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PROBLEMS

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**PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 46, NUMBER 2/2018**

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GENETIC SURVEY OF INVASIVE *S. PNEUMONIAE* SEROTYPES IN BULGARIA FOR A 5-YEAR PERIOD

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ABSTRACT

Streptococcus pneumoniae colonises the mucosal lining of the upper respiratory tract and is an important cause of invasive infections affecting young children, adults over 65 years of age, the immunocompromised and individuals with chronic diseases. Recent studies have shown variations in virulence based on the high rate of pneumococcal recombination. PCR-based molecular methods are highly sensitive, specific and are becoming the preferred tool for quick and accurate diagnosis of bacterial meningitis which is required to be defined within 2-3 hours.

During the 5-year survey period (2013-2017), 202 materials received as cerebrospinal fluid samples and pneumococcal strains isolated from patients diagnosed with meningitis, were examined by Real-time PCR in the reference laboratory at NCIPD. Serotyping of *S. pneumoniae*-positive materials was performed with conventional multiplex PCR and Real-time PCR with primers for 41 serotypes/serogroups.

There is a high incidence of *S. pneumoniae* serotypes not covered by the pneumococcal conjugate vaccine (PCV10) currently used

in Bulgaria. It was found that all cases of meningitis caused by *S. pneumoniae* vaccine serotypes occurred in patients that were not vaccinated.

KEYWORDS:

serotyping, invasive *S. pneumoniae*, PCR

INTRODUCTION

Streptococcus pneumoniae is a Gram-positive, extracellular, opportunistic pathogen colonising the mucosal lining of the upper respiratory tract in humans (1, 2). Pneumococci are the leading cause of a wide range of invasive and non-invasive diseases (3). Non-invasive diseases are considered less severe and they are more widespread (4). One of the most common non-invasive diseases is otitis media in children. *S. pneumoniae* is the leading bacterial agent of community-acquired pneumonia, both in children and adults. It is a major cause of mortality, hospitalisation (5, 6) and invasive infections affecting young children, adults over 65 years of age, the immunocompromised and individuals with chronic diseases. The spread of *S. pneumoniae* outside its niche, the nasal epithelium, can cause invasive diseases such as pneumonia, bacteraemia, sepsis and meningitis (7).

The diagnosis of invasive pneumococcal diseases is a highly responsible task. In the routine work it is performed by isolation of the microorganism from blood cultures or other normally sterile body fluids – cerebrospinal fluid (CSF), pleural or synovial fluid.

PCR-based molecular methods are gaining popularity in the diagnosis of invasive pneumococcal infections. They are highly sensitive and very specific. Molecular techniques are extremely valuable and becoming the preferred tool for quick and accurate diagnosis of bacterial meningitis which is required to be defined within 2-3 hours as an essential part of the proper and adequate treatment of the patient.

Determination of the capsule serotype is most often used to monitor the administered pneumococcal vaccines.

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The pneumococcal capsule has a polysaccharide composition and is defined as the major virulence factor involved in pathogenesis by various mechanisms. According to numerous studies characterising the capsule of *S. pneumoniae*, strains with thicker capsules such as serotype 3, 6A, 6B, 9N and 19F, have been associated with more aggressive invasive disease (8).

A small number of capsular serotypes cause invasive diseases in children and adults. Their prevalence has geographical and age specificities and may change depending on the administered vaccines (9). Recent studies have shown variations in virulence based on the high rate of pneumococcal recombination. In different clonal lines are observed differences in the gene content and phenotypic diversities, regardless of the capsular serotype (10).

PCR-based serotyping characterises the genes within the *cps* locus. Different serotypes have distinct construction of the genes within the cluster. The most important gene incorporated in the *cps* locus, the *wzy* gene, has multiple allotypes that determine the different construction of capsular polysaccharide chains. Approximately 95 pneumococcal serotypes are described so far (11, 12, 13, 14, 15, 16). The sequence of the *cps* locus and the location of the genes involved in biosynthesis and construction of the capsular polysaccharide chains, were determined in the last decade.

The introduced pneumococcal vaccines are an important measure for the prevention of bacterial meningitis in young children and adults.

MATERIALS

During the survey period from January 2013 to December 2017, the reference laboratory at NCIPD processed a total of 202 clinical materials sent from hospitals in the country for confirmation or determination of the etiological agent causing meningitis. Bacterial DNA isolation was performed from CSF samples and pneumococcal strains isolated from meningitis patients aged from 1 month to 83 years (Table 1).

Table 1. Number of analysed materials during the period 2013- 2017.

Year	2013	2014	2015	2016	2017
Number of analysed materials	39	41	55	30	37

Bacterial DNA was isolated using 5% Chelex 100 and Proteinase K, and stored at -20°C.

The positive result for *S. pneumoniae* DNA in CSF, blood and/or pleural fluid samples was considered indicative of the presence of invasive pneumococcal disease.

METHODS

Isolation of bacterial DNA was carried out using 5% Chelex 100 and 20 mg/ml Proteinase K. 200 ml of the examined sample (CSF) was added to a 1.5 ml tube containing 150 µl 5% Chelex 100 and 6 µl proteinase K. The tube was placed in a thermoblock for 15 minutes at 56°C and for another 15 minutes at 100°C. After that the tube was allowed to cool for 5 minutes at room temperature and centrifuged for 5 minutes at 14,000 rpm. The supernatant containing the extracted DNA was transferred to a sterile 1.5 ml tube and the sediment was discarded.

The target genes used for identification of *S. pneumoniae* were *cpsA* and *lytA*, containing conserved sequences. Some of the samples showed positive result for *S. pneumoniae* DNA only by Real-time PCR. The strains received as culture isolates were serotyped by conventional multiplex PCR with 41 oligonucleotide primers used in 13 separate assays for deduction of 70 possible serotypes. The strains from the positive samples were serotyped by Real-time multiplex PCR with oligonucleotide primers used in 21 assays for deduction of 37 pneumococcal serotypes. In the conventional multiplex PCR all primers were targeting the *cpsA* gene which also served as an internal control of the assay (17).

RESULTS AND DISCUSSION

This study employed probe-specific primers and probes to screen for *S. pneumoniae* DNA in 202 materials received as CSF samples

and strains isolated from CSF of patients diagnosed with meningitis. Sixty-two materials gave positive result for *S. pneumoniae*.

The examination of CSF samples in this survey demonstrates that *Streptococcus pneumoniae* is the dominant pathogen of purulent bacterial

meningitis in Bulgaria.

Multiplex PCR was used for determination of 41 serotypes/serogroups of *S. pneumoniae*. Electrophoresis results of DNA amplicons typed with conventional multiplex PCR are shown in Fig. 2.

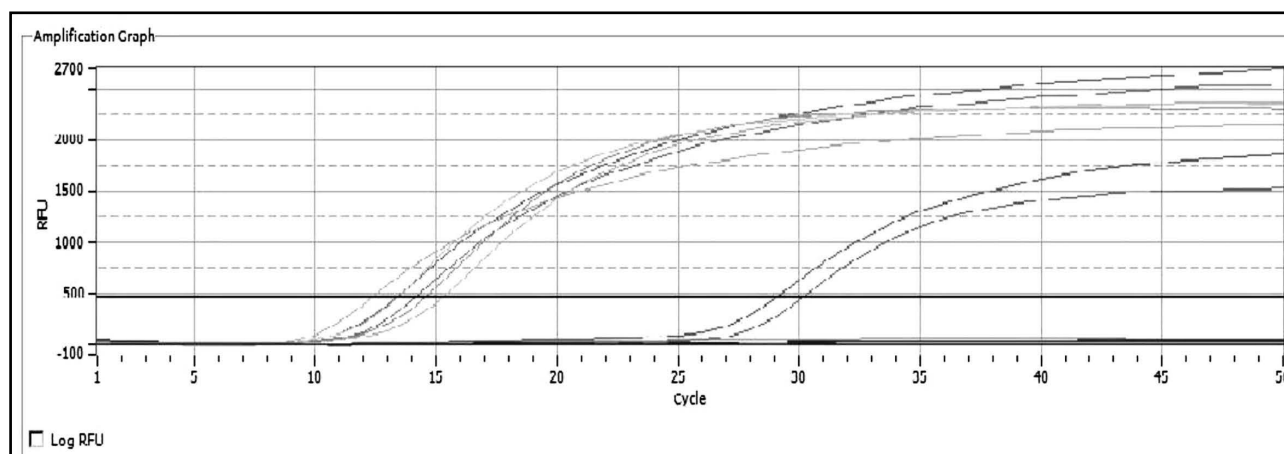


Figure 1. Real-time PCR results for identification of *S. pneumoniae*.

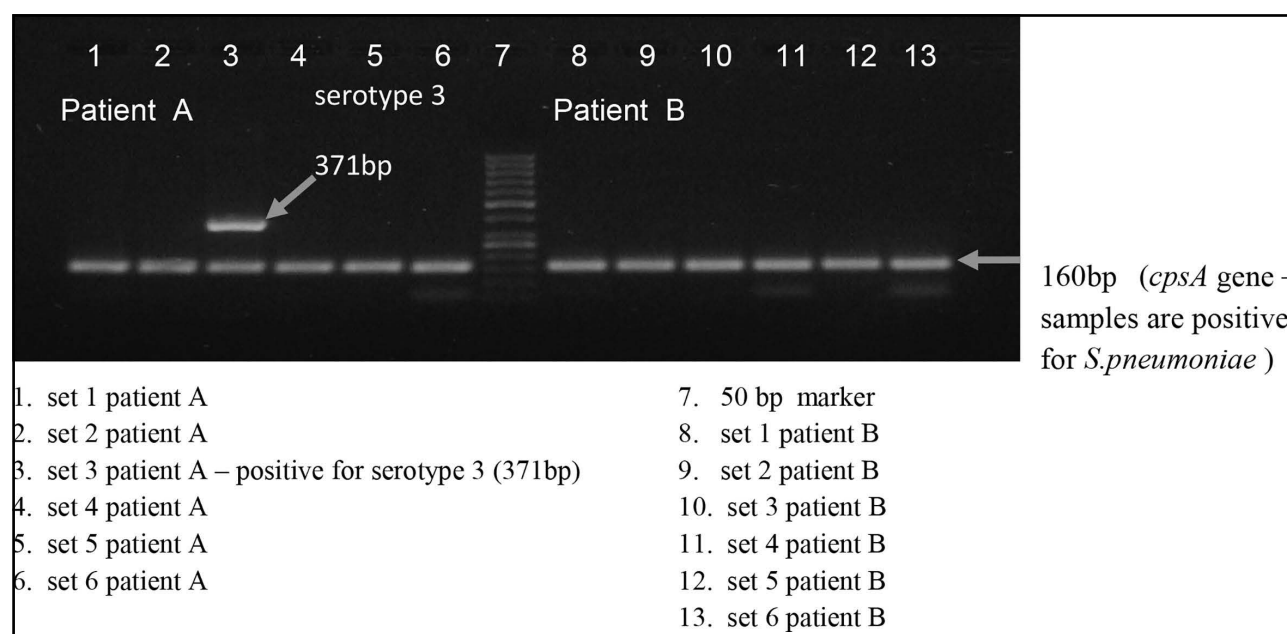


Figure 2. Electrophoresis results for samples of patients A and B tested by multiplex PCR with specific serogroup/serotype primers.

S. pneumoniae serotypes/serogroups identified in this study are presented in Table 2 and Fig. 3.

Table 2. Serogroup/serotype distribution of *S. pneumoniae*- positive samples.

Serogroup/serotype	Total number of positive samples	% of positive samples	Included in PCV10
3	13	21.0	
6A/B/C/D	4 (not vaccinated)	6.5	yes
7F/A	2 (not vaccinated)	3.2	yes
8	2	3.2	
9A/V	4 (not vaccinated)	6.5	yes

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Serogroup/serotype	Total number of positive samples	% of positive samples	Included in PCV10
9N/L	3	4.8	
10A/D	3	4.8	
10B	1	1.6	
11A/D	1	1.6	
14	1 (not vaccinated)	1.6	yes
15B/C	4	6.5	
15A/F	1	1.6	
18C	5 (not vaccinated)	8.1	yes
19A	8	12.9	
19F	2 (not vaccinated)	3.2	yes
23F	1 (not vaccinated)	1.6	yes
24 A/B/F	1	1.6	
Nontypeable	6	9.7	

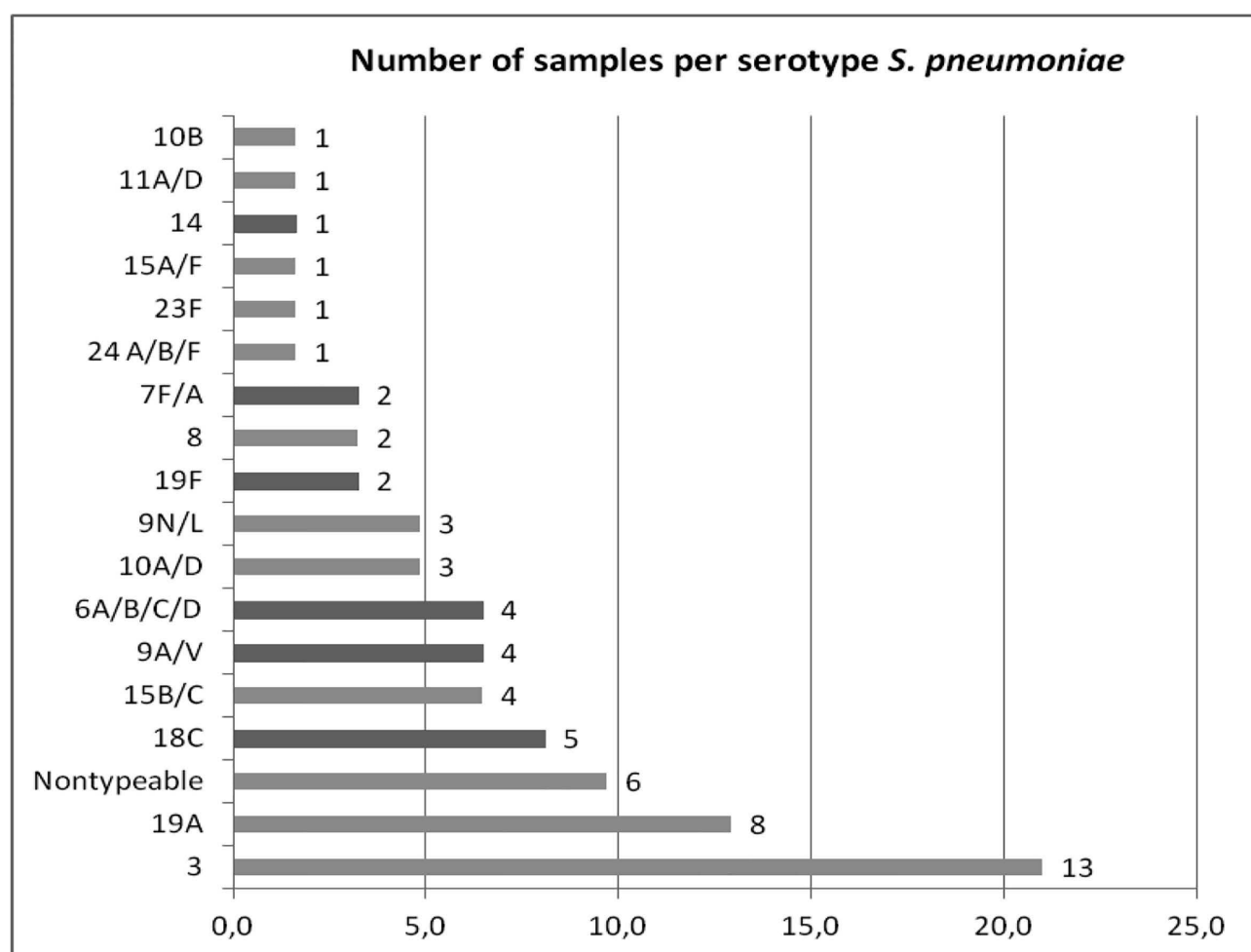


Figure 3. Number of samples per serotype/serogroup of *S. pneumoniae* from 62 positive cases of bacterial meningitis.

