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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 47, NUMBER 1/2019

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STREPTOCOCCUS PNEUMONIAE SEROTYPE DISTRIBUTION AFTER THE INTRODUCTION OF PNEUMOCOCCAL CONJUGATE VACCINES – REVIEW

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ABSTRACT

Streptococcus pneumoniae serotypes are changing due to the widely introduced pneumococcal conjugate vaccines. Surveillance studies have proven valuable in monitoring these vaccine effects. *S. pneumoniae* is highly adaptable to its human reservoir and colonises mucosal surfaces of upper airways mainly in children. Carriage decreases during the first 2 years of life because of the development of naturally acquired adaptive immune memory. Most of the serotypes do not cause serious illnesses but few of them are responsible for severe pneumococcal infections. Ten of the most common serotypes are estimated to cause over 60% of invasive diseases worldwide. The virulence factor of *S. pneumoniae* is the polysaccharide capsule as non-encapsulated strains are absent among the strains causing invasive pneumococcal disease. Prevalence of serotypes differs depending on the age group and geographic area of patients. Differences in PCV implementation lead to changes in serotype distribution and to significant reduction of disease caused by vaccine types.

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KEYWORDS:

Streptococcus pneumoniae, pneumococcal serotypes, PCVs

ABBREVIATIONS

AOM – Acute otitis media
CSF – Cerebrospinal fluid
Hib – *Haemophilus influenzae* type b
IgG – Immunoglobulin G
IPD – Invasive pneumococcal disease
NVT – Non-vaccine serotype
NP – Nasopharynx
PCV – Pneumococcal conjugate vaccine
PCV7 – 7-valent pneumococcal conjugate vaccine
PCV10 – 10-valent pneumococcal conjugate vaccine
PCV13 – 13-valent pneumococcal conjugate vaccine
Ply – Pneumolysin
RCT – Randomised controlled trial
U5 – Under five (years of age)
UNICEF – United Nations International Children's Emergency Fund
URT – Upper respiratory tract
VT – Vaccine serotype
WHO – World Health Organisation

INTRODUCTION

Streptococcus pneumoniae is a Gram-positive diplococcus and opportunistic pathogen colonising the human nasopharynx. Pneumococcal polysaccharide capsule and its chemical structure define the antigenicity and virulence of the strains. The host produces specific antibodies depending on the capsule type (1). This antibody-related immunity defines 98 different serotypes of *S. pneumoniae* by 2018 (2). Pneumococci are naturally competent and colonisation provides an opportunity for genetic recombination between serotypes or with other closely related species. This remarkable capacity of *S. pneumoniae* to remodel its genome may lead to antibiotic resistance or capsular switching (2, 3).

From 27% to 65% of children and more than 10% of adults are pneumococcal carriers. Normally the host`s innate and adaptive immune responses protect from invasion and retain the bacterium in a commensal state (3, 4). After colonisation pneumococci could be shed in the environment at levels allowing them to transmit

and colonise other hosts, where they may cause invasive pneumococcal disease (IPD) (3). The World Health Organisation (WHO) included *S. pneumoniae* as one of the 12 priority pathogens because of the remaining high burden of disease and the rising rates of antimicrobial resistance in pneumococci. The most common form of serious disease is pneumonia with highest mortality rates among children under the age of 5 (U5) worldwide (3, 4).

Development of pneumococcal vaccines started in the early 20th century but the first PCV was licenced in the year 2000 (1). This was PCV7 and it included purified capsular polysaccharides of 7 serotypes of *S. Pneumoniae* (4, 9V, 14, 19F, 23F, 18C, and 6B) conjugated to a non-toxic variant of diphtheria toxin (4). To date 10- and 13-valent formulations of PCV are used worldwide; the capsular polysaccharides are conjugated to a carrier protein, either protein D of *Haemophilus influenzae* type b (HiB), tetanus toxoid or diphtheria toxoid. In PCV10 protein D is used as the carrier protein for 8 serotypes (1, 4, 5, 6B, 7F, 9V, 14 and 23F), whereas 19F is conjugated to diphtheria toxoid and serotype 18C – to tetanus toxoid. PCV13 provides coverage also of serotypes 3, 6A and 19A. Additional products are expected to be licensed in the coming years (1, 4). PCVs effectively prevent the most serious forms of pneumococcal disease caused by the serotypes included in the vaccine and also reduce the risk of nasopharyngeal (NP) carriage of those serotypes (5). In Bulgaria PCV10 was introduced in the National Immunisation Calendar in 2010 with over 90% coverage in the period 2011-2017 according to data provided by WHO and UNICEF estimates of national immunisation (6).

Transmission, colonisation and carriage of *S. pneumoniae*

Until recently, all that was known about pneumococcal transmission was that it required close contact with one or more carriers, most probably young children. Colonisation in children was found to be frequent in fall and winter months, when viral infections of URL are common and nose secretions are abundant. (3). Animal models were examined and described – the ferret model and the infant murine model, both co-infected with influenza A virus (IAV) which increased transmission. “Shedding” from the host or leaving the human body, the survival of bacteria in the environment and the realisation of transfer between hosts, has been studied in detail with the infant mouse model. These studies have proven the need for large numbers of shed pneumococci so that at least one to be successful in reaching a new host (7). Another study with the same model, points out the necessity of the pneumolysin (Ply), the single toxin produced from *S. pneumonie*, as the key inflammatory and shedding agent in the colonized NP. This contributes to the transmission and the disease state by promoting the survival of bacteria in the environment and in the host at the same time (8). Studies of the *cps* promoter on the other hand showed that encapsulation may contribute to transmission of certain serotypes as capsules were more effective in facilitating shedding (7).

Transmission through secretions of carriers could involve direct person-to-person contact or spread involving bacteria on contaminated surfaces. *S. pneumoniae* is shown to survive on surfaces touched frequently by children carriers. The period of survival outside the human in saliva can reach a couple of days and bacterial expression of Ply increases survival in airway surface fluid (3). Ply-induced toxin-dependent inflammation and consequently, increased nutrients in secretions help bacteria survive outside the host (8). When the strain has a capsule it was shown it survives longer, since the capsule serves for nutrition through the carbohydrate reserve in it (7). Furthermore, pneumococci could survive drying up for many days, and biofilm bacteria retain viability *in vitro* better than planktonic bacteria (3).

According to data shown in murine models and experimental human carriage, colonisation increased antibody levels which had immunising effects and protected against subsequent disease (7). Colonisation increased nasal, lung and serum antibody levels and protected against reacquisition of the same strain up to 1 year. These protective events were found to be specific to the serotype and strain of *S. pneumoniae* that the volunteer was carrying and if different serotype was introduced there was no increased protection (9).

Correlation between NP carriage of pneumococci and disease was found mainly in studies with children U5 and as disease consequence – AOM manifestation being the most common. Although long periods of carriage were common with this

age group the acquisition of a new serotype was related to disease development (5). Several bacterial factors were required for colonisation and persistence in order for *S. pneumoniae* to effectively transfer to another host. Adherence was found to be compromised by the mucus, antimicrobial peptides, immunoglobulins of the NP and after overcoming these obstacles pneumococci might access and attach to the surface of epithelial cells (3). *S. pneumoniae* serotypes interact with the NP flora and this relationship is likely extensive and complex. Pneumococcal carriage was also related to the colonisation with other microbial species, usually in children during the first 2 years of life when the microbiome changes intensively (10). Dynamics in the relationships between the different serotypes colonising the same host were studied, and competitive pneumocins were found to be type–specific for pneumococci (3). Serotype distributions varied by syndrome, disease severity and carriage prevalence. There were factors found contributing certain serotype's colonisation – as the age of the host; also interactions with many children carriers daily, as in the family or in kindergartens (11).

Pneumococcal disease burden

Pneumococci could spread from the NP by contiguous extension in the respiratory tract to cause infection in the middle ear (otitis media), sinusitis or non-bacteremic pneumonia. *S.pneumoniae* could also invade the bloodstream and spread to other sites in the host causing secondary, more distal infection (Table 1.) (1, 4). IPD is defined as "isolation of pneumococcus from a normally sterile body site" (such as blood, CSF or pleural fluid) and can range clinically from bacteremia with fever but no focus of infection (the so-called occult bacteremia) to life-threatening infection. According to these definitions, Table 1 shows the different diseases caused by pneumococci classified as invasive or non-invasive (1). There are serotype-specific differences in the frequency and site of disease following NP colonisation. Some serotypes are more commonly found causing bacteremic pneumonia and others are more commonly found causing meningitis, for example (11). Recent studies show a link between pneumococcal bacterial load and invasiveness of a specific serotype compared to other carried serotypes. The higher NP concentration of a certain serotype compared to the others colonising the host suggests higher probability for the numerous serotypes causing IPD. This quantitative assay may potentially predict invasiveness and distribution of some of the S. pneumoniae serotypes according to their numbers in the nasopharynx (12).

