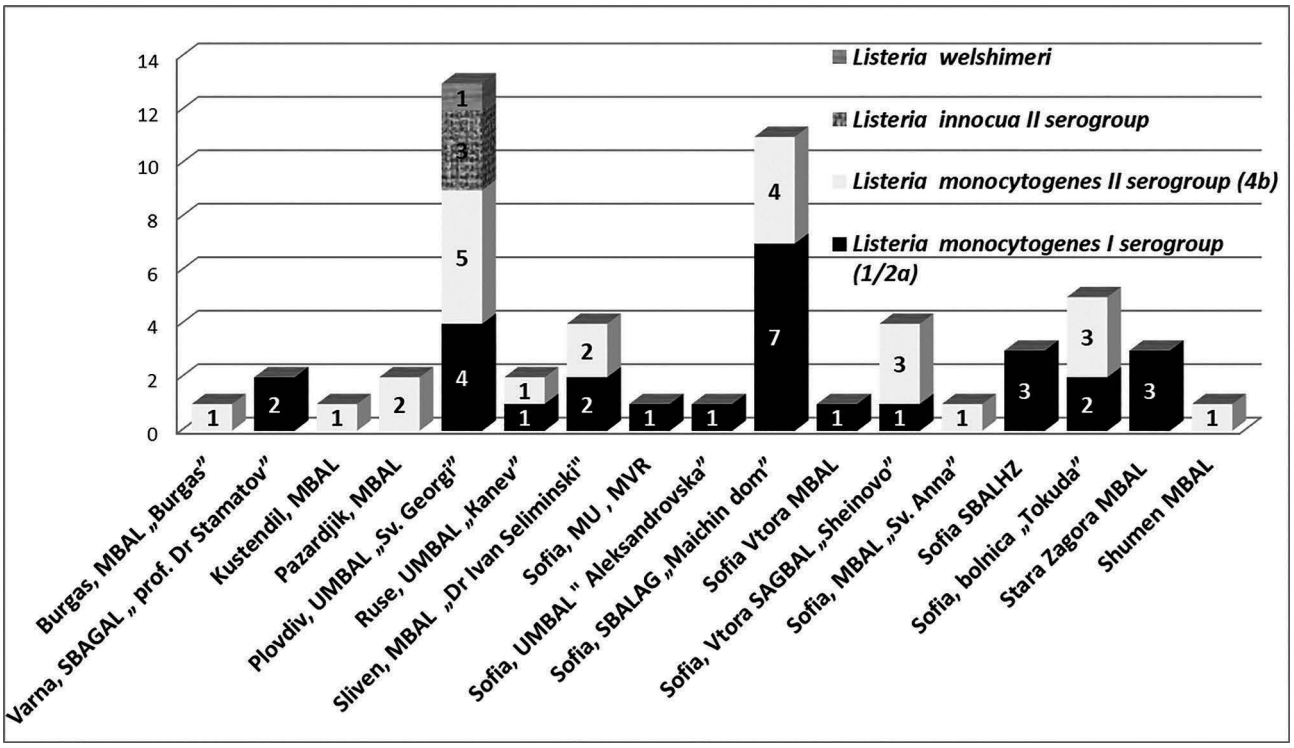

Figure 6. Distribution of Listeria strains by hospitals, 2010-2019.

The high percentage (21.43%) of isolated *Listeria* strains from newborns confirms the fact that they are a major risk group for Listeria infection (Fig.7).

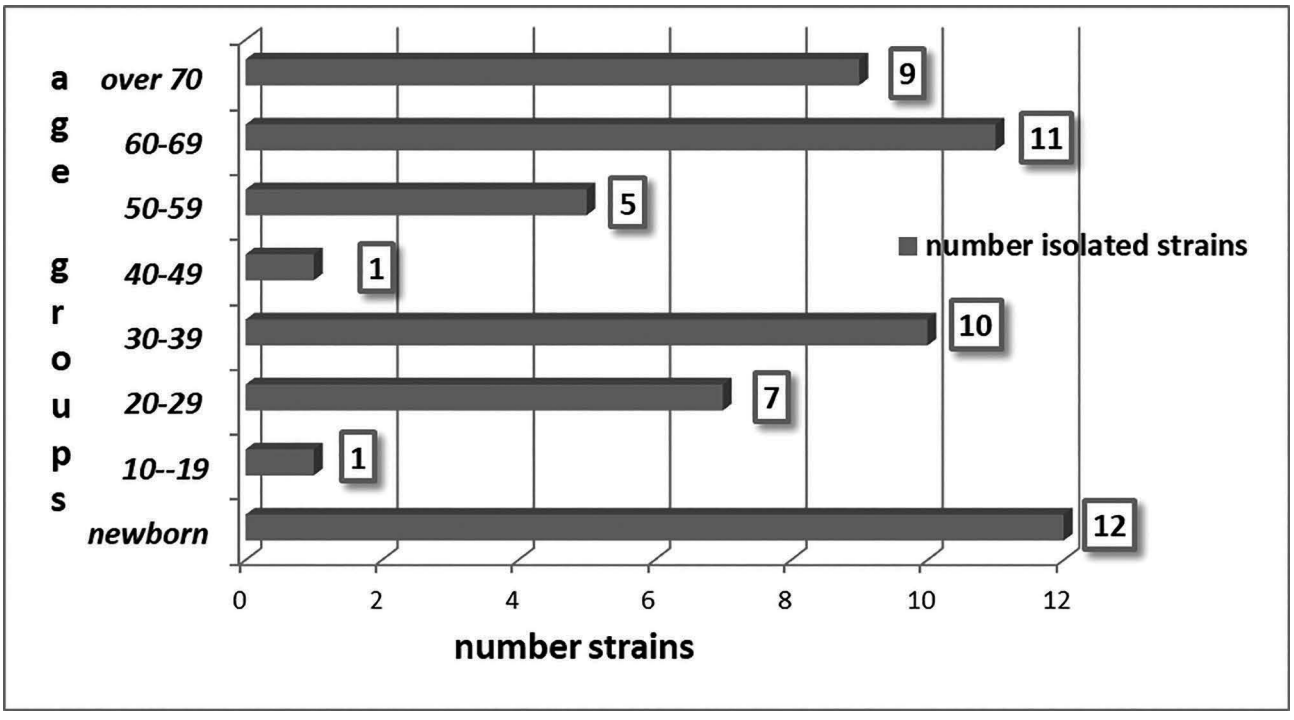

Figure 7. Distribution of Listeria strains by age groups, 2010-2019.

It is known that the risk of listeriosis is highest in vulnerable groups: pregnant women and their embryos, newborns, and immunosuppressed people with chronic liver disease, diabetes, cancer, AIDS or transplants, as well as people in old age (over 65 years). There is evidence lot of literature data for development of severe complications (abortions, premature birth, meningitis, meningoencephalitis, sepsis). They are observed both in animals and humans.Mainly risk groups such as pregnant women, newborns and immunocompromised individuals are affected (3,10).

CONCLUSIONS

Our study emphasizes the need to unite the efforts of microbiologists, immunologists, infectious disease specialists in search of appropriate tests for diagnosis of listeriosis. The emphasis should be on the development and implementation of risk groups screening, especially of women aged 20-40 years with evidence of miscarriage and stillbirth. It is necessary to collect, summarize and analyze information about feeding women of childbearing potential to determine the risk of infection with listeriosis through food.

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