The obtained results provide important data for the molecular epidemiology of circulating *S. pneumoniae* strains in Bulgaria. The frequency of serotype 3 differs significantly from serotype 14 ($p < 0.05$). The pneumococcal conjugate vaccine currently used in the immunisation calendar of Bulgaria is PCV10, which does not

cover *S. pneumoniae* serotypes showing the highest frequency according to this study. Serotype 3 is related with high mortality rates in different regions of the world (18, 19, 20). The high frequency of this serotype – 21.0% of positive samples, and its association with greater mortality requires continuous monitoring of its

incidence after the introduction of PCV10. In order to provide protection against serotype 3, PCV10 may be considered to be replaced by PCV13. In the present study, all cases of meningitis caused by *S. pneumoniae* vaccine serotypes (6A/B/C/D, 7F/A, 9A/V, 14, 18C, 19F, 23F) occurred in patients that were not vaccinated.

Vaccine prophylaxis with pneumococcal conjugate vaccines significantly reduces the incidence of invasive pneumococcal infections caused by serotypes included in PCV10.

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DISTRIBUTION OF *MYCOBACTERIUM AVIUM* COMPLEX IN BULGARIA

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ABSTRACT

The members of *Mycobacterium avium* complex (MAC) are opportunistic microorganisms, common in the environment (soil and water) and cause infections in birds, mammals and humans. The objective of the present study was to determine the prevalence of MAC representatives among suspected for tuberculosis patients in Bulgaria over an eight-year period – from 2010 to 2017. The exact epidemiology of pulmonary non-tuberculous mycobacterial (NTM) disease was difficult to determine because unlike cases of infection with *M. tuberculosis* complex, notification of NTM and MAC in particular, is not mandatory in terms of Ordinance 21 of the Ministry of Health (1) and there is no any accurate information about their prevalence in Bulgaria. The survey was conducted in the National Reference Laboratory of Tuberculosis at the National Centre of Infectious and Parasitic Diseases. MAC was identified using phenotypic methods, biochemical features and molecular genetic assay (LPA).

A total of 596 NTM strains were identified during the study period and 16.8% (n=100) of them were MAC. In EU countries, MAC representatives are prevalent among NTM isolates, but in Bulgaria they are significantly less in number.

KEYWORDS:

LPA, NTM, MAC

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INTRODUCTION

Non-tuberculous mycobacteria (NTM) are all *Mycobacterium* species other than *Mycobacterium tuberculosis* complex (MTC) and *Mycobacterium leprae*. There are currently more than 170 species of these environmental, mostly opportunistic pathogens (2). Recently, NTM organisms have gained attention due to increased isolation frequency (3-5), considered to be most pronounced in countries like Bulgaria, where the incidence of tuberculosis (TB) is declining (6).

Mycobacterium avium complex (MAC) consists of two main species: *M. avium* and *M. intracellulare*. *M. avium* can be further classified in subsp. *avium*, subsp. *silvaticum* and subsp. *paratuberculosis*. *M. avium* subsp. *paratuberculosis* is an obligate pathogen in ruminants and is associated with Crohn disease in humans (3). It is very slow-growing – primary isolation requires several months of incubation. Sequence analysis of 16S rRNA or hsp65 gene identified new species and subspecies within MAC. For example, *M. avium* subsp. *hominissuis* was separated from *M. avium* subsp. *avium* based on several differences determined with IS1245 RFLP, 16S-23S rDNA ITS and growth temperature (2, 3, 15).

Furthermore, recent reports describe serious infections in patients who had undergone open cardiac surgery using contaminated heater-cooler device during extracorporeal circulation. The infections occurred in patients in Europe, the United States, Australia, and were caused by *Mycobacterium chimaera*. This organism is a slow-growing NTM species ubiquitous in soil and water, and included in the MAC as it is closely related to *M. intracellulare*. *M. yongonense* associated with pulmonary infections is another newly identified species also closely related to *M. intracellulare* (2, 7).

MAC may be found both in natural and man-made environment. Routes of infection with MAC are pulmonary by inhalation of aerosols, gastrointestinal by ingestion of contaminated water/food, and direct inoculation through trauma or existing wounds. MAC bacteria can cause chronic pulmonary infection in patients with pre-existing pulmonary disease (e.g. COPD), cervical lymphadenitis in immunocompetent children, localised infections after traumatic inoculation, and disseminated

disease in the severely immunocompromised, e.g. HIV-infected, transplant recipients and those on immunosuppressive chemotherapy. *M. avium* infection is more often associated with the last listed patients. Worldwide, *M. avium* is the causative agent in more than 95% of MAC infections in patients with AIDS but rarely causes lung diseases among them (8). On the contrary, *M. intracellulare* is a pathogen responsible for pulmonary diseases in immunocompetent individuals.

Worldwide, pulmonary disease caused by MAC appears to be increasing (9). There is no evidence of human-to-human transmission (3). The organisms are common in the environment and there is substantial evidence showing that the environmental niche is in biofilms lining suburban water pipes. Many MAC have been isolated from drinking water (9).

Currently, in the United States MAC isolates represent 26% of the total mycobacterial isolates (9). In the European Union (EU) and the European Economic Area (EEA) Finland is the only country with mandatory notification of NTM. Several countries have published reports on the nationwide prevalence (10). According to a recently published snapshot of NTM in pulmonary samples collected in 2008 from 30 different countries, MAC predominated in most of them, followed by *M. goodii* and *M. xenopi* (10).

OBJECTIVE:

To determine the prevalence of MAC representatives among patients in Bulgaria suspected for tuberculosis for the period from 2010 to 2017.

MATERIAL AND METHODS

The study included patient isolates processed throughout the whole TB laboratory network in Bulgaria. Species identification was performed only in the National Reference Laboratory of Tuberculosis at the National Centre of Infectious and Parasitic Diseases (NRL TB, NCIPD). The quality of microbiological diagnosis was ensured through external quality assessment (EQA) at national level by NRL TB and by INSTAND e.V. on supranational level.

A total of 596 strains were specified as NTM. For the purpose of the study each patient was

represented by a single strain. Strains were isolated from the following clinical specimens: sputum, bronchoalveolar lavage (BAL), gastric washes (in children), cerebrospinal fluid (CSF), throat swabs, surgery material and urine of patients suspected for tuberculosis. The laboratories use methods optimised for cultivation of *M. tuberculosis* complex: on a solid and/or liquid media, respectively, Löwenstein-Jensen and/or MGIT (BACTEC MGIT 960 TB system) according to the relevant standard operating procedures in national and international guidelines (11, 12). The main methods used for isolation and identification of NTM at the NRL TB were: Ziehl-Neelsen staining, culture on Löwenstein-Jensen and MGIT media with determination of colony morphology, pigmentation and photoreactivity, temperature dependence and speed of growth; resistance to the p-nitrobenzoic acid; absence of MRT64 antigen by BD MGIT TBc Identification Test and line probe assay (LPA) Geno Type® Mycobacterium CM and AS (14). LPA are PCR tests designed to identify the most common and relevant to human pathology NTM. Species identification was performed using LPA after DNA isolation from pure culture with subsequent amplification and reverse hybridisation to specific oligonucleotides immobilised on a membrane strip. Geno Type® Mycobacterium CM and Geno Type® Mycobacterium AS can be performed independently and also complement each other. Isolates defined as genus *Mycobacterium* by GenoType® CM, were tested with GenoType® AS.

RESULTS AND DISCUSSION

In the beginning of the study period there is a gradually increasing trend in the number of NTM/MAC isolates until 2015. After that the number of isolates starts to decrease and reaches a plateau around 85 NTM per year. The total number of MAC isolates between 2010 and 2017 is 100 with a clear trend towards increase of NTM as a whole. The rise in NTM/MAC isolation could be a result of improved laboratory diagnosis. During the 2nd cycle of the External Quality Control evaluating the diagnostic proficiency of laboratories, NRL TB, NCIPD introduced quality assessment of culture-based diagnosis of tuberculosis, and in particular the differentiation between MTC and NTM strains. That may have made impact towards more

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precise laboratory diagnosis. However, the ageing forms of chronic lung diseases probably is another population with increasing prevalence of some important factor as well.

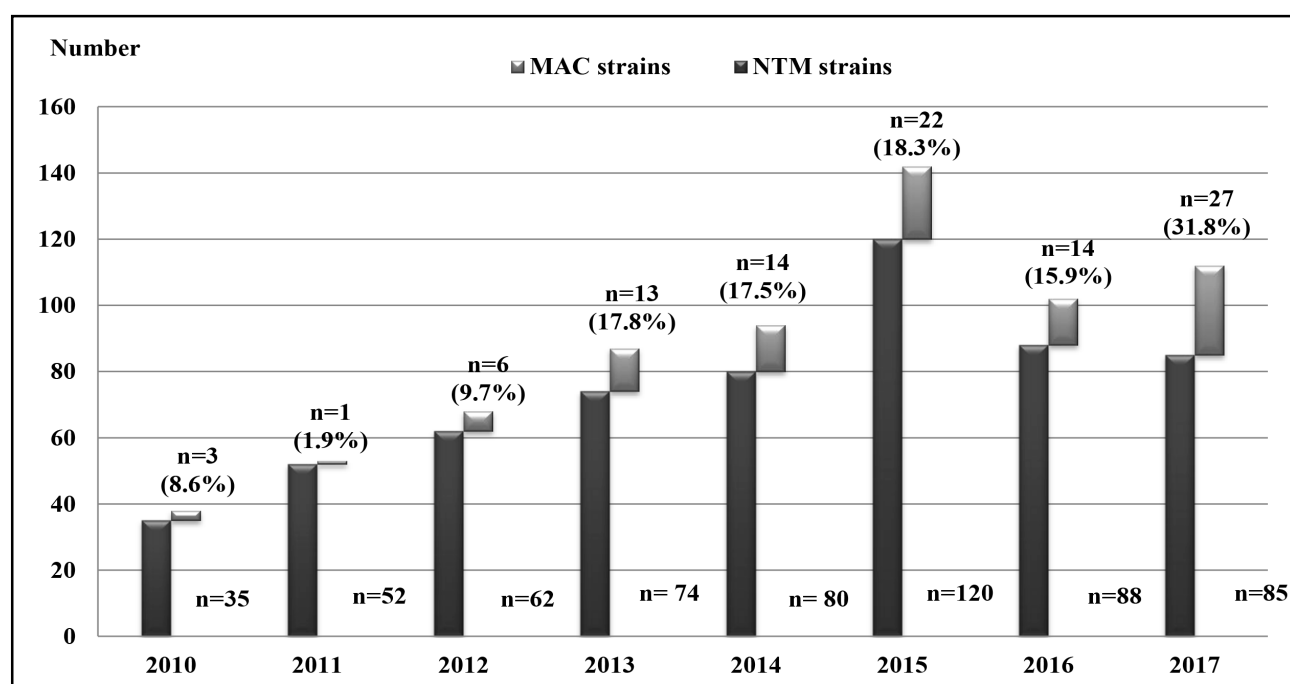


Figure 1. Distribution of NTM/MAC strains during the study period.

NTM were isolated in 24 out of 30 microbiology laboratories performing culture-based diagnosis of tuberculosis in Bulgaria. In 9 (30%) of them MAC was not reported.

We observed a marked contrast in the number of NTM/MAC isolates between the capital and other country districts. Most of the patients are

residents of Sofia and cities with large population like Plovdiv, Varna and Burgas. One of the reasons for the prevalence in urban areas may be the plumbing system and biofilm formation. NTM in biofilms are more resistant than those in natural water and can serve to repopulate plumbing pipes and instruments after disinfection.

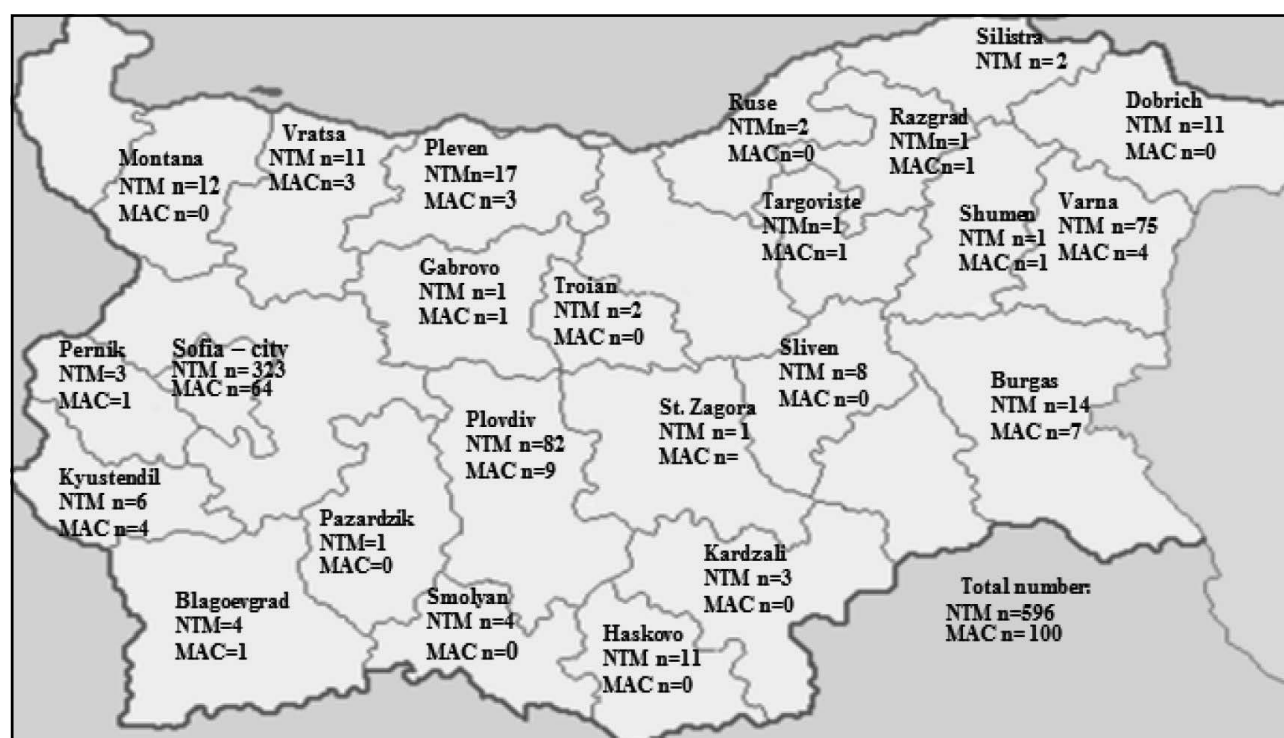


Figure 2. Geographic distribution of NTM/MAC strains.

The processed specimens in which MAC were detected are presented in Fig. 3. About a quarter of them – 23% (n = 23, all sputum) were with positive smear microscopy.

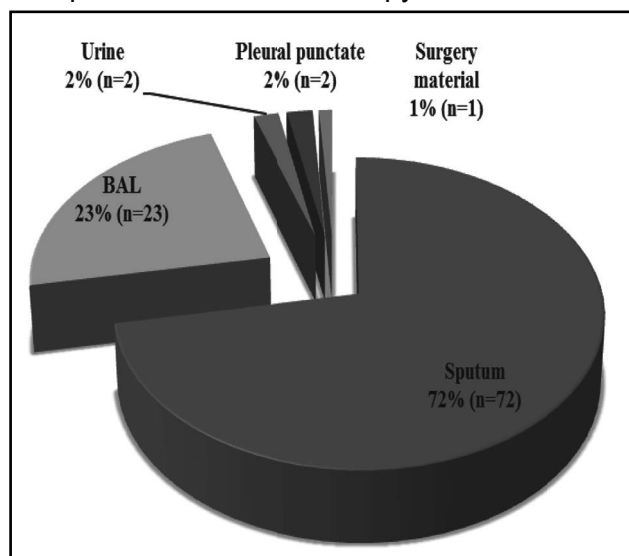


Figure 3. Clinical specimen types in which MAC were detected.