Table 1. Invasive and non-invasive pneumococcal diseases.

Invasive pneumococcal diseases	Non-invasive pneumococcal diseases
• Bacteremia without a clinical focus of infection • Bacteremic pneumonia • Cellulitis with bacteremia • Endocarditis • Pericarditis • Septic arthritis • Osteomyelitis • Peritonitis • Epiglottitis	• Acute otitis media • Non-bacteremic pneumonia • Bronchitis • Sinusitis • Conjunctivitis • Mastoiditis • Periorbital cellulitis

Pneumococcal mortality is a significant contributor to the U5 mortality rate worldwide. *Streptococcus pneumoniae* is the most common cause of bacterial pneumonia in children and accounted for 16% of all deaths of children U5 according to WHO 2015 report. Invasive disease accrues in younger children more commonly in developing countries – through the first year of their life, compared to developed countries were there were less common and at later age (1, 4). The leading IPD was meningitis, which leaded to severe consequences like neurological sequelae, hearing loss and mental retardation. Case fatality rates from IPD in children can be high, ranging up to 50% according to the disease type and the country of occurrence (11).

Effects of PCVs on invasive disease and carriage

Despite the high coverage of PCV's in Europe and the United States and the global vaccine funding for low income countries, *S. pneumoniae* remains the leading cause of childhood mortality worldwide (1, 11). Before the introduction of PCV's, studies have shown that around ten serotypes caused the majority of IPD in children. PCV7 included serotypes that were responsible for meningitis and were nonsusceptible to penicillin. In the United States, following PCV7,

only 5% of the clinical isolates were of serotypes included in the vaccine (4, 6B, 9V, 14, 18C, 19F, and 23F). Prevalence of NVT had then increased with 19A becoming a leading cause of IPD with a high percentage of MDR strains. The PCV13 with added coverage of 19A and other serotypes (1, 3, 5, 6A and 7F) was approved and by 2012-2013 and had the desired effect, with serotype 19A strains falling to 10%. The new PCV13 covered 19A serotype but not long after that the niche was replaced with 35B also not susceptible to penicillin (13).

Globally serotype 14 was known to cause IPD most frequently and 6B was the next common IPD serotype. In Africa there were differences in distribution – there was serotype 1 as most prevalent, together with serotype 5 on second place (11).

In Bulgaria a study during a period after the introduction of PCV10 demonstrates distribution of NVT 3 and 19A causing meningitis in vaccinated people. Other serotypes/serogroups in the study causing IPD are 8, 9N/L, 10A/D, 10B, 11A/D, 15B/C, 15A/F, 24 A/B/F that are not included in the PCV10 nor PCV13 (14). According to another study from Bulgaria, in Plovdiv region, serotypes 3 and 19A causing meningitis in the period 2013-2017 were confirmed as most abundant (15).

The major difference between the two PCVs used was the additional inclusion of serotypes 3, 6A and 19A. There were not any cross-reactive serotypes in PCV10 against serotype 3, but serotype 6A was assessed to have cross-reactive immunogenicity with serotype 6B and no effect with 6C. Serotype 19A was estimated to have cross-reactivity with 19F and was supposed to be covered by both the ten and thirteen – valent conjugate vaccines (11).

Most notably PCV7 had effects on AOM in vaccinated children. However, these effects were limited to primary infections of the middle ear, not the chronically accruing otitis media high risk group children (4). The vaccination with PCV protects against acquisition of VT in children but does not contribute to clearance of carriage. (1, 6). Random control trials report about 50% decrease in VT carriage and increase of NVT colonization and serotype replacement is observed in all countries implementing the vaccines (1, 11).

In conclusion, many factors are being responsible for the effects of PCVs on disease burden and related pneumococcal serotype carriage. Serotypes causing IPD changed throughout the years after PCV implementation and NVT are now responsible for invasive disease. However, the revue data showed severe reduction of VT carriage and VT pneumococcal diseases worldwide and definitely confirmed the positive outcomes of the IPD implementation (11, 14, 15).

REFERENCES

1. The Evidence Base for Pneumococcal Conjugate Vaccines (PCVs): Data for decision-making around PCV use in childhood. Johns Hopkins Bloomberg School of Public Health, January 2017. Available from: https://www.jhsph.edu/ivac/wp-content/uploads/2018/05/PCVEvidenceBase-Jan2017.pdf
2. Ndlangisa K, du Plessis M, Allam M, Wolter N, de Gouveia L, Klugman KP, Cohen C, Gladstone RA, von Gottberg A. *Invasive disease caused simultaneously by dual serotypes of Streptococcus pneumoniae.* J Clin Microbiol. 2018; 56:e01149-17.
3. Weiser J, Ferreira D, Paton J. *Streptococcus pneumoniae: transmission, colonization and invasion.* Nat Rev Microbiol. 2018; 16:355–367.
4. CDC - Immunology and Vaccine-Preventable Diseases – Pink Book – *Pneumococcal Disease.* Available from: https://www.cdc.gov/vaccines/pubs/pinkbook/pneumo.html
5. Simell B, Auranen K, Käyhty H, Goldblatt D, Dagan R, L O'Brien K. *The fundamental link between pneumococcal carriage and disease.* Expert Rev Vaccines. 2012; 11(7):841-855.
6. WHO vaccine-preventable diseases: monitoring system. 2018 global summary. Last update:15-Jul-2-18Availablefrom: http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tswucoveragedtp3.html
7. Ortigoza MB, Blaser SB, Zafar MA, Hammond AJ, Weiser JN. *An Infant Mouse Model of Influenza Virus Transmission Demonstrates the Role of Virus-Specific Shedding, Humoral Immunity, and Sialidase Expression by Colonizing Streptococcus pneumoniae.* mBio. 2018; 9(6):e02359-18.
8. Zafar A, Wang Y, Hamaguchi S, Weiser JN. *Host-to-Host Transmission of Streptococcus pneumoniae Is Driven by Its Inflammatory Toxin, Pneumolysin*. Cell Host Microbe. 2017; 21(1):73-83.
9. Pennington SH, Pojar S, Mitsi E, Gritzfeld JF, Nikolaou E, Solórzano C et al. *Memory B Cells Predict Protection against Experimental Human Pneumococcal Carriage.* Am J Respir Crit Care Med. 2016; 194(12):1523-1531.
10. Biesbroek G, Tsivtsivadze E, Sanders E, et al. *Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children*. Am J Respir Crit Care Med. 2014; 190(11):1283-1292.
11. World Health Organization - *Pneumococcal conjugate vaccines in infants and children under 5 years of age: WHO position paper*. February 2019. Weekly EpidemiologicalRecord Available from: http://www.who.int/iris/handle/10665/310970
12. Messaoudi M, Milenkov M, Albrich WC, van der Linden MP, Benet T, Chou M, et al. *The relevance of a novel quantitative assay to detect up to 40 major Streptococcus pneumoniae serotypes directly in clinical nasopharyngeal and blood specimens.* PLoS One. 2016; 11(3):e0151428.
13. Richter SS, Musher DM. *The ongoing genetic adaptation of Streptococcus pneumoniae*. J Clin Microbiol. 2017; 55:681-685.
14. Simeonovski I, Levterova V, Malcheva M, Kantardjiev T. *Genetic survey of invasive S. pneumoniae serotypes in Bulgaria for a 5-year period.* Probl Infect Parasit Dis. 2018; 46(2):5-9.
15. Kalchev Y, Kirina V, Mircheva M, Tsolova M, Setchanova L, Levterova V, Simeonovski I, Stoycheva M, Kantardjiev T, Murdjeva M. *Etiology and epidemiology of non-viral meningitis in the Plovdiv region*. General Medicine. 2018; 20(2):9-15.