The distribution of MAC by gender was similar to that of TB infection with slight male

prevalence – 55% (n=55), female – 45% (n=45). However, most studies identify preponderance of NTM among females. A large population-based estimate of trends in NTM in the USA was conducted by Prevots et al. from 2004 to 2006. Their data showed that NTM surpassed TB with an increasing prevalence. They also found that the prevalence of NTM was 1.1-1.6-fold higher among women relative to men across the different states (13).

A study in Europe conducted by the NTM-NET collaborative group showed predominance of NTM diseases in female patients (14).

Patient age structure is presented in Fig. 4. Our results indicate that the overall prevalence of MAC is relatively steady among the age groups of up to 14 years (about 1%). Thereafter, it increases considerably and reaches about 15% for people at the age of 25-44 years. Most frequently MAC were isolated from population aged over 45 years – 82% (n = 82). In accordance with worldwide reports, our results demonstrate a clear age-related prevalence.

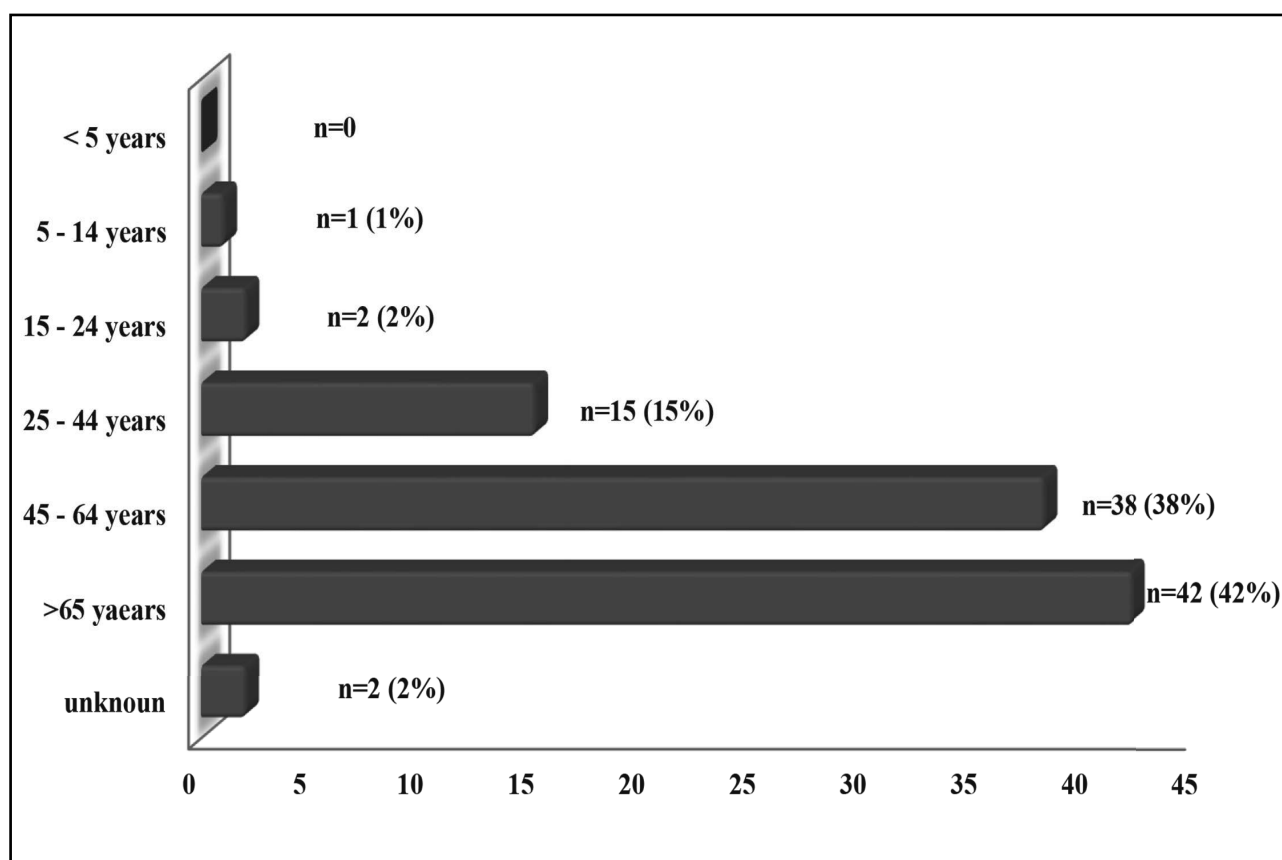


Figure 4. Age structure of patients with MAC isolates for the period 2010-2016.

During the eight-year study period in Bulgaria *M. intracellulare* showed prevalence rate of 56% (n=56) and *M. avium* ssp. representatives – 44% (n=44). Our results are dissimilar to those in other European countries where *M. avium* ssp. are predominant. Such ratio is proven to be typical of the Australian continent where its occurrence is explained with changes in the age and gender ratio of the population. One of the reasons for the observed results in this study may be the lack of detailed clinical information in our country. Therefore, differentiation between contamination of specimens and disease is difficult. *M. intracellulare* are ubiquitous in the environment and the environmental niche is in biofilms lining water pipes. Another reason may be the low incidence of *M. avium* ssp. among

immunosuppressed individuals in Bulgaria such as patients with HIV, cystic fibrosis and transplant recipients.

According to a study conducted by the NTM-Network, the relative frequency of *M. intracellulare* versus *M. avium* in different parts of the world is diverse. A noteworthy difference is the relative prevalence of *M. avium* in North and South America, whereas *M. intracellulare* is most common in Australia (57% of all cultured mycobacteria and 80% of MAC) and South Africa (40% of all cultured mycobacteria and 77.5% of MAC). In Europe MAC was found with the largest share among NTM species – 37%, and 47% of them were confirmed as *M. avium* ssp., 31% *M. intracellulare* and only 22% were identified as part of MAC (14).

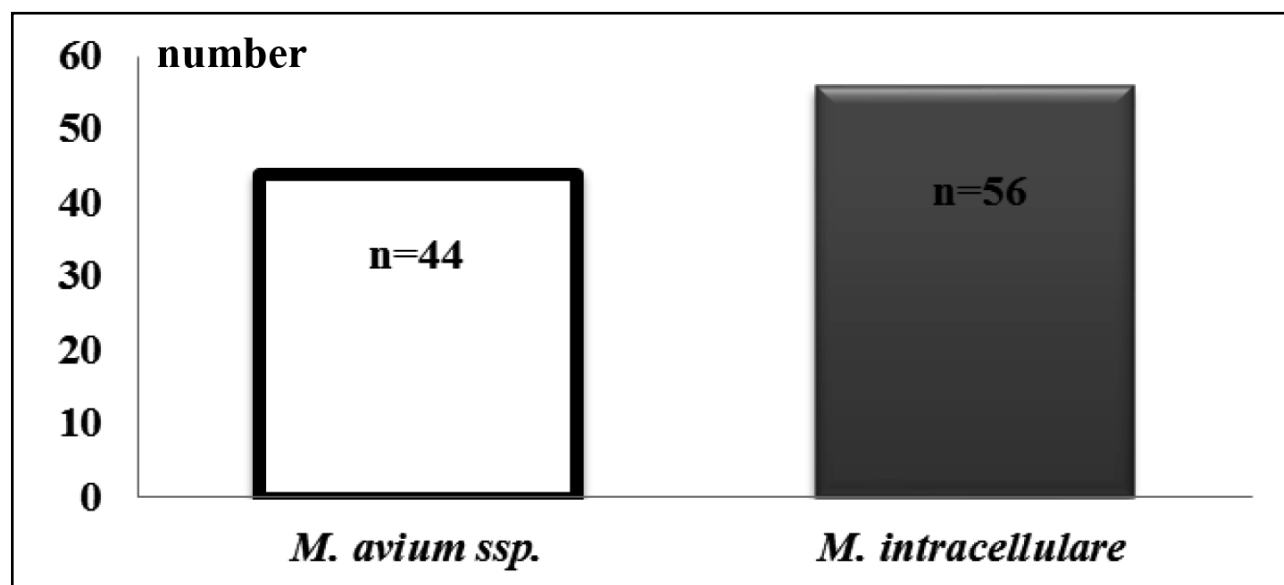


Figure 5. Distribution of MAC isolates by species.

In contrast to the growing body of literature supporting the distribution of MAC among immunosuppressed patients and in particular those with HIV, in Bulgaria for a period of eight years (2010-2017) only one patient was confirmed to be HIV-positive and infected with *M. avium* ssp. In Bulgaria *M. intracellulare* isolates predominated. It should be noted that *M. intracellulare* was detected in 35% (n=5) of BAL specimens included in our study, all of them originating from one hospital. In these cases the possibility of contamination should be considered.

CONCLUSION

Although the present study is not population-based, it clearly shows a tendency towards increase in the number of NTM/MAC isolates in Bulgaria, which is in line with results observed elsewhere, and provides a snapshot of the predominant types of MAC in our country. A significant number of MAC isolates are *M. intracellulare*. The clinical significance of the increased MAC isolation from human samples observed in this study makes it difficult to interpret whether these isolates are associated with colonisation or disease. Additional studies,

including detailed clinical data, are needed to better understand the changes in MAC epidemiology. NRL TB, NCIPD has the capacity and is actively looking for identification of *M. chimaera*, but so far this species has not been registered.

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UNEXPECTEDLY HIGH NUMBER OF WEST NILE NEUROINVASIVE DISEASES IN BULGARIA IN 2018

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ABSTRACT

West Nile virus (WNV) is an emerging arbovirus in Europe and America which disseminated widely in recent years. This study analysed epidemiological data and laboratory findings of probable and confirmed human WNV cases in Bulgaria in 2018. A total of 15 patients with WNV infection were detected in 2018, with 2 deaths among them. All patients were diagnosed in August or September. Four patients were from Sofia district, 4 from Burgas, 3 from Plovdiv and 1 from each of the following districts: Shumen, Yambol and Pazardzhik. Laboratory diagnosis for 7 of the patients was based solely on detection of specific antibodies in the serum samples. Eight patients met the criteria for confirmed WNV case. In 6 of them WNV genome was detected by RT-PCR in blood or urine sample and in 3 patients specific IgM antibodies were found in CSF samples which confirmed WNV neuroinvasive infection. The high number of diagnosed human WNV cases in 2018 in Bulgaria is a good sign of increasing recognition of the disease. More efforts are needed in proper transportation of clinical samples undergoing genetic detection of the viral genome. WNV neuroinvasive infection

should be suspected and included in the differential diagnosis of viral encephalitis or meningoencephalitis especially in late summer and early autumn.

KEYWORDS:

viral encephalitis, flavivirus, Bulgaria, West Nile virus

INTRODUCTION

West Nile virus (WNV) is an emerging arbovirus in Europe and America which disseminated widely in recent years.

WNV is a member of genus *Flavivirus* of the *Flaviviridae* family. The virus is maintained in nature by enzootic cycle involving wild or domestic birds as hosts and *Culex* mosquitoes as vectors. Migratory birds contribute to the transfer of the virus over long distances.

The disease in people is most often asymptomatic. About 20% of WNV infections result in diseases that are usually presented only with non-specific febrile syndrome. Less than 1% of WNV infections involve central nervous system, mostly with encephalitis but also with meningitis or myelitis (1). The fact that mainly neuroinvasive infections are recognised means that the actual number of infected people is at least a hundred times higher. The first recognised WNV outbreak was described in southern France in 1962-1963. The first significant epidemic in humans was registered in Romania in 1996 with 393 WNV infection cases. The largest outbreak of WNV in Europe was recorded between 2010 and 2013 in Greece with a total of 609 laboratory-confirmed cases and 73 deaths (2). The outbreak was caused by WNV lineage 2. The first time this lineage caused disease outside Africa was in Hungary in 2004 (3). In subsequent years WNV lineage 2 appeared in many countries of Central, East and South Europe (Hungary, Russia, Romania, Italy, Albania, Serbia, Greece) and now is the predominating lineage in Europe.

In Bulgaria, human WNV cases are reported every year since 2015, when the first laboratory-confirmed neuroinvasive case appeared (4). However, in 2018 unusually high number of WNV cases were recorded in the country. The aim of this study was to analyse epidemiological data and laboratory findings of these cases.

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MATERIAL AND METHODS

Patients and clinical samples

Serum, blood and CSF samples were drawn from suspected patients by physicians at the regional hospitals in Bulgaria. Laboratory investigations were performed at the National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria by ELISA and RT-PCR.

ELISA

Serum and CSF samples were tested for WNV-specific IgM and IgG antibodies using ELISA test (Euroimmun, Germany).

RT-PCR

Viral RNAs were extracted from blood and urine samples using QIAmp Viral Mini Kit (Qiagen, Hilde, Germany). WNV RNAs were detected by commercially available real-time RT-PCR kit (Sacace Biotechnologies, Italy).

RESULTS

A total of 15 patients with West Nile virus (WNV) infection were detected in 2018, with 2 deaths among them (case fatality ratio 13.3%). All except 4 were male (73.3%).

The age of the patients varied between 45 and 84 years. The most affected age groups were 70-79 (4 patients) and 50-59 (3 patients). Older age was a risk factor for more complications

and even lethal outcome (as for the 84-year-old patient).

All patients were diagnosed in August or September with 6 in August and 9 in September. This fact coincides with the known high activity of *Culex* mosquitoes in the country.

Analysis of case distribution according to the district of origin showed that 4 patients were from Sofia district, 4 from Burgas, 3 from Plovdiv and 1 from each of the following districts: Shumen, Yambol and Pazardzhik.

Serological diagnosis of serum samples was carried out in all except one case. However, this finding alone is suggestive only of a probable case. In our study, laboratory diagnosis in 7 patients was based solely on detection of specific antibodies in the serum samples.

Eight patients met the criteria for confirmed WNV case. In 6 of them WNV genome was detected by RT-PCR in blood or urine sample and in 3 patients specific IgM antibodies were found in CSF samples which confirmed WNV neuroinvasive infection. In one of these patients, antibodies in CSF and viral genome in the blood sample were detected simultaneously.

Detailed characteristics of the patients are presented in Table 1.

Table 1. Patients diagnosed with WNV infection in 2018.

No	Age	Sex	Month	District	Laboratory findings			Case category
					ELISA serum	ELISA CSF	RT-PCR	
1.	49	M	August	Sofia	IgM+ IgG+	n.p.	n.p.	Probable
2.	55	M	August	Plovdiv	IgM+	IgM+	+ blood	Confirmed
3.	57	M	August	Sofia	n.p.	IgM+	n.p.	Confirmed
4.	75	F	August	Burgas	IgM+ IgG+	n.p.	n.p.	Probable
5.	72	M	August	Pazardzhik	IgM+ IgG+	n.p.	n.p.	Probable
6.	-	M	August	Plovdiv	n.p.	n.p.	+ blood + urine	Confirmed
7.	51	M	September	Burgas	IgG+	n.p.	n.p.	Probable
8.	69	M	September	Shumen	IgM+ IgG+	n.p.	n.p.	Probable
9.	45	M	September	Plovdiv	IgM+ IgG+	n.p.	n.p.	Probable
10.	-	F	September	Sofia	IgM+ IgG+	IgM+ IgG+	n.p.	Confirmed
11.	84	M	September	Yambol	IgM+ IgG+	n.p.	n.p.	Probable

No	Age	Sex	Month	District	Laboratory findings			Case category
					ELISA serum	ELISA CSF	RT-PCR	
12.	70	F	September	Burgas	IgM+ IgG+	n.p.	+ blood + urine	Confirmed
13.	70	F	September	Burgas	IgM+/- IgG+/-	n.p.	+ blood + urine	Confirmed
14.	-	M	September	Sofia	IgM+ IgG+	n.p.	+ blood + urine	Confirmed
15.	66	M	September	Sofia	IgM+	n.p.	+ blood + urine	Confirmed

*n.p. – not performed

DISCUSSION

In 2018, WNV caused unusually high number of infections across Europe. According to ECDC data, the total number of confirmed and probable WNV infections reported in 2018 exceeds the total number in the previous seven years (5). A total of 2 083 autochthonous WNV infections in 2018 were reported in Europe – 576 in Italy, 415 in Serbia, 311 in Greece, 277 in Romania, 215 in Hungary, 53 in Croatia, 27 in France, 20 in Austria. Except Italy, the 3 most affected countries are Bulgaria's neighbours. This fact should be taken into account when analysing the situation in Bulgaria. Even though 15 patients in one year is much more than the number of cases reported in the last seven years in the country (a total of 7 cases: 2012-2, 2015-2, 2016-2, 2017-1), this number is much lower compared with the neighbouring countries. This means that the vast majority of the WNV infections were not recognised by physicians. Moreover, again according to ECDC data, 68% of WNV infections in 2018 were neuroinvasive (5), indicating that about one third of the recognised infections were with non-specific febrile syndrome. For comparison, all recognised WNV cases in Bulgaria were neuroinvasive. Therefore, more effort is needed to add WNV infection in differential diagnosis of viral encephalitis or meningoencephalitis.

More effort is also needed to ensure appropriate transportation of clinical samples. They should be sent on ice to the Reference laboratory as this is the only way to preserve the virus during transportation. It should be kept in mind that blood is a more suitable clinical sample for diagnosis early in the course of the disease and urine is preferable later and in case of neuroinvasive infection.

Detection of specific antibodies in CSF is also suggestive of confirmed WNV infections. For this reason blood, urine and CSF are appropriate clinical samples for reliable laboratory diagnosis of WNV infection.

Analysis of epidemiological data of our patients revealed that they were mostly men above 44 years, some of them with chronic diseases and other complications (like alcoholism). These could be identified as risk factors for occurrence of WNV infection and especially neuroinvasive form of the illness.

Regarding the location of the WNV cases, it should be noted that the disease is found where it is searched for. The territory around Sofia is an endemic area as shown by the seroprevalence study (6) and confirmed by detection of WNV genome in patients (4). The first and nationwide seroprevalence study revealed the highest seroprevalence rate of WNV-specific IgG antibodies in Sofia Province (10%). The study also showed high seroprevalence rates in districts along the river Danube (4-7.5%), low rates in the districts of Yambol and Plovdiv (2% and 1.6%, respectively) and 0% in the districts of Burgas, Shumen and Pazardzhik, where human WNV cases were detected in 2018. Obviously, much more cases could be found in districts along the river Danube provided that are properly and accurately diagnosed with the assumption that in late summer and early autumn WNV is not uncommon cause of febrile illnesses and viral encephalitis.

Etiology of the vast majority of neuroinfections in the country remains unknown (7). WNV infection of CNS should be suspected among cases presenting with clinical manifestation of viral encephalitis. Significant increase in the number

of WNV infections this year may lead to an outbreak next year (8).

In conclusion, the high number of diagnosed human WNV cases in 2018 in Bulgaria is a good sign of increasing recognition of the disease, a tendency that should be kept in the future. More efforts are needed in proper transportation of clinical samples undergoing genetic detection of the viral genome. WNV neuroinvasive infection should be suspected and included in the differential diagnosis of viral encephalitis or meningoencephalitis especially in late summer and early autumn.

ACKNOWLEDGEMENTS

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DETECTION OF MEASLES AND RUBELLA ANTIBODIES IN DRIED BLOOD SPOTS

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ABSTRACT

This study **aimed** to determine the frequency of detection of measles and rubella antibodies in dried blood spots in Bulgaria.

Material and Methods: Two types of clinical material, serum samples and dried blood spots (DBS), were tested from a total of 101 patients. Serological methods (indirect ELISA) were used for detection of specific viral markers (IgM and IgG antibodies) indicating acute or past measles and rubella infection.

Results: In the present study, the patients were with median age of 39 years and divided into 11 age groups. The majority of patients were under 30 years of age and from the capital of Sofia. In 3 patients acute measles infection was confirmed by positive ELISA-IgM results for the serum samples and DBS. No acute rubella infection was detected. Measles and rubella IgG seroprevalence was determined as 83/101 (82%, 95% CI: 74.51÷89.49) and 79/101 (78%, 95% CI: 69.92÷86.08) in serum samples, and 79/101 (78%, 95% CI: 69.92÷86.08) and 73/101 (72%, 95% CI: 63.25÷80.75) in DBS, respectively.

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In combination with immunoenzymatic testing for measles and rubella IgM/IgG markers, coincidence of results for both types of clinical material was found in >90% of cases. No significant differences were found in the results in terms of gender and age.

Conclusion: In recent years a variety of new and innovative applications of DBS are introduced in medicine, neonatology, virology, microbiology, etc. The optimisation of the DBS technique as an alternative approach to venepuncture in virology is very important for conducting seroepidemiological studies and to a certain extent for the surveillance of epidemic outbreaks.

KEYWORDS:

dried blood spots, measles, rubella, ELISA assay, IgM/IgG antibodies

INTRODUCTION

Ivar Christian Bang (1869-1918) is considered the founder of modern clinical microanalysis (1, 2) and the idea of using blood collected on paper map made of cellulose and filter paper technique. Subsequently, several researchers reported the use of dry blood spots (DBS) in serological tests for the diagnosis of syphilis (2), for the detection of antibodies against measles, mumps, polio virus, parainfluenza virus and respiratory syncytial virus in 1953 (2), and also for the identification of *Shigella* in stool (faeces) dried on filter paper (3). In 1924, Chapman (4) summarises the advantages of DBS technique, stressing four key points that are still valid today: (a) compared to conventional venepuncture, it requires less blood volume, which is especially important in areas such as pediatrics and neonatology; (b) blood collection procedure is easy, inexpensive and non-invasive; (c) the risk of bacterial contamination or haemolysis is minimal; and (d) DBS can be maintained for a long time with almost no impact on the quality of the analysis. In 1953 the application of this technique is considered for detection of syphilis, antibodies against measles, mumps, polio virus, parainfluenza virus and respiratory syncytial virus (2) and also for the identification of *Shigella* in stool dried on filter paper (3). In 1969/70s Guthrie published his method of neonatal screening for phenylketonuria with DBS obtained by pricking the heel of newborns (5) and surveillance of congenital

hypothyroidism and sickle cell disorders (5, 6). The approach of using capillary blood obtained from heel or finger and soaked on filter paper, was conducted to screen for metabolic disease in a large population of newborns in Scotland in 1963 (5).

There are many reports on the application of DBS in medicine, toxicology, pharmacokinetics, metabolic exchange, therapeutic drug monitoring, forensic toxicology studies, in clinical laboratory diagnostics and chemistry (7, 8).

Viral infections weaken the immune system and open the door to secondary health problems such as pneumonia, blindness, diarrhoea, encephalitis, etc. The main methods for diagnosis of viral infections are based on detection of specific antiviral antibodies in blood specimens. Measles and rubella infections are vaccine-preventable diseases and are a major cause of morbidity and mortality in children worldwide. The high contagious index (>90% for measles and rubella, and >50% for mumps) and the occurrence of debilitating complications with high frequency determine their health and socioeconomic importance. Approximately 30% of reported measles cases have one or more complications, such as disabling effects that are most common in children under 5 years of age. The importance of rubella infection for the public health is determined by the teratogenic effects of the virus during pregnancy. There is high percentage of miscarriages, stillbirths or congenital rubella syndrome.

The collection of blood, particularly from children, is very often difficult and transportation of the samples to the laboratory in a cold chain is not always achievable. In these cases using DBS as an alternative sampling technique is suitable. DBS have been used for a range of epidemiological studies as an alternative to serum. Antibodies are stable in this form and therefore this method is particularly valuable where the lack of a cold chain is an issue. DBS technique has recently been applied successfully in measles cases, and there is accumulating evidence that it will work for rubella cases as well (9, 10, 11).

This study **aimed** to determine the frequency of evidence of measles and rubella antibodies in dried blood spots in Bulgaria.

MATERIAL AND METHODS

Material - Study Area and Sample Collection

Two types of clinical material, serum samples and dried blood spots, were tested from a total of 101 patients. The specimens were collected under the terms of a research project funded by the National Science Fund, Bulgaria, Contract № DM 03/1,12.12.2016, and tested at the National Reference Laboratory (NRL) "Measles, Mumps and Rubella" of the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria. Blood specimens were collected from all patients by venepuncture and by pricking a finger or heel using sterile automatic lancets for preparation of dried blood spots (1.5 - 2 mm). Blood was centrifuged at 4000 x g for 10 minutes and the serum was aliquoted and frozen at -20°C until analysed. Blood spots were stored on the cards of filter paper, labelled, dried at room temperature for 30 minutes and put in ziploc bags with desiccant for storage at 2°C - 8°C.

The median age was 39 years. The majority of patients were under 30 years of age and from the capital of Sofia.

Methods

• Serological analysis

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

• Statistical Analysis

For the statistical processing of the results obtained we used relative percentages (%), confidence interval (95% CI), graphical and table analysis.

RESULTS

In the present study, the tested patients were aged from 1 to 82 years with median age of 39 years. Due to this wide range they were divided

in 11 age groups (Figure 1). The majority of patients were under 30 years of age and from Sofia (62/101, 61.38%, 95% CI: 51.88÷70.88) and Burgas regions (18/101, 17.82%, 95% CI: 10.36÷25.28). The rest were from the regions of Plovdiv, Stara Zagora, Pazardzhik and Montana.

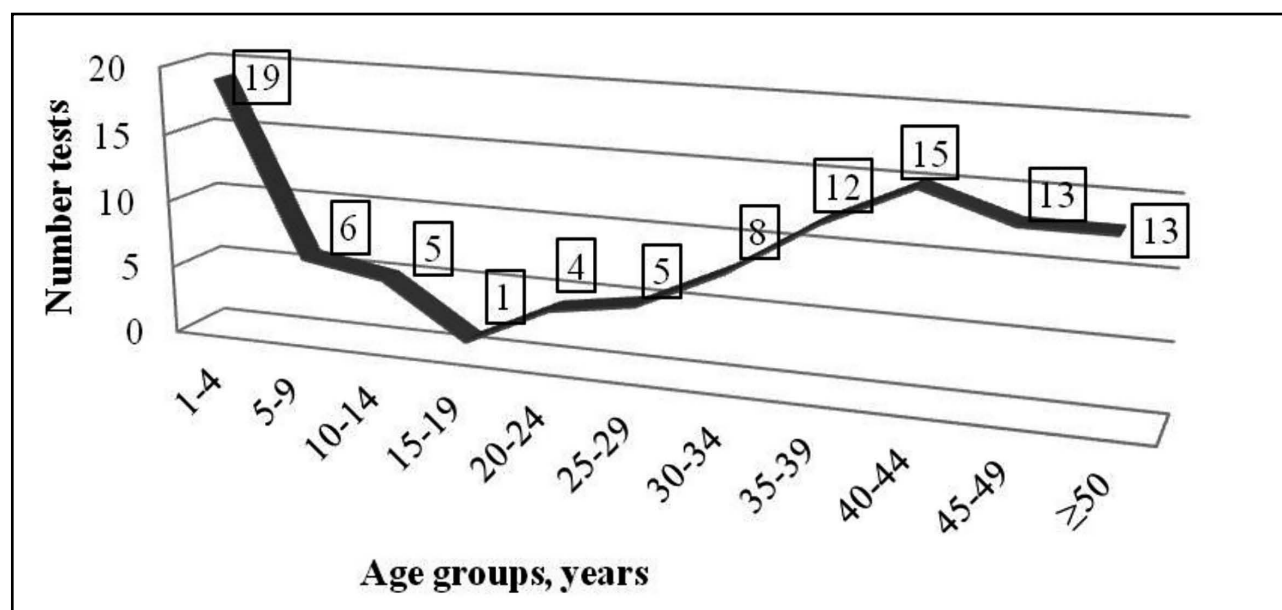


Figure 1. Age group distribution of the patients (n=101).

In 3 patients acute measles infection was confirmed with ELISA-IgM test of the serum samples and DBS. No acute rubella infection and IgM viral marker were detected. The obtained results corresponded with the clinical manifestation, as 12 out of 101 patients were diagnosed with a possible measles infection and none with rubella infection. The samples were collected using case-

based surveillance of these infections in Bulgaria. Immunoassay analysis of all 101 patients showed the presence of measles and rubella IgG in 83/101 (82%, 95% CI: 74.51÷89.49) and 79/101 (78%, 95% CI: 69.92÷86.08) serum samples, and in 79/101 (78%, 95% CI: 69.92÷86.08) and 73/101 (72%, 95% CI: 63.25÷80.75) DBS, respectively (Figure 2).

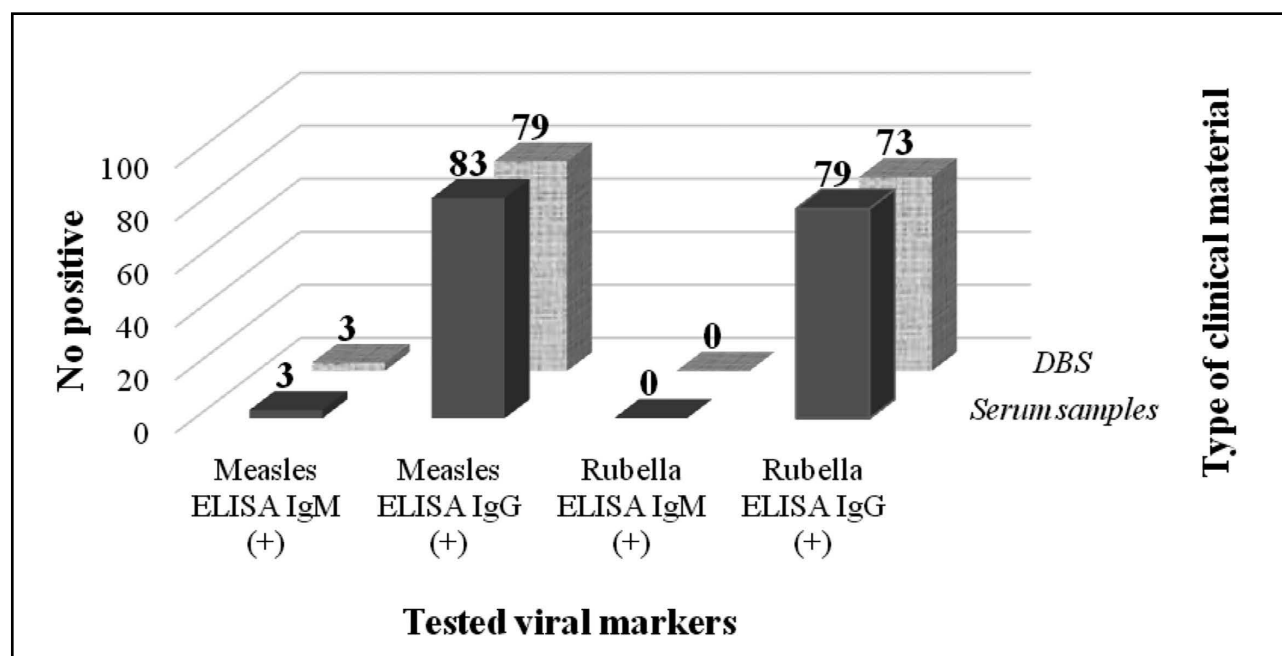


Figure 2. Frequency of detection of immunoenzymatic measles and rubella diagnostic markers (ELISA IgM/IgG) in serum and DBS.

The calculated percentage for coincidence of results obtained with the two types of clinical materials was more than 90%. Different ELISA IgG results were found for 4 DBS tested as negative for measles and rubella, compared with positive results of the serum samples from the same person. When determining the frequency of viral markers detection in DBS and serum samples, no significant differences in the results were found in terms of gender and age of patients. DBS collection was successful in young children and infants as well as in adults, including pregnant women, which is useful for their application in clinical practice.