AETIOLOGICAL STRUCTURE OF ACUTE RESPIRATORY TRACT INFECTIONS AMONG CHILDREN YOUNGER THAN 5 YEARS IN BULGARIA

I. Trifonova, S. Angelova, N. Korsun

National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria

ABSTRACT

Acute respiratory tract infections (ARI) are a leading cause of morbidity and hospital admissions among infants and young children. This study aims to determine the viral aetiology of ARI and the clinical significance of the most common respiratory viruses in children aged <5 years in Bulgaria.

During the period October 2017-March 2019, nasopharyngeal specimens were collected from children younger than 5 years in different country regions. Real-time PCR analysis was performed for detection of influenza viruses A/B, respiratory-syncytial virus (RSV), human metapneumovirus (HMPV), parainfluenza viruses (PIV) types 1, 2 and 3, rhinoviruses (RV), adenoviruses (AdV) and bocaviruses (BoV).

Of the 953 children examined, 663 (69.6%) were positive for at least one virus. The number of detected A(H1N1)pdm09, A(H3N2), B/Yamagata, B/Victoria, RSV, HMPV, PIV-1, PIV-2, PIV-3, RV, AdV and HBoV viruses was as follows: 150 (15.7%), 51 (5.6%), 50 (5.2%), 1 (0.1%), 193 (20.3%), 38 (4%), 15 (1.6%), 5 (0.5%), 17 (1.8%), 101 (10.6%), 60 (6.3%), 77 (8.1%), respectively. Co-infections with two and three viruses were found in 95 (14.3%) of the infected children. AdV, RV, BoV and PIV-3 were the most common pathogens in co-infections. Respiratory viruses were detected in 80%, 79.3%, 61.2% and 53.1% of children with laryngotracheitis, bronchiolitis, pneumonia and central nervous system (CNS) complications.

The results show that RSV, influenza viruses, RV and BoV were the most frequently detected viruses in children <5 years with ARI during the study period. These viruses were also leading causative agents of serious illnesses of the respiratory tract and CNS.

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KEYWORDS:
viral infection, respiratory virus, bronchiolitis, pneumonia

INTRODUCTION

Acute respiratory infections (ARI) are associated with a large number of doctor visits, hospital admissions, significant mortality, serious health and social consequences. With respect to the anatomical localisation, ARIs are classified as upper and lower respiratory tract infections. Upper respiratory tract involvement usually occurs as a mild or moderate illness. The high incidence of these infections is associated with prolonged circulation of pathogens in the community. Lower respiratory tract involvement leads to severe complications as bronchitis, bronchiolitis, pneumonia, often requiring hospitalisation. In 2010, 11.9 million episodes of severe and 3 million episodes of very severe ARI have led to the admission of infants and young children to hospital worldwide (1). Bronchiolitis occurs in children <2 years of age and most often has viral aetiology, whereas pneumonia can be caused by bacteria, viruses or other pathogens. Pneumonia is the leading infectious cause of mortality in children <5 years of age (2).

A large spectrum of viruses is associated with ARI and the major causative pathogens are influenza viruses, respiratory syncytial virus (RSV), human metapneumovirus (HMPV), parainfluenza viruses (PIV) 1/2/3, rhinoviruses (RV), adenoviruses (AdV) and bocaviruses (BoV).

This study aims to determine the role of influenza viruses and 8 other respiratory viruses in the development of ARI in children younger than 5 years of age during two consecutive seasons in Bulgaria.

MATERIAL AND METHODS

A total of 953 children aged <5 years, ambulatory-

treated or hospitalised for influenza-like illness (ILI) or acute respiratory illness (ARI) in different regions of the country were examined in the study. Of them, 444 (46%) had complications – laryngotracheitis, bronchiolitis, pneumonia or central nervous system (CNS) infections (febrile seizures, brain oedema, meningitis, encephalopathy, encephalitis).

Viral nucleic acids were automatically extracted from respiratory specimens using a commercial ExiPrep Dx Viral DNA/RNA Kit (Bioneer) according to the manufacturer's instructions. Laboratory testing was conducted at the National Laboratory "Influenza and Acute Respiratory Diseases" recognised by WHO as a National Influenza Centre. Detection and typing/subtyping of influenza viruses was carried out by real-time RT-PCR method with SuperScript III Platinum® One-Step Quantitative RT-PCR System (Invitrogen). All samples were initially tested for influenza A and B viruses using primers and probes donated by CDC Atlanta and those positive for influenza A and B were subsequently tested for A(H1N1)pdm09 and A(H3N2), B/Yamagata and B/Victoria, respectively. All samples were also examined by singleplex real-time RT-PCR assays for RSV, HMPV, PIV 1/2/3, RV, AdV and BoV using specific primers/probes and AgPath-ID One Step RT-PCR Kit (Applied Biosystems). Primers and probes were identical to those previously described (3).

RESULTS

Respiratory virus detection

The present study included two consecutive epidemic seasons in Bulgaria – 2017/2018 and 2018/2019. The study population consisted of 953 children aged < 5 years presenting with ILI or ARI: 459 in the first and 451 in the second season, respectively and 43 outside the season in 2018. Most of the patients – 831 (87.2%) were hospitalised and 122 (12.6%) were outpatients. Age of the patients varied from 30 days to 60 months (average 21.53 ± 12.54 months). Males were 59% and females – 41%. Virus infections were laboratory-confirmed in 663 (69.6%) samples. Influenza viruses were found in 252 (26.4%) of the tested children. Influenza B/ Yamagata virus was predominant during the 2017/2018 winter season, while A(H1N1)pdm09 prevailed during the next winter season. In the first season, the proportion of each influenza virus among influenza-positive samples was as follows: B/Yamagata – 57.5%, B/Victoria – 1.1%, A(H1N1)pdm09 – 35.6% and A(H3N2) – 5.7%; in the second season: A(H1N1)pdm09 – 72.1% and A(H3N2) – 27.9%.

Table 1. Distribution of respiratory viruses in outpatients and hospitalised patients.

	Number of detected respiratory viruses											
	A(H1N1) pdm09	A(H3N2)	B/ Yamagata	B/ Victoria	RSV	HMPV	PIV-1	PIV-2	PIV-3	RV	AdV	BoV
2017-2018	31	5	50	1	91	27	15	2	11	72	35	48
2018-2019	119	46	-	-	101	9	-	2	4	16	20	26
Summer 2018	-	-	-	-	1	2	-	1	2	13	5	3
Outpatients	16	11	13	1	9	8	3	1	1	11	4	7
Inpatients	134	40	37	-	184	30	12	4	16	90	56	70

Among the non-influenza viruses, RSV was the most frequently detected respiratory virus found in 193 (20.3%) of the tested samples, followed by RV – 101 (10.6%), BoV – 77 (8.1%) and AdVs – 60 (6.3%). The number of detected HMPV and PIV type 1, 2 and 3 was as follows: 38 (4%), 15 (1.6%), 5 (0.5%) and 17 (1.8%), respectively (Fig. 1).

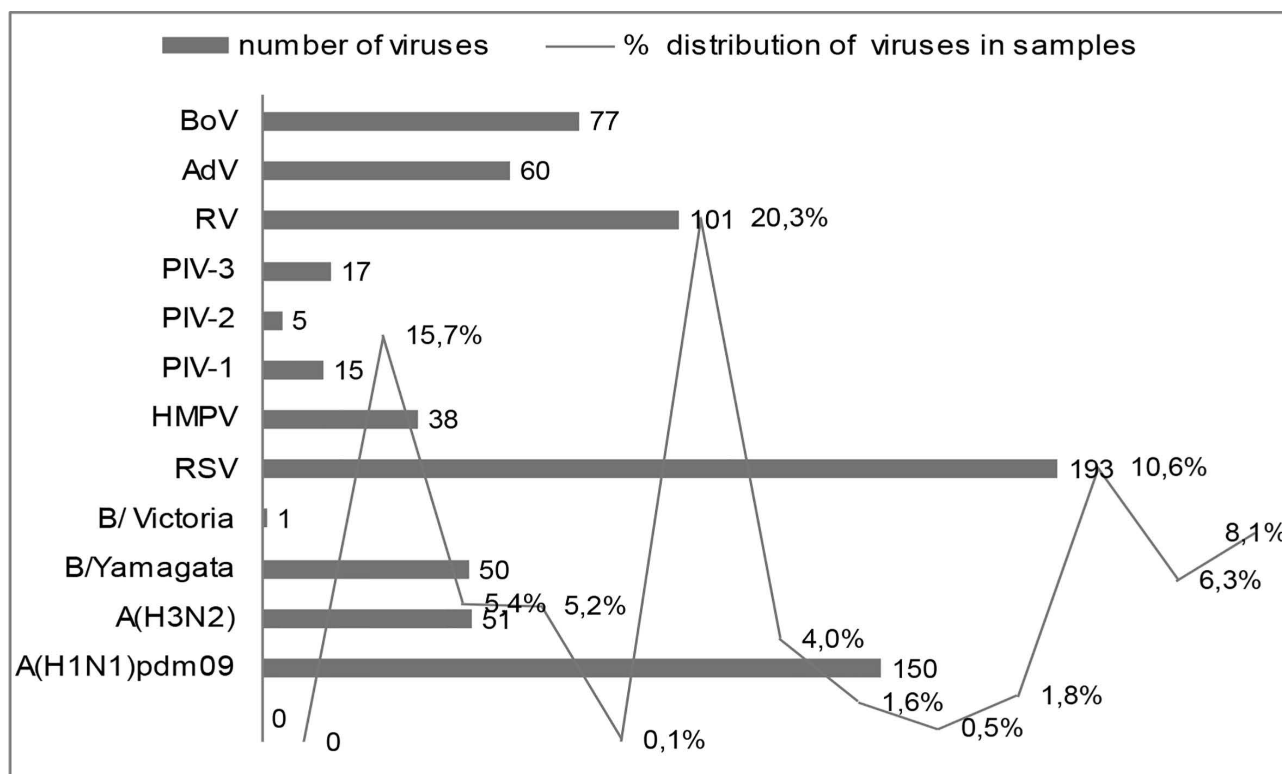

Figure 1. Distribution of respiratory viruses detected in Bulgaria during the 2017/2018 and 2018/2019 seasons and in the summer of 2018.

Single infections were detected in 568 (59.6%) patients; 88 (9.2%) children were co-infected with two and 7 (0.7%) children – with three viruses. Among the children with mono-infections, the most commonly determined pathogen was RSV, followed by influenza A(H1N1)pdm09. AdV, RV, BoV and PIV-3 were the most frequently detected pathogens in co-infections – the proportion of mixed infections was 61.7%, 52.5%, 41.6% and 41.2%, respectively (Fig. 2). The most common combinations in mixed infections were RSV+RV, RV+BoV, RV+AdV and RSV+AdV (Table 2).

Table 2. Number of respiratory viruses, detected as a single pathogen or co-pathogen.

Viruses	A(H1N1) pdm09	A(H3N2)	Inf typeB	RSV	hMPV	PIV1	PIV2	PIV3	RV	AdV	BoV
A(H1N1)pdm09	**134**	-	-	4	-	-	-	2	1	5	3
A(H3N2)		**44**	-	4	-	-	1	-	-	-	1
Inf type B			**46**	2	-	-	-	-	1	1	-
RSV				**147**	-	-	-	2	15	10	3
hMPV					**35**	-	-	-	2	-	1
PIV1						**11**	-	-	2	-	2
PIV2							**3**	-	1	-	-
PIV3								**10**	-	1	2
RV									**53**	11	15
AdV										**23**	4
BoV											**45**
Co-pathogen in dual infection	15	6	4	40	3	4	2	7	48	32	31
Co-pathogen in triple infection	1	1	1	6	-	-	-	-	5	5	1

*Single pathogens are indicated in bold.

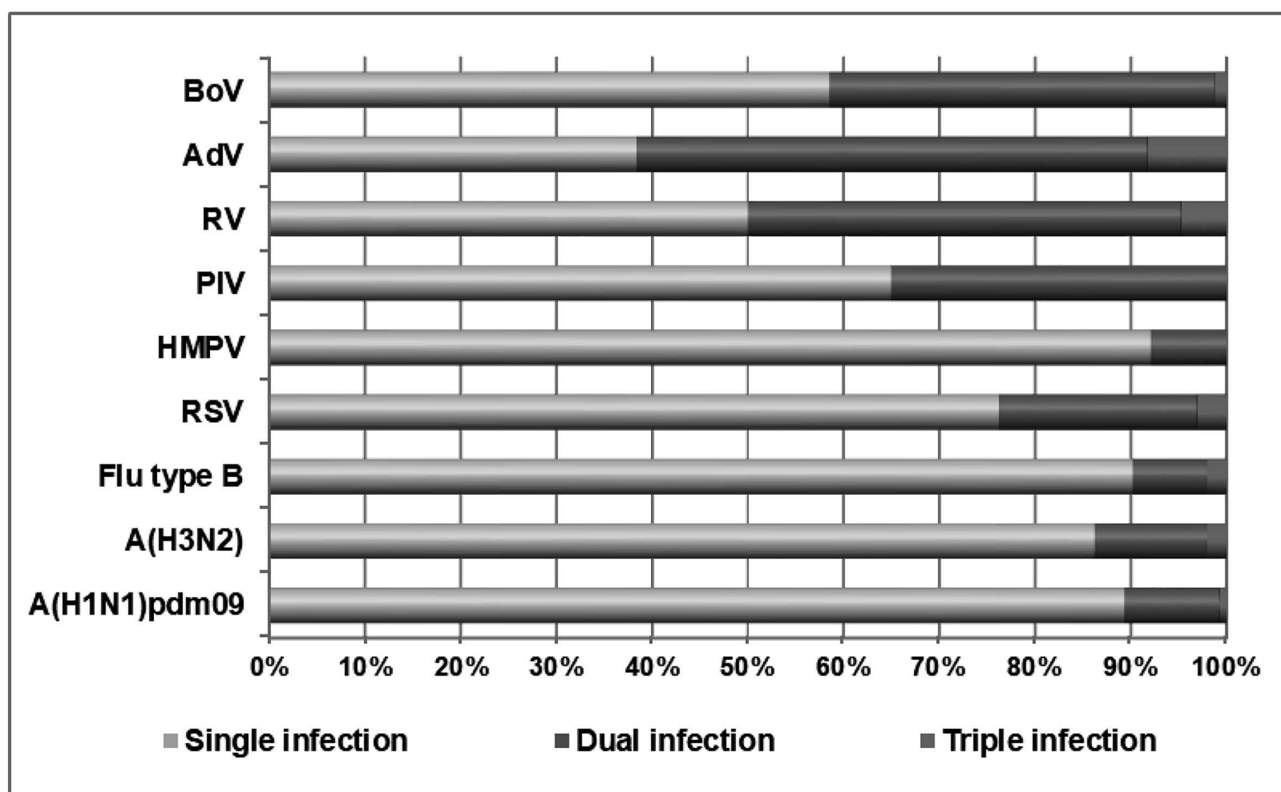

Figure 2. Proportion (%) of respiratory viruses detected in single, dual or triple infections during the 2017/2018 and 2018/2019 seasons and in the summer of 2018.

Seasonal distribution of viral agents

The greatest number of respiratory viruses was detected in specimens obtained in December 2018, February 2017 and May 2018 (Fig. 3). Influenza and RSV infections were more prevalent in winter. RV, BoV and HMPV infections occurred predominantly during fall and spring in Bulgaria.