DISCUSSION

In the beginning of the 21st century, the world, Europe and Bulgaria are taking the path of elimination of measles and rubella (12). This process is difficult to implement because of many epidemic outbreaks and low immunisation coverage of certain population groups. According to European Centre for Disease Prevention and Control (ECDC) data, between 1 July 2017 and 30 June 2018, 29 EU/EEA Member States reported 13 234 cases of measles. During the period, most cases were reported by Italy (3 341), Greece (3 193), France (2 740) and Romania (1 354), accounting for 25%, 24%, 21% and 10%, respectively, of all cases reported by EU/EEA countries (13). Between 1 July 2017 and 30 June 2018, 13 EU/EEA Member States reported a total of 624 cases of rubella with Poland (490), Germany (60), Italy (27) and Austria (21) (13). Collecting venous blood samples is the “gold standard” in laboratory detection of measles and rubella. The collection of blood samples, particularly from children, is a problem as it is an invasive technique, often creates discomfort (pain, injection vacuum system, trained staff, etc.) and maintaining a cold chain when transporting samples to the laboratory is not always achievable. This necessitates the search for alternative samples (dried blood spots and oral fluid) and techniques to detect the viruses and the specific antibodies against them (11). Recently in the WHO Measles and Rubella Laboratory Network the use of DBS begins to be validated as a useful tool alternative to serum

for the measles/rubella program in a range of epidemiological studies (10, 14, 15, 16).

The viral antibody remains stable in DBS which is particularly valuable where the lack of a cold chain and logistics are an issue. Helfand et al. (16) and Karapanagiotidis et al. (10) reported a successful application of this technique in measles cases, and there is accumulating evidence that it will work for rubella cases as well. Uzicanin et al. (17) and Helfand et al. (9) published studies on the seroepidemiological importance of DBS in the control and monitoring of measles and rubella infection. The authors prove DBS as a real alternative to serum samples, particularly in epidemic situations. Our results confirm the potential use of this technique in the laboratory diagnosis of acute and past (presence of protective immunity) measles and rubella infection. In combination with immunoenzymatic testing of measles and rubella IgM/IgG markers, coincidence of results for both types of clinical materials was found in >90% of cases. The study covered a two-year period in which 2 measles outbreaks and 0 rubella cases were reported in Bulgaria. Patient samples were selectively collected, mainly from healthy vaccinated people. Despite the comparatively small number of tested samples, the study showed the potential role of DBS in proving viral markers with standard immunoassay, and optimised the serum elution protocol. The study focuses on the enrichment of the laboratory range for detection of measles and rubella virus in Bulgaria and provides an easy-to-carry test of the immune status of the population in the phase of elimination of these infections.

CONCLUSION

DBS approach is non-aggressive and more acceptable to the public, including young children, pregnant women, etc. In recent years a variety of new and innovative applications of DBS are introduced in medicine, neonatology, virology, microbiology, etc. The optimisation of DBS technique as an alternative approach (non-invasive, inexpensive, not requiring trained staff and cold chain for transport and storage) to venepuncture in virology, is very important for conducting of seroepidemiological studies and to a certain extent for the surveillance of epidemic

outbreaks. Serum-based technology remains a major approach in the immunoenzymatic diagnosis of viral infections.

Competing Interest

The authors do not have any competing interest.

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MIXED INFECTION - HANTAVIRUS HAEMORRHAGIC FEVER WITH RENAL SYNDROME AND HERPES SIMPLEX VIRUS ENCEPHALITIS: A CASE REPORT

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ABSTRACT

Introduction: This case report describes a patient treated at the Clinic of Infectious Diseases of University Hospital "St. George" Plovdiv, with two infections occurring at the same time – haemorrhagic fever with renal syndrome (HFRS) and herpes simplex virus (HSV) encephalitis. **Results:** A 53-year-old male presented with fever, flu-like syndrome and renal impairment with mild nitrogen elevation. The patient was treated at the Clinic of Nephrology for one week. Several hours after discharge he had three generalised tonic-clonic seizures and was admitted to the intensive care unit with status epilepticus and acute respiratory failure requiring intubation and mechanical ventilation for 4 days. Lumbar puncture results showed: white blood cells – $1.10^6/l$, glucose – 5.3 mmol/l, total protein – 1.6 g/l, increased immunoglobulin levels. The patient was transferred to the Clinic of Infectious Diseases with suspicion of viral encephalitis. HSV type 1 was detected in the cerebrospinal fluid by PCR. Due to suspected hantavirus infection, serum was examined and IgM and IgG

antibodies against the HFRS virus were detected by ELISA. Specific treatment with acyclovir was started and there was a rapid improvement in the clinical condition. Convulsions did not recur. Nitrogen levels normalised and there was a short period of polyuria. Thrombocytopenia quickly resolved without occurrence of haemorrhagic syndrome. In the course of the disease, the patient developed mild diarrhoeal syndrome and left thrombophlebitis. He was discharged clinically well with negative PCR results for HSV. In conclusion, we can assume that as a result of immunosuppression due to HFRS, HSV type 1 reactivated and led to herpes encephalitis.

KEYWORDS:

encephalitis, hantavirus, seizures, PCR, ELISA, herpes simplex virus

INTRODUCTION

Herpes encephalitis represents about 10-20% of all cases of sporadic viral encephalitis, with an incidence of about 2.3 cases per million per year and it has no seasonality (1). There is a bimodal age distribution – peak in the interval from 5 to 30 years and over 50 years of age. Herpes simplex virus (HSV) type 1 causes over 95% of cases (2). PCR examination for viral deoxyribonucleic acid (DNA) in the cerebrospinal fluid (CSF) is the gold standard for diagnosis (3). Mortality is about 70% without etiological treatment. Even with treatment, mortality remains high – 18%, and some of the patients develop severe neurological deficits (1, 4).

Haemorrhagic fever with renal syndrome (HFRS) starts with flu-like symptoms but it can progress to shock, haemorrhage and renal failure. Seizures or focal neurological symptoms occur in 1%. Diagnosis is confirmed by serological tests and PCR. Mortality is 6-15% (5). The disease occurs mainly in Asia and Europe, accounting for about 100,000 cases per year (6). Severe forms of HFRS (caused by Hantaan, Seoul, and Dobrava viruses) are characteristic of Korea and the Balkan Peninsula countries (5). In Bulgaria the disease has been reported since 1953. HFRS cases are mainly reported in the mountainous regions of the country (7). For the period 2013-2014, 23 cases of HFRS were observed and 69.6% were caused by the Dobrava-Belgrade virus (DOBV)

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and 30.4% by the Puumala virus. The clinical presentation due to DOBV was more severe (8). In Bulgaria the seroprevalence of hantaviruses is estimated to be 3.1% (9). Although the disease is well-known in endemic regions, it can be found in non-endemic as well (10).

MATERIAL AND METHODS

This case report describes a patient with hantavirus haemorrhagic fever with renal syndrome and herpes encephalitis occurring simultaneously. The patient was treated at the Clinic of Infectious Diseases, University Hospital "St. George" Plovdiv from 6 March to 22 March, 2017. The following methods were used – clinical observation, laboratory, microbiological, serological, molecular tests and imaging techniques.

RESULTS

I.I.C., a 53-year-old male diagnosed with nephritis tubulointerstitialis was treated between 23 February and 2 March 2017 at the Clinic of Nephrology. The patient was admitted with fever of 39°C, chills, oliguria and asthenodynamia. Laboratory examinations showed proteinuria and erythrocyturia, creatinine 264 mg/dl, urea 17.8 mg/dl; kidney ultrasound revealed diffuse bilateral parenchymal process; lung computed tomography (CT) indicated small pleural effusions predominantly in the right side.

On the day of discharge, the patient had three generalised tonic-clonic convulsions with oral secretion and tongue injury. He was transported to the Emergency Department of the University Hospital "St. George" Plovdiv, where he had another seizure with focal onset affecting the left side of the face, rapid generalisation, stertorous breathing, cyanosis and severe tonic phase. The patient was admitted to the ICU with status epilepticus, acute respiratory failure, Glasgow Coma Scale (GCS) - 5 points, requiring intubation and mechanical ventilation for 3 days, Depakine, Dormicum and phenobarbital infusions. Lumbar puncture (LP) was performed and the patient was transferred to the Clinic of Infectious Diseases on 6 March due to suspected viral encephalitis. Physical examination showed severe medical condition. The patient was afebrile, conscious,

orientated, with bradypsychia and bradylalia, no signs of meningeal irritation, normal symmetrical tendon reflexes, without Babinski sign and no cranial nerve palsy. There was a massive subconjunctival haemorrhage in the left eye. Respiratory system – normal breathing; cardiovascular system – normal sinus rhythm, RR 150/90 mmHg, 80 beats/min; abdominal status – painless, hepatosplenomegaly, Pasternacki's sign – negative.

Laboratory tests showed:

- mild anaemia in the course of disease with decreased haemoglobin of 101.109 g/l;
- leucocytosis of $16.10^9/l$ with neutrophilia and lymphopenia;
- transient thrombocytopenia of $56.10^9/l$;
- increased erythrocyte sedimentation rate (ESR) – 52 mm/h and C-reactive protein (CRP) – 157 mg/dl;
- mild nitrogen elevation during the first days of hospital stay with 207 mg/dl creatinine and 15 mg/dl urea. Significant electrolyte disturbances were not observed. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were slightly elevated – 105 and 77 UI/l, respectively, and low albumin levels were recorded throughout the whole hospital stay of up to 28 mg/dl;
- coagulation tests – slightly decreased prothrombin time (PT) and activated partial thromboplastin time (APTT); normal fibrinogen and transient increase of D-dimers. Multiple urine tests showed proteinuria, presence of erythrocytes, leukocytes and single granular casts;
- CSF findings are presented in Table 1 and 2.

Table 1. Cerebrospinal fluid examination.

CSF findings	I lumbar puncture (3 March)	II lumbar puncture (20 March)
Total protein (g/l)	1.6 ↑	0.37
Glucose (mmol/l)	5.4 ↑	2.7
White blood cells ($\times 10^6/l$)	1	3

Table 2. Immunoglobulin results in the initial lumbar puncture and serum (3 March)

Immunoglobulins	CSF (mg/l)	Serum (g/l) - within normal range
IgA	121.28 ↑	3.84
IgM	31.34 ↑	2.21
IgG	375.17 ↑	13.9
IgE	-	5.5

Microbiological examination yielded negative CSF culture; *Enterococcus faecium* was isolated from urine and ESBL-producing *Serratia marcescens* from blood culture. The patient was further investigated for HBsAg and HIV-Ab which were negative.

Two etiological agents were identified at the National Centre of Infectious and Parasitic Diseases, Sofia, as follows:

- 9 March: first serum sample – IgM antibodies against the HFRS virus were not detected by ELISA;
- 20 March: second serum sample – IgM and IgG antibodies against the HFRS virus were detected by ELISA;
- 9 March: first CSF sample – positive PCR results for HSV-1 and negative for HSV-2;
- 22 March: second CSF sample – PCR results for HSV-1 after acyclovir treatment were no longer positive.

Imaging: on the day of admission was performed head CT scan showing brain oedema. An electroencephalogram (EEG) was also performed in the middle of the hospital stay indicating no abnormalities.

The treatment was prolonged and complex comprising of etiological, pathogenetic and symptomatic drugs. Administration of acyclovir was adjusted according to renal function to 400 mg IV 3 times daily for 23 days; Depakine infusions during the first days were replaced with oral administration for 2 weeks; antibiotics - ceftriaxone 2 g daily for 10 days, vancomycin 1 g/8 hours for 10 days, metronidazole 500 mg/8 hours for 5 days and ciprofloxacin 200 mg/12 hours for 7 days targeting the microorganisms isolated from blood and urine as well as the

diarrhoeal syndrome, superficial and deep thrombophlebitis that developed during the hospital stay.

DISCUSSION

The mixed infection with HFRS and herpes encephalitis occurred as a severe disease but with rapid clinical improvement. Seizures did not recur. Nitrogen elevation associated with HFRS was overcome and short-term polyuric stage ensued for 3-4 days with 6 l/daily. There was a mild diarrhoeal syndrome. Thrombocytopenia quickly resolved without occurrence of haemorrhagic syndrome. During the course of the disease the patient developed iatrogenic phlebitis on the right cubital vein and deep left thrombophlebitis. He was discharged fully recovered with negative PCR results for HSV. Based on the clinical presentation, serological and molecular tests, it was assumed that the patient had mixed infection with HSV type 1 and HFRS virus. To our knowledge, no other cases of such mixed infection were described in the literature. In a study by Shin et al. it was proven that hantaviruses are involved in the modulation of innate immune responses in the brain (11). Host immune responses affect the onset of HSV disease, the severity of infection and recurrence rates (2).

CONCLUSION

In the case of our patient, we can assume that as a result of immunosuppression caused by HFRS, HSV-1 reactivated and led to herpes encephalitis.

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EXITUS LETALIS OF A GIRL WITH HIV/ AIDS INFECTION AND HIV-ASSOCIATED CARDIOMYOPATHY (CLINICAL CASE REPORT)

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ABSTRACT

This report describes the clinical case of an 8-year-old girl with very late HIV diagnosis, AIDS-defining diseases and fatal outcome.

Material and methods

Patient examination was performed with clinical and epidemiological analysis, haematological, biochemical, microbiological tests and computed tomography (CT) scan. Virological and immunological monitoring of HIV was conducted by ELISA, western-blot, Real-time PCR and flow cytometry.

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Results

An 8-year-old girl with anaemia was hospitalised at the Clinic of Paediatrics, University Hospital "St. George" - Plovdiv. The child was with delayed neurophysiological development, wasting syndrome, severe oropharyngeal candidiasis, hepatomegaly and diarrhoeal syndrome. On 28 March 2018 she was diagnosed with HIV and hospitalised at the Clinic of Infectious Diseases in poor general condition, with fever and progressive cardiac failure. Blood tests showed anaemia, neutrophilia, elevated aminotransferase activity, hypoalbuminemia, viral load of more than 10,000,000 cop/mL and CD4⁺ T-lymphocyte count of 1/mm³. CT scan revealed congestive changes in the lungs, hepato-splenomegaly and para-aortic lymphadenopathy. Antiretroviral therapy was initiated with zidovudine, lamivudine and lopinavir/ritonavir. On 14 May 2018 the child was discharged with improved general condition and laboratory results. On 3 July 2018 she was hospitalised again with fever, sore throat and inability to swallow. On 4 July 2018 the grandmother took the girl and left the Clinic without leave. On 5 July 2018 she was admitted in a terminal condition. *Exitus letalis* was registered within hours.

Conclusions

The factors that led to this rapid, progressive course of HIV infection were the late diagnosis and the delayed antiretroviral treatment. The direct cause of the fatal outcome was HIV-associated cardiomyopathy. Regular screening for HIV in families of HIV-positive patients is required together with well-timed diagnosis and therapy.

KEYWORDS:

HIV, children, HIV-associated cardiomyopathy, exitus letalis

INTRODUCTION

According to UNAIDS, in 2017 1.8 million children up to 14 years of age live with HIV, 180 000 are newly infected and 110 000 have died (1). About 75-90% of paediatric HIV infections are transmitted peri- or intrapartum and 10-25% in utero and/or while breastfeeding (2). The unspecified HIV status of the mother and the lack or incomplete viral suppression are the most common causes of HIV infection in children (2). Without antiretroviral therapy, in 75-

90% of children HIV infection advances faster, AIDS-defining diseases develop with aggressive progression, often leading to fatal outcome (2). Precise cardiologic and pulmonary baseline examinations are important because individuals with advancing HIV disease experience worsening cardiac and lung complications (3). In HIV-positive patients, HIV-associated dilated cardiomyopathy is of major interest and is defined as reduced systolic function with dilated and less contractile left ventricle (4, 5). HIV-1 is known to cause cardiomyopathy (6). In addition to being a result of the direct impact of HIV, dilated cardiomyopathy is reported to be related with autoimmune mechanisms; cardiac-specific autoantibodies (anti- α -myosin antibodies) are described in up to 30% of HIV-positive patients with cardiomyopathy (2). Several studies indicate that dilated cardiomyopathy is associated with cardiotoxic agents (pentamidine, interleukin-2, doxorubicin) or caused by malnutrition (7). Furthermore, it is under discussion whether antiretroviral drugs may induce cardiac dysfunction due to mitochondrial toxicity (8, 9).