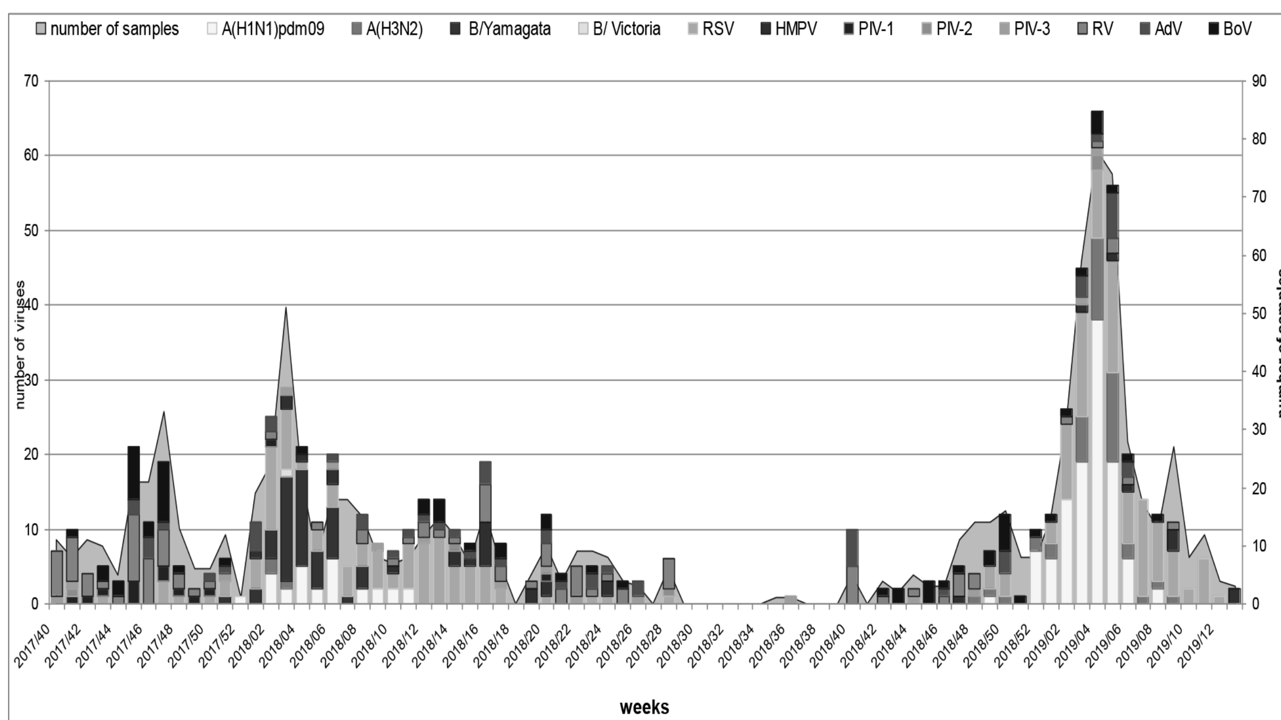

Figure 3. Weekly distribution of respiratory viruses detected during the two consecutive epidemic seasons in Bulgaria.

Clinical characteristics

During early childhood respiratory viruses may cause serious complications affecting the respiratory tract (laryngotracheitis, bronchiolitis, pneumonia) or CNS (febrile seizures, brain oedema, meningitis, encephalopathy, encephalitis). This study also analysed the involvement of influenza viruses, RSV, HMPV, PIV1/2/3, RV, AdV and BoV in development of the complications mentioned above. A total of 85, 198, 121 and 32 cases of laryngotracheitis, bronchiolitis, pneumonia and CNS complications were investigated. At least one virus was detected in 80%, 79.3%, 61.2% and 53.1% of cases with these syndromes, respectively. Fig. 4 represents the number and proportion (%) of patients infected with respiratory viruses in the groups with different clinical diagnosis.

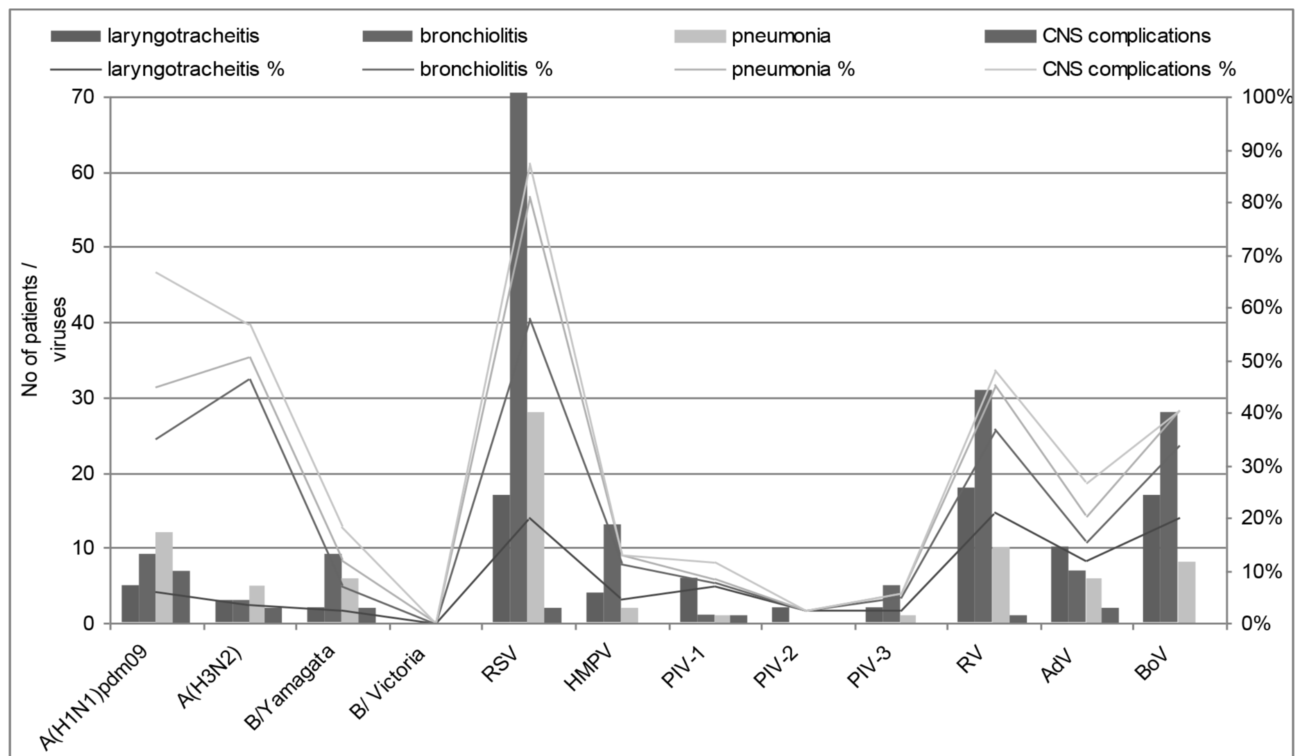

Figure 4. Number and proportion (%) of patients with different clinical diagnosis and detected respiratory viruses.

Among patients with laryngotracheitis, RVs were the most frequently identified viruses (21.2%), followed by RSV (20%) and BoV (20%). Among patients with bronchiolitis, RSV, RV and BoV were the most common pathogens – 37.9%, 15.7% and 14.1%, respectively. RSV was the most commonly identified virus in patients with pneumonia (23.1%) followed by influenza A(H1N1)pdm09 (9.9%) and RV (8.3%). In total, influenza viruses were responsible for 19% of pneumonia cases. Most (34.4%) neurological complications were associated with influenza infections.

DISCUSSION

Respiratory tract infections are a leading cause of morbidity and mortality in children <5 years around the world. This study aimed to describe the circulation of 12 respiratory viruses among infants and young children in Bulgaria during the 2017/2018 and 2018/2019 seasons. The involvement of these pathogens in the development of some serious diseases of the respiratory tract and CNS was also analysed.

The high level (69.6%) of detection of respiratory viruses found in this study is comparable to other surveys (4, 5). Mixed infections were identified at a lower percentage (14.3%) compared to other studies (6, 7). In a systematic review, Goka et al. reported incidence of mixed viral infections ranging from 5% to 62% (8). A high percentage of mixed infections is likely to be observed in settings with high population density and a large number of children attending childcare

facilities. Some authors suggest that there is a link between co-infection and disease severity, especially co-infections involving RSV (9, 10, 11). The relationship between clinical severity and infection status with single vs. multiple respiratory pathogens remains inconclusive.

Influenza epidemics are characterised by variations in the types/subtypes of influenza viruses involved, their virulence and clinical manifestation. In Bulgaria, during the 2017/2018 winter season, B/Yamagata was the predominantly circulating influenza virus, while during the next 2018/2019 season A(H1N1) pdm09 was prevalent. Similar predominance of B/Yamagata and A(H1N1)pdm09 viruses was observed in most European countries (12).

RSV is the most important pathogen of ARI among infants and young children and the major causative agent of bronchiolitis and pneumonia (13, 14). In our study, RSV was the most frequently detected virus in all examined patients and a leading cause of bronchiolitis (37.9% of cases) and pneumonia (23.1% of cases).

RV infections occur early and periodically in life, mainly as a common cold. However, they are also associated with severe illnesses in infants and young children (15, 16). In this study, RVs were the 3rd most frequently identified viruses; they were also the most common pathogen in cases of laryngotracheitis (21.2% of cases) and one of the leading causative agents of bronchiolitis and pneumonia.