MATERIAL AND METHODS

This clinical case report presents an 8-year-old girl with late diagnosis of HIV infection, in the stage of advanced immune deficiency, with AIDS-defining illness and fatal outcome.

ELISA testing was repeated two times at the Laboratory of Microbiology, University Hospital "St. George" Plovdiv, to determine the presence of HIV infection. Afterwards it was verified with specific confirmatory western blot and PCR at the National Reference Confirmatory Laboratory of HIV, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria. Flow cytometry was used in immunological monitoring of the patient to determine CD4⁺ T cells at the time of initiation of antiretroviral therapy and to track their dynamics; the assays were carried out in the Department of Microbiology and Immunology and the Research Immunology Centre, Medical University Plovdiv.

Patient monitoring was performed also with: 1) laboratory tests; 2) microbiological examinations including a) culture of biological samples – blood, urine and faeces; b) serological tests for

anti-HAV IgM, HBsAg, anti-HCV, anti-cytomegalovirus (CMV) IgM and IgG, anti-Epstein-Barr virus IgM and IgG, anti-*Toxoplasma gondii* IgM and IgG; c) T-SPOT.TB; 3) radiography, abdominal sonography, echocardiography, computed tomography (CT) of the chest and abdomen, orthopantomography.

RESULTS AND DISCUSSION

I.R.B. was a seven-and-a-half-year-old girl, born from third, normal pregnancy by caesarean section, full term and with normal neonatal period. Family history: the father of the child was HIV-positive, the mother died 4 years ago from stomach cancer, the grandfather had epilepsy and liver cancer.

The child had not been tested for HIV. Since the age of 3, the girl had recurrent rash with severe pruritus, erosions and hyperpigmentations. For more than a year she experienced repeated abdominal pain, headache, vomiting and reduced appetite. For the last 3 months, the girl had pain in the leg, refused to play with other children and could not speak clearly.

From 10 February to 28 March 2018, she was hospitalised at the Clinic of Paediatrics and Genetic Diseases with diagnosis of anaemia. Her relatives reported that she had a high temperature with vomiting and pain in the epigastrium since 3 days before admission. Physical examination revealed impaired general condition, pale, dry skin with hyperpigmentation of the limbs, scars left after a healed skin infection, cervical lymphadenopathy, abdominal distension with visible subcutaneous veins on the abdominal wall, epigastric pain, hepatosplenomegaly, delayed neurophysiological development, vague speech and restricted movement of the two ankle joints.

In children, generalised melanoderma is more common in endocrine and systemic diseases, and is observed less frequently in patients with AIDS (10). Chronic generalised enlargement of the liver, spleen and lymph nodes in all age groups may be associated with chronic infections involving the reticuloendothelial system (RES), collagenosis, chronic allergies, chronic metabolic disturbances and malignant diseases of blood and RES (10). Furthermore, some of the

characteristic symptoms and signs of chronic HIV infection in children are severe rash, unexplained persistent hepato-splenomegaly and persistent generalised lymphadenopathy (2).

Haematological tests showed anaemia, neutrophilia, lymphopenia and elevated erythrocyte sedimentation rate (ESR) (Table 1). Anaemia manifests in the final stages of HIV/AIDS infection (3). There is also a gradual decrease in lymphocytes in the chronic and terminal stage of HIV/AIDS infection (3). Leukocytosis with neutrophilia is observed in patients with generalised bacterial infections (11). The platelet count of the child was within the reference range, regardless of the expected and common in AIDS thrombocytopenia (15). The highly elevated ESR is a very specific biomarker for AIDS, thus for HIV-induced chronic inflammation (10).

Biochemical tests showed elevated Cu^{2+} , ferrum, NH_4 and lactate dehydrogenase (LDH) (Table 2). In children, elevated LDH is detected in abnormal cell lysis (10). Elevated ammonia levels occur in severe liver diseases, metabolic disorders and gastrointestinal bleeding (10). Elevated Cu^{2+} excretion in urine is characteristic of Wilson-Konovalov disease (10).

Serological testing was negative for anti-HAV IgM, HBsAg, anti-HCV, anti-CMV Ig and anti-EBV Ig. No pathogenic bacteria were isolated from faeces, blood, and urine. *Giardia lamblia* was identified from the faecal sample. Radiological imaging revealed right hilar congestion, sonographic examination showed structural chang-

es characteristic of diffuse liver disease, and there was also echocardiographic mitral valve prolapse.

Complex therapy was initiated with administration of antibiotics (ceftriaxone, amikacin, metronidazole) and symptomatic treatment.

The child presented with persistent fever and diarrhoeal syndrome, abdominal pain, anaemia and remained non-communicative. She was treated for 12 days with metronidazole due to the positive faecal sample for *Giardia lamblia*. Cuprenil therapy was started as the elevated levels of Cu^{2+} in the urine suggested Wilson-Konovalov disease.

During the hospital stay serological testing showed positive result for HIV and for the period between 28 March and 14 May 2018 the child was transferred to the Clinic of Infectious Diseases. She was admitted with persistent complaints, general pathological changes, viral load (VL) of 10,000,000 cop/mL, and CD4^+ T-lymphocytes – $1/\text{mm}^3$. Antiretroviral treatment was started with zidovudine, lamivudine and lopinavir/ritonavir. The therapy included also trimethoprim/sulfamethoxazole, isoniazid, ciprofloxacin, fluconazole, nystatin-glycerin, hepatoprotectors and vitamins.

Haematological abnormalities continued to persist (anaemic syndrome, neutrophilia and lymphopenia). Biochemical findings showed hypoalbuminemia, elevated aspartate aminotransferase (ASAT) and gamma-glutamyltransferase (GGT) (Table 1 and Table 2).

Table 1. Haematological findings.

Date	HGB g/l	RBC T/l	HCT	MCV fl	WBC G/l	PLT G/l	ESR mm/h	Neu %	Ly %
10.02.2018	78	3.02	0.24	81	9.99	422	72	90.7	6.2
12.02.2018	123	4.5	0.38	79.5	5.68	383	36	83.5	8.5
19.02.2018	100	3.8	0.31	83.6	6.55	215	56	77	16.1
05.03.2018	94	3.7	0.27	76.1	7.64	392		89.1	7.0
19.03.2018	65	2.4	0.19	82.1	7.76	353		86.6	5.4
28.03.2018								73	6.0

Table 1 Haematological findings

1. Haemoglobin (HGB), g/l.
2. Red Blood Cells (RBC), T/L.
3. Hematocrit (HCT).
4. Mean Corpuscular Volume (MCV), fL.
5. White Blood Cells (WBC), G/L.
6. Platelets (PLT), G/L.
7. Erythrocyte sedimentation rate (ESR), mm/h.
8. Neutrophils (Neu), %.

9. Lymphocytes (Ly), %.

Table 1 Haematological findings

1. Haemoglobin (HGB), g/l.
2. Red Blood Cells (RBC), T/L.
3. Hematocrit (HCT).
4. Mean Corpuscular Volume (MCV), fL.
5. White Blood Cells (WBC), G/L.
6. Platelets (PLT), G/L.
7. Erythrocyte sedimentation rate (ESR), mm/h.

8. Neutrophils (Neu), %.

9. Lymphocytes (Ly), %.

Abbreviations: Haemoglobin (HGB), g/l.; Red Blood Cells (RBC), T/L; Hematocrit (HCT); Mean Corpuscular Volume (MCV), fL; White Blood Cells (WBC), G/L; Platelets (PLT), G/L; Erythrocyte sedimentation rate (ESR), mm/h; Neutrophils (Neu), %; Lymphocytes (Ly), %.

Table 2. Biochemical findings.

Date	CRP mg/l	AST U/l	GGT U/l	LDH U/l	ALB g/l	Ferrum μmol/l	NH ₄	Cu ²⁺ in urine μmol/l
10.02.2018	23	104	58	1151	35	1025	86	2.0
19.02.2018	25			909	31			
19.03.2018				1099				
28.03.2018				1356				

Abbreviations: C-reactive protein (CRP), mg/l; Aspartate Aminotransferase (AST), U/l; Gamma Glutamyltransferase (GGT), U/l; Lactate Dehydrogenase (LDH), U/l; Albumin (ALB), g/l; Iron (Ferrum), μmol/l; Ammonia (NH₄), μmol/L; Copper (Cu²⁺ in urine), μmol/l

Pathogenic bacteria were not detected from faeces and urine samples. Parasite examination was negative for toxoplasmosis and intestinal parasites. T-SPOT.TB was also negative.

Computed tomography scan showed stasis in the lungs (Fig. 1), hepato-splenomegaly and para-aortic lymphadenomegaly (Fig. 2). Orthopantomography showed no indications of bone lesions of dental origin.

During hospitalisation, the condition of the girl worsened with progressive heart failure – tachycardia, shortness of breath, oedema of the lower limbs. She was examined by cardiologist and diagnosed with cardiomyopathy, followed by initiation of appropriate therapy.

The echocardiography performed on 19 April 2018 demonstrated increased size and volume of the left ventricle. Spironolactone was added to the therapy. On 25 April 2018 left ventricular congestion was observed with deterioration of systolic function. Administration of Furantril, Captopril, Lanitop was also started. On 9 May 2018 control echocardiography showed persistent slight dilation of the two ventricles with mildly depressed global systolic function.

Therapy was maintained and control check-up was recommended after 2 months.

There was a gradual improvement in the general condition, fever control, correction of anaemia with substitution therapy, and amelioration of the cardiovascular disease. On 14 May 2018, at the time of discharge, the VL of the patient was 3936 cop/mL and CD4⁺ T-lymphocytes were found to be slightly increased – 45/mm³.

On 3 July 2018 the child was hospitalised for the second time at the Clinic of Infectious Diseases in critical condition, high temperature, sore throat, swallowing difficulties, cachexia, severe oropharyngeal candidiasis, hepato-



Figure 1. Computed tomography scan showing stasis in the lungs.



Figure 2. Computed tomography scan showing hepato-splenomegaly and para-aortic lymphadenomegaly.

splenomegaly, physical and neuropsychiatric impairment.

Blood tests found anaemic syndrome, thrombocytopenia, neutrophilia and lymphopenia. Biochemical examinations showed

hypoproteinemia, hypoalbuminemia, increased C-reactive protein (CRP), increased creatine kinase and creatine kinase-MB fraction. *Candida albicans* was isolated from faeces.

On 4 July 2018 the grandmother took the child and left the clinic without leave. On 5 July 2018 the girl was hospitalised again, with prostration. She could not eat, drink or take the prescribed medicines. Blood gas analysis showed severe metabolic acidosis with pH 7.15 and base excess of -11. Nasogastric tube and urethral catheter were inserted. The condition of the child progressively deteriorated and it passed away from heart and respiratory failure on 7 July 2018.

In conclusion:

Early diagnosis and timely antiretroviral therapy are crucial for the course and outcome of HIV infection. The cause of this severe HIV/AIDS infection was the late diagnosis and the delayed and interrupted treatment leading to a rapid, progressive course of the infection. The direct cause of the fatal outcome was HIV-associated cardiomyopathy. Regular screening for HIV in families of HIV-positive patients is required, together with well-timed diagnosis and therapy.

Competing interests

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in

the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

Declaration of authorship

Authors (PV, TV, LCh, IB, AD, IA, PG, MI, MM, MS) participated in the design, organisation, and implementation of the study. PG and MI performed the microbiological and immunological tests. IA performed western blot and HIV assays. PV, IA, MM and TV prepared the article. MS gave the idea to present the clinical case and guided the project. All authors (PV, TV, LCh, IB, AD, IA, PG, MI, MM, MS) contributed to the writing of the second and final draft. All authors approved the final draft.

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CHANGES OF THE GALL BLADDER WALL IN CHILDREN WITH *SALMONELLA* GASTROENTERITIS

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ABSTRACT

This study examined 102 children admitted to the hospital with acute gastroenteritis. *Salmonella* spp. was identified as the etiological agent in 38 of them. Abdominal ultrasonography monitoring changes of the gall bladder was performed in all patients. Thickening of the gall bladder wall was found in 6 children, 2 of whom developed acute acalculous cholecystitis. All children with thickening of the gall bladder wall in the course of acute *Salmonella* gastroenteritis were with prolonged hospital stay.

INTRODUCTION

It is well known that *Salmonella* spp. can persist in the bile ducts and the gall bladder during acute illness and after recovery. Some of the convalescent patients remain carriers for different periods of time. The walls of the bile ducts and the gall bladder are likely to be affected as a result of the pathological features of *Salmonella* spp. infection (1, 2). Acute acalculous cholecystitis (AAC) is defined as acute inflammation of the gall bladder which is not associated with the presence of gallstones. It is most often diagnosed by a combination of clinical symptoms and imaging findings, but still there are no uniform criteria for diagnosis in the

literature (3). AAC is a rare condition, approximately 5-15% of all cases of acute cholecystitis, and develops most often in patients with systemic infections, autoimmune disorders, hepatic and haematological diseases. Although rarely reported, there are cases of AAC in children in the course of *Salmonella* gastroenteritis (4).

MATERIAL AND METHODS

In the period April – September 2018, faecal samples of 102 children hospitalised with acute diarrhoea were examined for the presence of *Salmonella* spp.

Clinical samples were incubated at 37-38°C for 24 hours in a selective and differential medium –MacConkey and Apocholate Citrate Agar. Biochemical identification was determined with API 20E. Isolates positive for *Salmonella* serovar were identified by slide agglutination tests using O and H antigen-specific antisera. Serotyping of the isolates was carried out with anti-*Salmonella* sera, Bul Bio-NCIPD Ltd. (BG).

Abdominal ultrasonography was performed in all children with proven *Salmonella* gastroenteritis using Convex Array Ultrasound Transducer 3-7 MHz, Samsung Medison.

RESULTS

Culture results showed 38/102 (37.25%) positive samples for non-typhoid *Salmonella*. Of these, 11/38 isolates belonged to group B, 12/38 to group C, and 15/38 to group D. The children were between 1 and 12 years of age, 23 boys and 15 girls. Abdominal ultrasonography was performed in all children diagnosed with *Salmonella* gastroenteritis based on laboratory findings between day 3 and 4 of the hospital stay, immediately after the microbiological diagnosis. Thickening of the gall bladder wall with more than 4 mm was found in 6/38 (17.78%) children; in 2 of these children the increase in the gall bladder transverse diameter was more than 5 cm (Table 1). Two children were diagnosed with acute acalculous cholecystitis based on clinical findings and ultrasonographic examination. The mean hospital stay for children with *Salmonella* gastroenteritis was 5.8 days, whereas for the 6 children with thickened gall bladder wall (including those with AAC), the average hospital stay was 9 days.

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Table 1. Demographic data and clinical characteristics of children with thickening of the gall bladder wall.

Age (years), sex	<i>Salmonella</i> group	Gall bladder wall (mm)	Hospital stay (days)
4, m	B	8	13
9, m	C	6	15
6, f	C	6	6
11, m	C	5	5
3, f	D	7	8
5, m	B	8	7

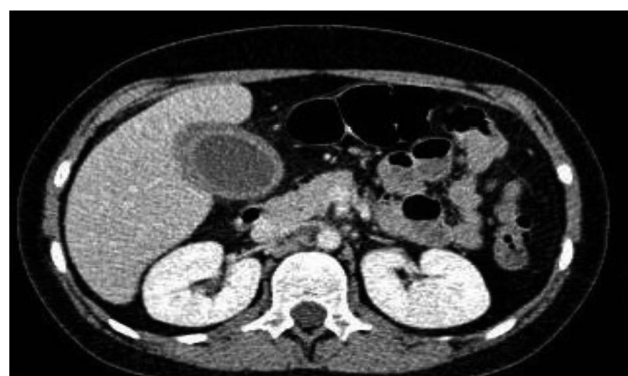
Case Report 1

A 4-year-old boy fell ill with watery diarrhoea, vomiting and fever. He was admitted to the hospital approximately 4 hours after the onset of complaints. The only finding of laboratory tests was leukocytosis with white blood cell count reaching the upper limit of $11 \times 10^9/L$. Intravenous rehydration was initiated by administration of glucose-electrolyte solutions. On day 2 of the hospital stay the child complained of diffuse abdominal pain, which was more severe upon palpation of the ileocecal region. Diarrhoea and fever persisted, vomiting became more frequent. Microbiological examination of faecal sample showed positive result for *Salmonella* group B at the 72nd hour of hospital stay. Abdominal ultrasonography revealed thickening of the gall bladder wall (8 mm) (Fig. 1).

Intravenous administration of amikacin and metronidazole was initiated, as there was evidence of acute acalculous cholecystitis. After 10 days of antibiotic treatment, the child was discharged without complaints.

**Fig. 1.** Ultrasonographic image of gall bladder wall thickening and perivesical oedema.**Case Report 2**

A 9-year-old boy having watery diarrhoea with admixture of blood, fever and vomiting received symptomatic treatment at home for 2 days. Upon hospital admission, the child was intoxicated, dehydrated and with active diarrhoea. The abdomen was soft but the child complained of diffuse pain. Laboratory test findings showed leukocytosis with white blood cell count $16 \times 10^9/L$ and elevated erythrocyte sedimentation rate of 65 mm. Intravenous rehydration was initiated by administration of glucose-electrolyte solutions. On the 3rd day, *Salmonella enteritidis* was isolated from faecal samples. Abdominal ultrasonography revealed enlarged gall bladder (transverse diameter of 6.5 cm) with thickening of the wall (6 mm). Vomiting and fever persisted, abdominal pain became more severe upon palpation of the right subcostal area (positive Murphy's sign). Computed tomography was also performed, showing enlarged gall bladder with thickened walls (Fig. 2). Intravenous administration of amikacin and metronidazole was initiated, as there was evidence of acute acalculous cholecystitis based on clinical findings and ultrasonographic examination. After 12 days of antibiotic treatment, the patient was discharged without complaints and with intact gall bladder.

**Figure 2.** Computed tomography of a gall bladder with thickened wall and minimal amount of perivesical fluid.

DISCUSSION

A number of authors describe ultrasonographic changes of the gall bladder wall, enlarged gall bladder as well as acute acalculous cholecystitis in patients with systemic infections. Although AAC and other gall bladder abnormalities are described mainly in typhoid fever, they are also reported in common *Salmonella* gastroenteritis. There are many reports on the potential of *Salmonella* spp. to survive in the bile juice and to persist in the gall bladder. Shetty described ultrasonographic changes of the gall bladder in a group of 62 patients with typhoid fever (4, 5). Both Shetty and Stuart used similar criteria to diagnose AAC – ultrasonographic (enlarged gall bladder, thickened wall, sonographic Murphy sign, perivesical fluid) and clinical findings (fever, abdominal pain, vomiting). In most cases, patients present with a combination of these symptoms but the occurrence of all symptoms is very rare. Shetty and Stuart assert that AAC can be diagnosed if most of the symptoms are present. Some authors support diagnostic criteria that are not very strict (6, 7). According to Ruiz-Rebollo, diagnosis can be based only on 2 ultrasonographic signs, without any clinical symptoms (8). However, in our opinion, the diagnosis of AAC should be based on combination of ultrasonographic and clinical evidence.

With regard to our patients with ultrasonographic changes of the gall bladder but no clinical signs of AAC, we believe that in *Salmonella* gastroenteritis cases the ability of the bacteria to survive in the gall bladder without causing symptoms is associated with the observed abnormalities. Probably visible ultrasonographic changes may also be found in disease carriers (9, 10).

CONCLUSION

AAC is a rare disease in children; most often it occurs in men over the age of 50. The exact pathogenetic mechanism remains unclear, but local ischemia, impaired mucosal resistance, etc. are also considered as predisposing factors besides infectious genesis. Eventually, the consequences can be severe, even fatal. Cases of gangrene and perforation of the gall bladder have been reported in the literature (5). The risk of these complications and the fact that in cases with *Salmonella* gastroenteritis AAC may develop several days after diarrhoea ceases, emphasise the need to perform abdominal ultrasonography in every patient with *Salmonella* infection and persisting abdominal pain, vomiting, and fever. The fact that the disease is more protracted, mainly because of the prolonged fever, is likely to indicate persistence of the infection in the gall bladder of children with thickening of the gall bladder wall and is probably associated with long-term carriage of the bacteria.

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SYSTEMIC MYCOSES - SEROLOGICAL AND MOLECULAR DIAGNOSTIC METHODS IN LABORATORY PRACTICE

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ABSTRACT

The aim of this review is to present the main laboratory methods for diagnosis of the majority of invasive fungal diseases – candidosis, aspergillosis and cryptococcosis. Some studies show an increasing range of infectious agents such as *Trichosporon* and the endemic fungal pathogens *Histoplasma*, *Blastomyces* and *Coccidioides*.

The most common methods for detection of antigens and antibodies in body fluids (serum, plasma, BAL) are indirect immunofluorescence, ELISA Platelia (enzyme-linked immunosorbent assay), latex agglutination, immunodiffusion and PCR-based molecular techniques.

KEYWORDS:

systemic fungal disease, *Candida*, *Aspergillus*, *Cryptococcus*, endemic mycoses

INTRODUCTION

Systemic fungal diseases are leading cause of mortality in immunocompromised individuals such as ICU, cancer and AIDS patients, transplant recipients, patients on chemotherapy, with catheters, heart prosthesis and others.

Routine microscopic and culture-based methods (blood culture) are basic but delay diagnosis with 48-72 hours (14, 16). For this reason, rapid and reliable screening methods are necessary for early detection of pathogenic fungi (10, 11).

The majority of invasive fungal diseases are

still caused by *Candida* (12) and *Aspergillus* species, but recent studies indicate an increasing incidence of species, such as *Cryptococcus*, *Trichosporon* (20). In some areas (USA) are reported endemic fungi – *Histoplasma*, *Blastomyces* and *Coccidioides* (25).

The method we use for determination of specific antibodies against *Candida*, *Aspergillus* and *Cryptococcus* is indirect immunofluorescence (IIF). In this assay a microscopic slide is prepared from culture suspension (10^6 - 10^7 CFU/ml) of *Candida* strain (CIP 628), *Aspergillus* (*A. fumigatus*) and *Cryptococcus neoformans*(fig.1). The slides are fixed and stored at -20°C (26). The principle of the method includes 2 steps. In the first step diluted serum sample (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1: 640 and 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 – for *Cryptococcus neoformans*) is placed on a slide for 30 minutes. In the second step after washing the slides, the attached antibodies are treated with fluorescein-labelled anti-human immunoglobulin antibodies and results are visualised on fluorescence microscope (2, 3, 9).

1:80 1:160 1:320 1:640

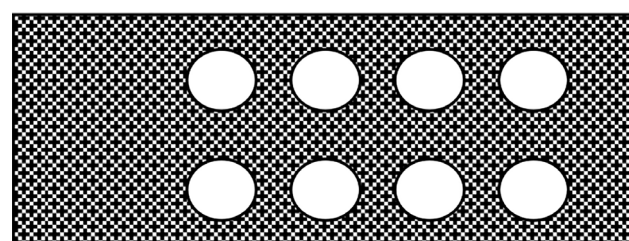


Fig.1. Indirect immunofluorescence (IIF) method for detections of antibodies in serum

Among the advantages of indirect immunofluorescence is the greater sensitivity than direct immunofluorescence.

In our laboratory we also have experience with serological antigen detection by ELISA (enzyme-linked immunosorbent assay).

ELISA Platelia (Bio-Rad) is an immunoenzymatic sandwich microplate assay for detection of circulating antigen, mannan in *Candida* or galactomannan in *Aspergillus*, in human serum and bronchoalveolar lavage fluid (BAL). Mannan and galactomannan antigens are polysaccharides non-covalently bound to the yeast cell wall

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and they appear to be the main biomarkers for diagnosis of invasive fungal disease. The assay uses rat monoclonal antibody directed against the *Candida* or *Aspergillus* antigen (4, 19).

Serum, plasma or BAL samples are heat-treated in the presence of EDTA in order to dissociate the immune complexes and to precipitate the serum proteins which could possibly interfere with the immunoassay reaction. The treated samples and the conjugate are added to the wells of the microplate coated with the monoclonal antibody. After incubation at 37°C the strips are washed. If there is circulating mannan or galactomannan antigen in the human samples, a complex is formed from anti-mannan monoclonal antibody – mannan or galactomannan antigen (Ag) – monoclonal antibody/peroxydase. Next, the chromogen solution containing the peroxidase substrate is added and incubated at room temperature allowing the detection of any complex bound to the microplate well. The enzymatic reaction is stopped by the addition of a stopping solution.

The optical density of human samples and calibrators is determined with a spectrophotometer set at 450/620 nm wavelength.

A negative test cannot rule out the diagnosis of invasive fungal diseases because of the very low concentration and the rapid elimination of the antigen during infection. For this reason, patients with high risk of invasive diseases should be tested twice a week. Regular monitoring increases the sensitivity and early positivity of the test.

Another serological test for detection of circulating antigens using a sample technique is the latex agglutination test (Bio-Rad). The test is qualitative and detects the polysaccharide antigen mannan (*Candida*) and glucuronoxylomannan (*Cryptococcus neoformans*) in biological fluids (serum, BAL, CSF). This procedure uses latex particles coated with monoclonal rat antibody directed against the fungal antigen. These particles react with the fungal antigen resulting in agglutination visible to the naked eye (13).

Limits of the test are associated, as for all immunological techniques, with the possibility of cross-reactions and should always be considered. For example, cross-reaction may

occur with antibodies to galactomannan of *Penicillium* in patients infected with *Penicillium marneffe*, or contaminated with spores from the environment (15).

Immunodiffusion (ID) is another method for detection of antibodies (fig.2). In our laboratory we have experience in determination of antibodies against *Aspergillus* and endemic fungal pathogens (*Histoplasma*, *Blastomyces* and *Coccidioides*). Phenol agar is poured on petri plates and immediately after the agar is set, the plates are placed in the refrigerator at 4°C. In the assay, the agar is cut to form wells with edges as smooth as possible, and the following reagents are added to the wells:

- Control serum (commercial kit)
- Antigen (commercial kit)
- Patient's serum

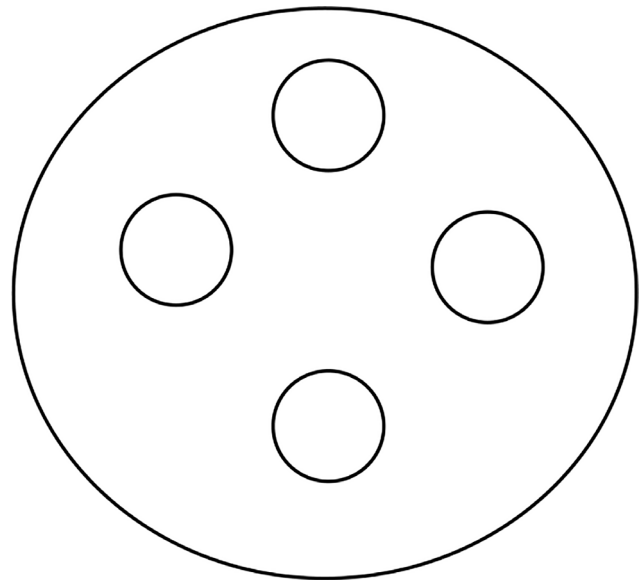


Fig.2 Immunodiffusion method (ID) for detection of antibodies.

The plates are left in a moist chamber at 25°C and reaction results are read after a few days. Precipitation lines are visualised if there is a formation of immune complex (26).

Histoplasmosis, coccidioidomycosis and blastomycosis are fatal infectious diseases in the USA. Pathological features involve invasion of blood vessels causing organ necrosis (cirrhosis, splenomegaly, affection of the lungs, anaemia) (23, 24).

PCR is a non-culture-based method for rapid diagnosis of infections by amplification of a specific DNA fragment with short, artificially

synthesised oligonucleotides (primers) that hybridise specifically with a particular target DNA sequence. The initial stage of denaturation is followed by hybridisation of primers and synthesis of a new DNA with the enzyme Taq polymerase, generating millions of specific DNA molecules (26).

Amplification techniques offer increased sensitivity over traditional staining and culture methods but may give positive results in asymptomatic individuals with colonisation (6, 8). Real-time PCR has the advantages of enhanced speed and sensitivity, and wider range of detectable organisms that cannot be cultivated. The different methods for laboratory diagnosis of invasive fungal infections are with different specificity and sensitivity.

Antibody titres to germ tube antigens of *Candida albicans* show high specificity and sensitivity. Many authors also confirm the method in the diagnosis of systemic mycoses as a tool to distinguish between systemic infection and colonisation with *C. albicans* which is very important for empiric therapy in patients (1, 11, 26).

Detection of galactomannan in serum is a feasible approach in adult neutropenic patients for the early diagnosis of invasive aspergillosis. ELISA Platelia (Bio-Rad) tests are more sensitive and more appropriate for the analysis of a great number of serum samples however latex agglutination tests are easier to perform in laboratory conditions (17). We must not forget also the high costs of antigen detection tests.

Real-time PCR is currently one of the fastest diagnostic methods for detection of fungal species that are the most frequent causative agents of systemic infections (21, 22).

The laboratory examination of invasive fungal diseases should be carried out with several diagnostic procedures such as culture methods, histological examination of biopsy samples and CT imaging, in order to aid the diagnosis of invasive fungal infections (18, 19).

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DETECTION OF ORAL MICROBIAL FLORA USING POLYMERASE CHAIN REACTION

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ABSTRACT

Periodontitis is an inflammatory disease of the oral cavity caused by anaerobic bacteria. In some cases it is related with other common chronic diseases, including cardiological and rheumatological diseases and therefore its diagnosis and therapy is very important. Some of the most common periodontal pathogens are *Porphyromonas gingivalis*, *Prevotella intermedia* and *Treponema denticola*. Classical microbiological methods for their cultivation and detection are cultural methods, which are time consuming, need expensive equipment and trained staff with specific skills. In this study we developed PCR-based technique using dead bacteria useful for diagnosis of these three species. In 83 patients we identified in 8 all of these 3 species, in 11 *P. gingivalis* and *P. intermedia*, in 7 *P. intermedia* and *T. denticola*, in 6 *P. gingivalis* and *T. denticola*, in 7 *P. gingivalis*, in 10 *P. intermedia*, in 4 *T. denticola*. DNA isolation for PCR was optimized without transport media for anaerobes in first time in this study and satisfactory results were observed. We developed a protocol for identification of these three pathogens which is much cheaper than the commercial available kits.

KEY WORDS:

Anaerobes, oral microflora, PCR

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INTRODUCTION

Oral microbiota is represented by many species of bacteria, fungi, archaea, and viruses. Some of them are part of the normal microflora and others are causative agents of infectious diseases of the teeth and gums. Anaerobic bacteria causing inflammations of the oral cavity don't have superoxide dismutase in their enzyme system and they are not able to survive in presence of oxygen. Culturing and manipulation of these pathogens requires anaerobic atmosphere containing high level of carbon dioxide and hydrogen and need expensive equipment. *Porphyromonas gingivalis* is one of the most common bacterium involved in the periodontal disease which is found also in the upper part of the gastrointestinal tract. This microorganism causes inflammation in the oral cavity based on its collagenase activity damaging the soft tissue. *P. gingivalis* sometimes is related with rheumatoid arthritis because its peptidyl-arginine deiminase activity is citrullizing the arginine contained in the proteins (1). *P. gingivalis* is a member of bacterial vaginosis microbiota in women (2). *P. intermedia* is related often with necrotizing ulcerative gingivitis and noma presented in higher concentration in pregnant women (3). *T. denticola* is related to the syphilis-causing obligate human pathogen, *Treponema pallidum* subsp. *pallidum* (4). All of these three species are involved in the vaginosis in women (2). We optimized in this investigation fast and easy method to detect *Porphyromonas gingivalis*, *Prevotella intermedia* and *Treponema denticola* in the oral cavity using tooth pins transported in sterile tubes without medium. This investigation is based on our experience from the work we have done earlier when protocols for identification of *P. gingivalis* from tooth pins provided by dentists and atheromatous plaques provided by cardio surgeons were optimized. All samples were delivered from the dental office or the operation room to the microbiological laboratory in Stuart transport medium (5,6). In the present study we used tooth pins delivered in sterile tubes without medium. The materials in present study were taken from patients with periodontal disease, provided by the Faculty of Dental Medicine of the Medical University – Sofia, Bulgaria.