According to literature data, HBoVs were found as relatively frequent respiratory pathogens in children younger than 5 years of age (17, 18, 19). In this study, BoVs ranked fourth in frequency among other respiratory viruses. In agreement with other studies, they were involved in co-infections at a significant rate – 41.6% (17). Spread of BoVs was observed throughout the year, reaching peaks in the autumn and spring months.

The proportion of AdV infections found among Bulgarian children with ARI was 6.3% which was similar to the findings of other researchers (20). AdV were characterised by a significant incidence of co-infections.

HMPV is a relatively new pathogen with prevalence in the paediatric population ranging from 5-25% (21). The results of this study showed 4% frequency of HMPV infections and 6.6% incidence in cases of bronchiolitis.

PIV type 1, 2, 3 and 4 show different clinical and epidemiological characteristics. PIV types 1 and 2 are leading causes of laryngotracheobronchitis (croup), while PIV-3 is frequently associated with bronchiolitis and pneumonia in infants and young children (22). PIVs were identified at low frequency – 3.9% of the studied children, mainly in cases of laryngotracheitis.

A limitation of this study is that human coronaviruses, which also cause ARI, were not included in the examinations.

The study highlights the role of 11 respiratory viruses in the aetiology of paediatric respiratory infections. RSV followed by influenza viruses, RVs and BoVs were found to be the most common causative agents of ARI in children younger than 5 years during two consecutive seasons (2017/2018 and 2018/2019) in Bulgaria. These pathogens were the main cause of complications such as laryngotracheitis, bronchiolitis and pneumonia that require hospital treatment. Timely and accurate diagnosis of viral respiratory infections is important in order to reduce the need for unnecessary lab tests and antibiotic use and to improve infection prevention and control measures.

REFERENCES

1. Nair H, Simões EA, Rudan I, et al. *Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis.* Lancet. 2013; 381(9875):1380-1390.
2. Walker CLF, Rudan I, Liu L, et al. *Global burden of childhood pneumonia and diarrhoea*. Lancet. 2013; 381(9875):1405-1416.
3. Kodani M, Yang G, Conklin LM et al. *Application of TaqMan Low-Density Arrays for Simultaneous Detection of Multiple Respiratory Pathogens*. J Clin Microbiol. 2011; 49(6):2175-2182.
4. Bicer S, Giray T, Çöl D, et al. *Virological and clinical characterizations of respiratory infections in hospitalized children.* Ital J Pediatr. 2013; 39:22.
5. Schlaudecker EP, Heck JP, Macintyre ET, et al. *Etiology and seasonality of viral respiratory infections in rural Honduran children.* Pediatr Infect Dis J. 2012; 31(11):1113-1118.
6. Cilla G, Oñate E, Perez-Yarza EG, et al. *Viruses in community-acquired pneumonia in children aged less than 3 years old: High rate of viral coinfection.* J Med Virol. 2008; 80(10):1843-1849.
7. Zhang G, Hu Y, Wang H et al. *High incidence of multiple viral infections identified in upper respiratory tract infected children under three years of age in Shanghai, China.* PLoS One. 2012; 7(9):e44568.
8. Goka EA, Vallely PJ, Mutton KJ, Klapper PE. *Single and multiple respiratory virus infections and severity of respiratory*

disease: a systematic review. Paediatr Respir Rev. 2014; 15(4):363-370.

9. Tregoning JS, Schwarze J. *Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology.* Clin Microbiol Rev. 2010; 23(1):74-98.
10. Franz A, Adams O, Willems R, et al. *Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection*. J Clin Virol. 2010; 48(4):239-245.
11. Asner SA, Rose W, Petrich A, et al. *Is virus coinfection a predictor of severity in children with viral respiratory infections?* Clin Microbiol Infect. 2015; 21(3):264.e1-6.
12. Adlhoch C, Snacken R, Melidou A, et al. *Dominant influenza A(H3N2) and B/Yamagata virus circulation in EU/EEA, 2016/17 and 2017/18 seasons, respectively.* Euro Surveill. 2018; 23(13).
13. Hall CB. *Respiratory syncytial virus and parainfluenza virus.* N Engl J Med. 2001; 344(25):1917-1928.
14. Pavlova S., Hadziolova T, Kotzeva R. *Diagnostic studies on the etiological role of respiratory syncytial virus and influenza viruses in hospitalized children.* Probl Infect Parasit Dis. 2006; 34(2):29-31.
15. Toivonen L, Schuez-Havupalo L, Karppinen S, et al. *Rhinovirus Infections in the First 2 Years of Life*. Pediatrics. 2016; 138(3) pii:e20161309.
16. Iwane MK, Prill MM, Lu X, et al. *Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children.* J Infect Dis. 2011; 204(11):1702-1710.
17. Naghipour M, Cuevas LE, Bakhshinejad T, et al. *Human bocavirus in Iranian children with acute respiratory infections.* J Med Virol. 2007; 79(5):539-543.
18. Wang M, Cai F, Wu X et al. *Incidence of viral infection detected by PCR and real-time PCR in childhood community-acquired pneumonia: a meta-analysis.* Respirology. 2015; 20(3):405-412.
19. Chow BD, Esper FP. *The human bocaviruses: a review and discussion of their role in infection*. Clin Lab Med. 2009; 29(4):695-713.
20. Hong JY, Lee HJ, Piedra PA, et al. *Lower respiratory tract infections due to adenovirus in hospitalized Korean children: epidemiology, clinical features, and prognosis.* Clin Infect Dis. 2001; 32(10):1423-1429.
21. Sloots TP, Mackay IM, Bialasiewicz S, Jacob KC, et al. *Human metapneumovirus, Australia, 2001-2004.* Emerg Infect Dis. 2006; 12(8):1263-1266.
22. Xiao NG, Duan ZJ, Xie ZP, et al. *Human parainfluenza virus types 1-4 in hospitalized children with acute lower respiratory infections in China.* J Med Virol. 2016; 88(12):2085-2091.

THE USE OF DRIED BLOOD SPOTS FOR MEASURING THE IMMUNITY AGAINST HBV: THE SIGNIFICANCE OF ANTIBODY DILUTION

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ABSTRACT

Background: Dried blood spots (DBS) have been used to study the prevalence of hepatitis B virus (HBV) infection in endemic areas and in high-risk groups. However, detection of HBV serological markers in DBS by ELISA assays has not yet been fully optimised. The aim of the present study is to evaluate the dilution level of anti-HBs when DBS cards are used as storage matrix implemented for ELISA.

Material and methods: Antibodies against hepatitis B surface antigen (anti-HBs) were detected by ELISA. The following specimens were examined: serum samples from 20 patients paired with 20 DBS; serum samples from 20 HBV-vaccinated healthcare workers paired with 20 dried serum spots (DSS); and four different dilutions of Immunovenin. Different elution protocols were used in order to study the problem with sample dilution.

Results: Specificity of 100% and sensitivity of 45% were established for DBS versus the "gold standard". Dilution of the eluted DBS/DSS samples was established and in some cases the measured anti-HBs titre dropped under 10 mIU/ml. Correlation was not observed between the positive initial anti-HBs serum titres and the obtained values of DBS/DSS testing. Also, 20- to 50-fold dilutions were measured for eluted DSS samples when testing Immunovenin. Increasing of the eluted sample concentration raised DSS anti-HBs titre.

Conclusions: In order to resolve the problem of dilution, it is necessary to validate different elution protocols because the small amount of sample in DBS showed lower titres.

KEYWORDS:
DBS, anti-HBs, HBV

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INTRODUCTION

Hepatitis B virus (HBV) infection represents a major health burden worldwide with 248 million people chronically infected in 2010 (1). In 2014, 22442 newly diagnosed HBV infection cases were reported from 30 EU Member States (2). In 2017, 249 cases of viral hepatitis B were reported from Bulgaria, an incidence of 3.51$\%_{000}$, with 10 reported deaths (mortality of 0.14$\%_{000}$, lethality of 4.02%). All reported cases were laboratory confirmed (3). Up to 90% of HBV transmissions can be prevented with administration of immunoglobulin within 48 hours after birth or with implementation of HBV vaccine series, beginning at birth and completed within 12 months (4). In 1992, the World Health Organisation recommended all countries to include HBV vaccination in the universal childhood vaccination program. Since 1991, an obligatory vaccination has been implemented for all newborns in Bulgaria (5).