MATERIALS AND METHODS

Subgingival bacterial plaque of 83 patients was obtained with the help of paper pins. Subsequently DNA from clinical materials was isolated. QIAamp® DNA Mini Kit (250) was used in the isolation of bacterial DNA. Manufacturer's protocol for DNA isolation from tissue was applied. The pins were incubated for 2h in 180 µl ATL tissue lysis buffer + 20 µl proteinase K included in the kit at 56 °C and vortexed each 20-30 min. Than 200µl of AL lysis buffer was added and samples were vortexed and incubated 10 min at 56 °C. 200 µl of 96% ethanol were added. The samples were vortexed, moved to QIAamp Mini Spin Column and centrifuged at 8000 rpm for 1 min. DNA bound to the columns was washed twice – first time with AW1 Wash buffer 1 at 8000 rpm for 1 min and second time with AW2 buffer 2 at 13000 rpm for 3 min. The washed DNA was eluted with 20 µl AE Elution buffer at 8000 rpm for 1 min. Primer pairs for PCR amplification were constructed targeting specific regions of 16S rRNA coding gene for investigated three species. Primer sequences were 5'-AGGCAGCTTGCCATACTGCG-3' and 5'-ACTGTTAGCAACTACCGATGT-3' for *P. gingivalis*; 5'-CAAAGATTCATCGGTGGA-3' and 5'-GCCGGTCCTTATTCGAAG-3' for *P. intermedia*; 5'-TAATACCGAATGTGCTCATTTACAT-3' and 5'-TCAAAGAAGCATTCCCTCCTTCTTCTTA-3' for *T. denticola*. One *Taq*® DNA Polymerase, New England BioLabs® Inc. is used in the polymerase chain reaction. Thermodynamic parameters of the amplifications were following: *P. gingivalis* 5 min 94°C and after that 30 cycles including three steps each: 94°C for 30 sec, 60°C for 45 seconds, 25 sec for 68°C and final extension of 5 min for 68°C. Conditions for *P. intermedia* were 5 min 94°C and after that 30 cycles including three steps each: 94°C for 45 seconds, 54°C for 45 sec for 68°C for 18 sec and final extension of 5 min for 68°C. Conditions for *T. denticola* were 5 min 94°C and after that 30 cycles three steps each: 94°C for 45 seconds, 61°C for 45 sec for 68°C for 19 sec and final extension of 5 min for 68°C. Reaction mix included 2.5 µl DNA, 0.125

µl NEB *Taq* polymerase [5 U/µl], 5µl [5x] reaction buffer, 0.5µl dNTP [50mM each], 0.2 µl primers [100 pmol/µl] and PCR grade water to final volume of 25µl. The amplicons were visualized on ethidium bromide stained 1.5% agarose gel electrophoresis on 100V for 40 min. The sizes of the amplicons were compared vs. 50 bp DNA ladder. Expected size of the amplicons was 404 bp for *P. gingivalis*, 296 bp for *P. intermedia*, and 318 bp for *T. denticola*.

RESULTS AND DISCUSSION

The amplicons were visualized on fig. 1

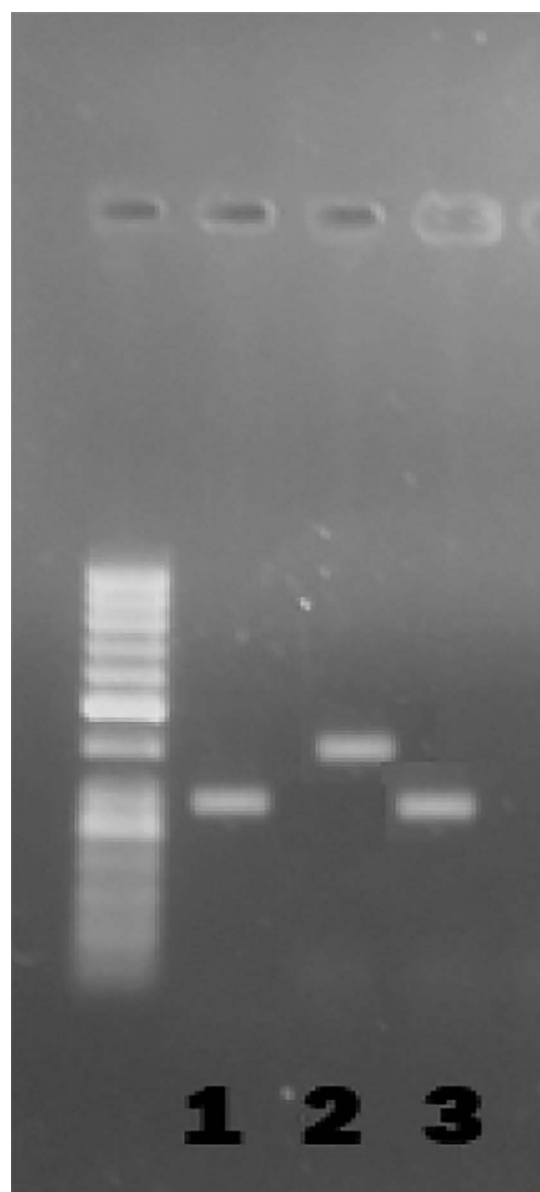


Fig. 1 On the line 1 is amplified *P. intermedia*, 2 – *P. gingivalis*, 3 – *T. denticola*, used marker 50 bp DNA ladder

The results are present in Table 1.

Tab.1.Number of patients by detected species.

Detected species	Number of Patients
<i>P. gingivalis</i> , <i>P. intermedia</i> , <i>T. denticola</i>	8
<i>P. gingivalis</i> , <i>P. intermedia</i>	11
<i>P. intermedia</i> , <i>T. denticola</i>	7
<i>P. gingivalis</i> , <i>T. denticola</i>	6
<i>P. gingivalis</i>	7
<i>P. intermedia</i>	10
<i>T. denticola</i>	4
No	30
Total	83

We developed successful protocol for detection of tooth pathogens not using transport medium or life bacteria. This protocol optimization allows the dentists to take from their patients and to provide for microbiological investigation samples without specific equipment and additional skills using tooth pins and sterile tubes only for first time in our laboratory. No additional equipment or training is needed for the collection and the delivery of the samples. The dentists do not need any help from an assistant. Commercial PCR test for detection of five oral anaerobic pathogens available – Micro-Ident® (Hain Lifescience GmbH) (<https://www.hain-lifescience.de/en/products/microbiology/dental-diagnostics/micro-ident-und-microdentplus.html>) is identifying *Tannerella forsythia*, *Agregattibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Treponema denticola*. Other kit of the same manufacturer - Micro-Ident® plus11 is identifying eleven pathogens - *Parvimonas micra*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Eubacterium nodatum*, *Eikenella corrodens*, *Capnocytophaga* spp. and the five listed above species. The protocol developed in this study is much cheaper

than available PCR based commercial kits for detection of such microorganisms. For detection of more pathogens further PCR optimization for the other species is required.

ACKNOWLEDGEMENT

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In Memoriam

Assoc. Prof. Irina Haydushka

Associate Professor Dr. Irina Atanassova Haydushka, PhD, age 73, born July 11, 1945 in the town of Pazardzhik, passed away Saturday, January 19, 2019, after a prolonged illness.

She studied at the English Language School in Plovdiv and then graduated medicine in 1973 at the Higher Medical Institute in Plovdiv. As a PhD student at the Department of Pediatric Diseases Dr. Haydushka defended a PhD thesis in 1979 on the immunological effects of the anti-measles vaccine in children. She had two medical specialties - Microbiology and Clinical Immunology. Since 1980 she was an assistant professor at the Department of Microbiology, Higher Medical Institute in Plovdiv, chief assistant - since 1984, and since 1990 she became an associate professor. She has been for many years head of the diagnostic Microbiological Laboratory and the Department of Microbiology and Immunology in the Medical University of Plovdiv (1993 – 2011).

The main directions in her scientific activities were: diagnosis of corineform bacteria, resistance of clinically significant bacteria to antibiotics, cellular immunity, immunomodulation in cancer patients. She published numerous scientific articles and was a co-author of a textbook on medical microbiology for students. A member of the Union of Scientists in Bulgaria, Deputy President of the Bulgarian Association of Microbiologists, member of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the American Society of Microbiology. Assoc. Prof. Haydushka was a respected clinical microbiologist, a strong and spiritual personality, confronting life's challenges with courage and boldness.

In Memoriam

Prof. Dimitar Strahilov

Prof. Dr. Dimitar Strahilov passed away on 24 November 2018. He was Director of the National Centre of Infectious and Parasitic Diseases (NCIPD) for eight years and Deputy Director for another eight years. Prof. Strahilov was head of the Centre at the time when NCIPD produced 85% of the vaccines for Bulgaria, 15% of the vaccines for the Federal Republic of Germany, 90% of the reagents for microbiological laboratories in the country and 75% of the blood products. He pioneered the introduction of immunofluorescence and monoclonal antibodies in microbiology diagnosis in the country as well as rapid immunological assays. We bow to the bright memory of Prof. Strahilov!

PROF. MORITZ ALBERT YOMTOV – a scientist, a teacher and a writer 5 November 1921 – 15 June 1992

In the beginning of the 1970s two medical students at the Medical University of Sofia started working in the field of clinical immunology under the guidance of Dr. Mihail Levov Ognyanov (later professor) in the Laboratory of the First Clinic of Internal Medicine at the University Alexandrovska Hospital. These two students were Dr. Krasimir Nikolov (later associated professor) and Dr. Marta Baleva (later professor). The two young scientists graduated in 1973 and started working in the same laboratory of Alexandrovska Hospital. In the next two years, under the guidance of Prof. Ognyanov, they specialised in immunology at the Research Institute of Epidemiology and Microbiology, RIEM, now - National Centre of Infectious and Parasitic Diseases (NCIPD) in Sofia.

A memory of Moritz Yomtov

Here we are, standing, in front of the marble stairs of RIEM. We are headed towards the study of Prof. Moritz Yomtov. We do not know him, actually we know nothing about him. Before we open the door of the Biochemical laboratory we could smell fine tobacco. A smiling and chubby middle-aged man with a smoking pipe greets us. This is prof. Moritz Yomtov. Our immunology specialisation at the RIEM begins. With short breaks, the duration of this specialisation will be two years. At that time, very few scientists worked in the field of immunology, mainly in ISUL (now, Queen Jovanna Hospital), Pirogov Hospital – in the field of organ transplantation, in RIEM – in the field of vaccines and vaccine prophylaxis, in several Institutes at the Bulgarian Academy of Sciences – Biochemistry, Microbiology, Immunology of Reproduction, and in some Departments of Sofia University.

The atmosphere in prof. Yomtov's lab is friendly and detached. All staff are meticulous and competent in their work, despite the remarkable workload, and show us many different techniques. Gradually we start working unaided. Our first task is to purify alpha-1-antitrypsin from human serum. We fill up columns, measure extinctions, and finally purify some substance that we examine electrophoretically ... YES! There is alpha-1-antitrypsin in this product, but still many impurities are present. Prof. Yomtov smiles and says: "Right! Now, pour this down the drain and start all over again!" After several attempts and after many advices, the pure product is extracted and we go on with our next tasks – we have to learn the immune diffusion methods of Ouchterlony and Mancini, immunoelectrophoresis, complement binding reaction, passive hemagglutination, we try to mark antisera with peroxidase and to reproduce immunoenzyme techniques ... We read a lot in the huge

Library at the RIEM and everywhere we could sense the care of prof. Yomtov for our problems. Gradually we understand that he is not only a famous immunochemist, but a screenplay writer and a novelist, one of the Mormarevi brothers. We learn a lot about his life.

Prof. Moritz Yomtov was born on 5 November 1921 in Sofia. He finished his primary school in the city of Burgas, and his high school – at the American College in Sofia in 1940 and started his university studies at the Chemical Faculty of Sofia University St. Kliment Ohridski. He graduated from Sofia University in 1949. He worked in the Editorial office for Broadcasting abroad at the Bulgarian National Radio. As an exceptionally erudite scientist, he was elected Professor of Biochemistry and a long-term member of the management staff of the Federation of European Biochemical Societies (FEBS) and Secretary of the latter for two consecutive mandates. From July 1948 until March 1949 he worked as press attaché at the Bulgarian legacy in Washington.

For 36 years, from 1951 until his retirement in 1987 he created and directed the Biochemical laboratory at RIEM. Under his direction, this laboratory became methodical and educational centre for qualification of RIEM staff and physicians, biologists, chemists and biochemists from Bulgaria. In 1968 he was elected Senior Research Associate I degree, and in 1971 – Professor of Immunochemistry at the Department of Biochemistry at the Biological Faculty of Sofia University. He specialised biochemistry of microorganisms and immunology at the Pasteur University in Paris (at the Laboratory of Prof. Grabar) and at the Gamaleya Institute in Moscow, Russia. His researches were in the field of the immunochemical characteristics of antigens and their purification, immunochemistry of enzymes, etc. Prof. Yomtov was the first to introduce in Bulgaria agar-agar immunodiffusion and immunoelectrophoresis. He participated in the introduction of methods for purification of bacterial antigens, methods for characterisation of toxins and sera production in Bulgaria. With the help of Prof. Yomtov, Prof. D. Strahilov and Prof. Hr. Taskov, was started the first monoclonal antibody production in Bulgaria. All activities of Prof. Yomtov are original, very precise and important. He was a long-time member of the Specialised Scientific Council of Microbiology, Virology, and Immunology at the Higher Attestation Committee in Bulgaria. He has more than 100 scientific publications and is a co-author of the monographs “Experimental microbiology” and “Dysproteinemias”. Prof. Yomtov participated in many scientific meetings, congresses and symposia in Bulgaria and abroad and has multiple citations. He was fluent in English, French, German and Russian language.

In his speech delivered at Sofia University for receiving the award *Doctor honoris causa* Acad. E. Golovinski added Prof. Yomtov to the long list of chemist-writers, along with Theodor Fontane, Robert Neumann, Erwin Chargaff, Isaac Asimov, Agatha Christie, Elias Canetti, Carl Djerassi, Asen Zlatarov, Georgi Danailov, Emil Zidarov, Georgi Markov.

IN MEMORIAM

The films of Moritz and Marko (the Mormarevi brothers) are unforgettable: “The golden coin”, “Two dioptries of long-sightedness”, “The hedgehogs are born without prickles”, “At the seaside with children”, “Exams out of time”, “The doppelganger”, and many others. Unforgettable are their novels: “Vasco Dagama of the Rupcha village”, “Raponcho”, “A task with many unknowns”, “The war of the hedgehogs”, “The diary of Asen Glazurkov”, etc. The Mormarevi brothers’ motto was “Life is funny and that is why it is worth living!”.

In the next decades we remained in touch with Prof. Yomtov. He was always there to help us and to give us advice. He was not afraid to take a stand in public, to say what he thought, despite the fact that his position was often outside the mainstream lines of the time. We can still remember his golden words “There are litres but there are no titres, there are titres but there are no litres.” in relation to the shortage of antisera in the end of the 1950s and the beginning of the 1960s, that was later surmounted.

Prof. Yomtov was easily accessible, amicable and loved by everyone, the soul of each company, with a vast culture and beautiful sense of humour, a scientist, a writer and a friend. Now that we have worked for more than 45 years as physicians and clinical immunologists, we would like to express once again our gratitude to Prof. Moritz Yomtov and to remind the Bulgarian public of this amazing man who left us on 15 June 1992.

Marta Baleva and Krasimir Nikolov

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