Serological markers used in the diagnosis of HBV infection are HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc IgM/IgG (6). Anti-HBs is a neutralising antibody and serves as a marker for long-term immunity. In vaccinated patients, anti-HBs is the only serological marker detected in serum. In the case of past HBV infection these antibodies appear with anti-HBc IgG. Occasionally, the simultaneous appearance of HBsAg and anti-HBs has been reported in HBsAg-positive patients (7). Anti-HBs becomes detectable during convalescence after the disappearance of HBsAg in patients who do not progress to chronic infection. The presence

of anti-HBs following acute infection generally indicates recovery and immunity to re-infection (8). At the same time rapid anamnestic increase of anti-HBs titres in HBV-vaccinated persons is a strong evidence of resent HBV infection (9).

Dried blood samples (DBS) on filter paper were originally used for the measurement of phenylalanine in newborns for detection of phenylketonuria (10). DBS or Dry Plasma Spot (DPS) is a recent technology used for collection, storing and transportation of blood samples. DBS provides a number of advantages over whole blood, serum or plasma sample collection. Sampling is simple and minimally invasive versus whole blood venipuncture. The biggest advantage of DBS is shipping and storage requirements. Samples are small, there are no requirements for cold chain and samples can be transported in standard post envelop (11). Only eight studies have evaluated the use of DBS for diagnosis of hepatitis viruses. Their potential was estimated for HCV, HAV and HBV serology (12). DBS have been used to study the prevalence of HBV infection in endemic areas and in high-risk groups. However, detection of HBV serological markers in DBS by immune-linked immunosorbent assays (ELISA) has not yet been fully optimised (13), especially anti-HBs. The aim of the present study is to evaluate the dilution level of anti-HBs when DBS is used as storage matrix implemented for ELISA.

MATERIAL AND METHODS

Patients and specimens: A total of 40 persons with two types of clinical material were tested: serum samples from 20 patients paired with 20 DBS (study group I); serum samples from 20 HBV-vaccinated healthcare workers paired with 20 dried serum spot samples (DSS) (study group II). The specimens were collected according to the requirements of the working program from research project Contract № DM 03/1,12.12.2016 funded by the National Science Fund, Bulgaria. The blood was collected by venipuncture and after centrifugation at 4000 rpm for 10 min the serum was separated, aliquoted and frozen at -20°C until analysed. In the case of study group I, 150 μl fresh blood were spotted on filter paper cards (HiMedia`s InstaDNA TM Cards) before centrifugation. For study group II, 150 μl serum sample was spotted on the cards. The filter cards were labelled appropriately, air dried at room temperature for 1 hour and stored individually in zip-locked bags with desiccant at 4°C for 1 month. Additionally, undiluted, 2-fold and 10-fold diluted Immunovenin intact 5% IgG (Bul Bio-NCIPD, Bulgaria) was applied on the filter cards and prepared following the same protocol. The protocol design and the study were performed in the National Reference Laboratory (NRL) ”Hepatitis viruses” at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria.

Elution of DBS: A 6-mm diameter disc was punched from each filter paper. One or two discs were incubated at room temperature in 200 μl or 500 μl elution buffer (PBS with 0.05% Tween) for 1 hour with agitation on a laboratory shaker. After that samples were incubated overnight at 4°C at rest. On the next day the samples were tempered and centrifuged at 10 000 rpm for 2 min according to the method by Mössner BK, et al (14). The filter paper discs were removed and DBS/DSS eluates were tested with anti-HBs ELISA.

Detection of anti-HBs by ELISA: Quantitative ELISA assays were performed and interpreted according to the manufacturer’s instructions (HBsAb ELISA, Dia.Pro, Italy). The amount of antibodies was quantitatively defined by means of standard curve calibrated against the WHO reference preparation. The ELISA assay had 98.9% diagnostic specificity and 100% diagnostic sensitivity. Samples with results of ≥10 mIU/ml were considered positive. All samples included in the study were retested in duplicate.

Statistical Analysis: All statistical calculations were performed using the Analysis ToolPak of Microsoft Office Excel 2010. For the statistical analysis we used relative percentages (%), correlation analysis, graphical and table analysis.

RESULTS

Detection of anti-HBs in serum and in DBS/DSS

Firstly, 40 serum samples were tested for the presence of anti-HBs. Half of the samples were from patients with different liver disorders and unknown anti-HBs status (study group I), and

the other 20 samples were from healthcare workers vaccinated against HBV (study group II). The median age of the patients was 34 ± 14 years and for the healthcare workers was – 45 ± 13 years. A total of 31 serum samples (78%, 95% CI: 65.16÷90.84) were anti-HBs-positive, with minimal antibody titre of 12 mIU/ml and maximal of 254 mIU/ml. Of these 31 positive samples, 11 were from study group I and 20 from study group II (Table 1). Next, the paired DBS samples of study group I and the DSS samples of study group II were tested for anti-HBs. For both groups elution was carried out with a 6-mm disc incubated in 500 µl elution buffer. Positive results with anti-HBs titre of >10 mIU/ml were found in 10 (25%, 95% CI: 11.58÷38.42) of all 40 filter card samples (DBS and DSS) with 5 anti-HBs-positive results in each of the study groups, respectively. The established sensitivity for both types of matrices was under 50%. The specificity of DBS was 100%. It was not possible to establish the specificity for DSS as all initially tested serum samples were anti-HBs-positive.

Table 1. Sensitivity and specificity of serological testing by HBsAb ELISA (Dia.Pro) of DBS/DSS compared with serum samples.

	Serum, n		DBS / DSS, n		Sensitivity [%]	Specificity [%]	PPV [%]	NPV [%]
	negative	positive	negative	positive				
Study group I	9	11	15	5	45	100	100	60
Study group II	0	20	15	5	33	NA	100	NA

Legend: PPV – positive predictive value; NPV – negative predictive value; NA – not applicable.

DBS/DSS were measured as anti-HBs-positive when the initial serum anti-HBs titre was over 200 mIU/ml (Table 2). At the same time, negative anti-HBs titres (<10 mIU/ml) were measured for the initial serum samples with anti-HBs titres between 225mIU/ml and 12 mIU/ml for DBS and between 226 mIU/ml and 20 mIU/ml for DSS.

Table 2. DBS/DSS anti-HBs results according to the initial serum titre.

Serum anti-HBs [mIU/ml]	DBS (n=20)		DSS (n=20)	
	Positive, n	Negative, n	Positive, n	Negative, n
10 - 50	0	3	0	3
50 - 100	0	1	0	2
100 - 150	0	1	0	5
150 - 200	0	0	0	2
200 - 250	5	1	5	3

Detection of anti-HBs in Immunovenin intact 5% IgG and in discs punched from filter cards

The possible dilutions of the samples were calculated before DBS/DSS testing. By following the designed protocol, 150 µl of the tested sample were dropped on HiMedia`s InstaDNA TM filter cards. When 6-mm discs were punched the sample volume decreased to approximately 9 µl (8.64µl). It was calculated that when one 6-mm disc (1 × 6 mm) was eluted in 500 µl elution buffer the sample is diluted 58-fold; in the case of two discs (2 × 6 mm) the dilution was 29-fold. In order to decrease the dilution, the design of the working protocol was modified and the discs

were eluted in 200 µl buffer with estimated 23- and 12-fold dilution, respectively.

The measured anti-HBs titres of the initial Immunovenin intact 5% IgG samples were 1440 mIU/ml, 880 mIU/ml, 1100 mIU/ml and 720 mIU/ml, respectively. For filter card samples anti-HBs decreased to 56 mIU/ml, 44 mIU/ml, 23 mIU/ml and 26 mIU/ml, respectively, when two 6-mm discs were eluted; and to 33 mIU/ml, 19 mIU/ml, 9 mIU/ml and 7 mIU/ml – when one 6-mm disc was eluted. As can be seen, anti-HBs titres obtained by the DBS collection method were decreased to extremely low levels – under10 mIU/ml (Fig. 1). Comparison of the results for the initial sample and for the two 6-mm discs demonstrated dilution ranging from 20- to 50-fold.

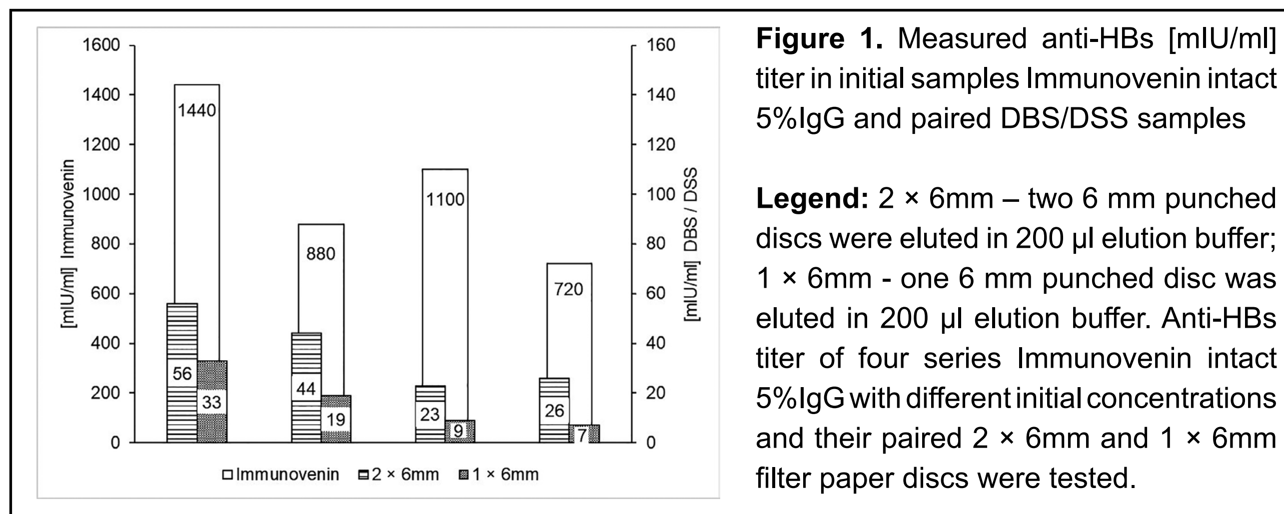

Figure 1. Measured anti-HBs [mIU/ml] titer in initial samples Immunovenin intact 5%IgG and paired DBS/DSS samples

Legend: 2 × 6mm – two 6 mm punched discs were eluted in 200 µl elution buffer; 1 × 6mm - one 6 mm punched disc was eluted in 200 µl elution buffer. Anti-HBs titer of four series Immunovenin intact 5%IgG with different initial concentrations and their paired 2 × 6mm and 1 × 6mm filter paper discs were tested.

The positive correlation for anti-HBs antibody titres obtained with two 6-mm discs and one 6-mm disc samples is shown in Fig. 2 with a correlation coefficient of 0.97. As can be seen, when the sample concentration increased from 9 µl (one 6-mm disc) to 18 µl (two 6-mm discs) the anti-HBs titre almost doubled.

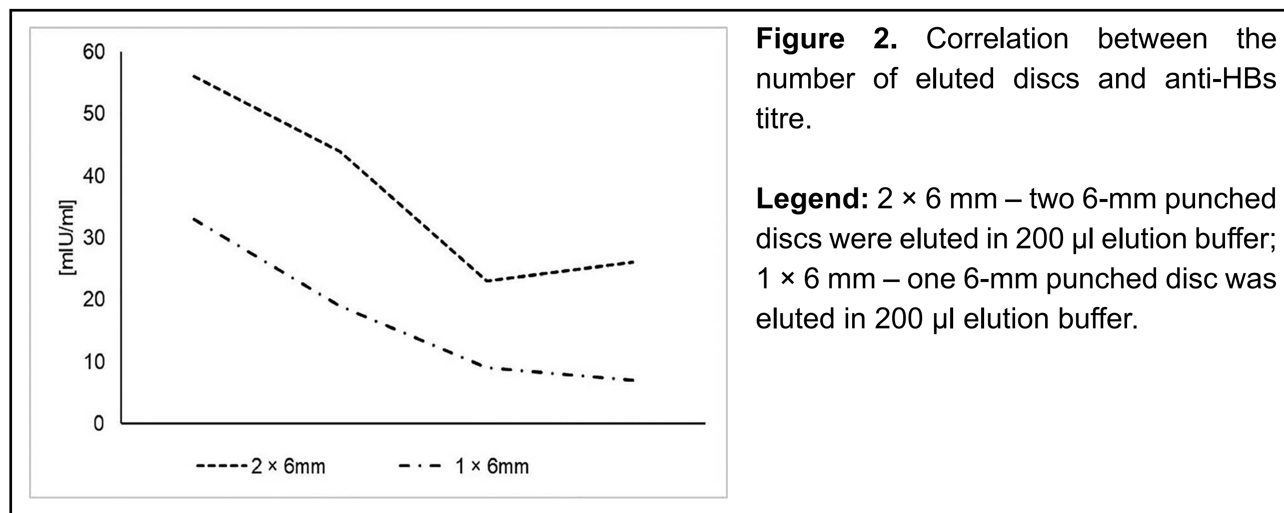

Figure 2. Correlation between the number of eluted discs and anti-HBs titre.

Legend: 2 × 6 mm – two 6-mm punched discs were eluted in 200 µl elution buffer; 1 × 6 mm – one 6-mm punched disc was eluted in 200 µl elution buffer.

DISCUSSION

In many aspects DBS is a convenient method for clinical sample collection (15). Although they can be used as sample storage matrix in HAV and HCV diagnosis and detection (16), their relevance in detection of some HBV markers, such as anti-HBc and anti-HBs, is limited (14). Although this study established 100% specificity for DBS, the sensitivity is too low – 45%. The low sensitivity for anti-HBs in DBS versus plasma was observed even when using automated diagnostic platform (17). There are two possible explanations: first, the number of tested samples in the present study is too small; and second, the different starting

titres of the samples can be the reason for the different dilution factor with elution (14). During the study it was established that the eluted DBS/DSS samples were diluted 12- to 23-fold and in some cases the measured anti-HBs titre dropped under 10 mIU/ml. Mössner, et al observed a similar correlation showing decreasing anti-HBs titre when adding elution buffer (14). Similar dilution was established when the initial sample was Immunovenin intact 5% IgG with eliminating the influence of hematocrit and other host factors on sample absorption and spread across the filter paper. As it is already known, hematocrit has an impact on different variables when using the DBS protocol (18). We established a positive correlation when the volume of the eluted sample is increased by using two instead of one disc. False negative results were eliminated. To resolve the problem of dilution, it is necessary to validate different elution protocols because the small amount of sample in DBS led to lower titres in comparison with the "gold standard" for anti-HBs detection.

CONCLUSIONS

DBS/DSS can serve as an alternative option in anti-HBs screening, epidemiological studies and sample storage. The test shows high specificity but additional studies are needed to overcome the problem with anti-HBs dilution after elution.

Competing Interest

The authors have declared that they have no conflicts of interest.

Acknowledgements

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REFERENCES

1. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. *Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013*. Lancet. 2015; 386(10003):1546–1555.
2. European Centre for Disease Prevention and Control. Hepatitis B Surveillance in Europe – 2014. Stockholm: ECDC, 2016.
3. KurchatovaA, VladimirovaN, MinkovaA, KamenovG, Stoitsova S, Parmakova K. *Acute infectious diseases in Bulgaria in 2017 (Key Epidemiological Indicators).* https://www.ncipd.org/index.php?option=com_docman&view=list&slug=analysis-2017&Itemid=1127&lang=bg
4. Børresen ML, Koch A, Biggar RJ, Ladefoged K, Melbye M, Wohlfahrt J, Krause TG. *Effectiveness of the targeted hepatitis B vaccination program in Greenland*. Am J Public Health. 2012; 102(2):277-284.
5. Todorova TT, Tsankova G, Lodozova N. *Hepatitis B infection in eastern regions of Bulgaria*. J IMAB. 2016; 22(1):1033-1035.
6. Golkocheva-Markova E, Alexiev I, Teoharov P. *Relation between hepatitis B surface antigen kinetic and viral replication in patients with resolved hepatitis B virus infection*. Probl Infect Parasit Dis. 2013; 41(2):24-28.
7. Song JE, Kim DY. *Diagnosis of hepatitis B*. Ann Transl Med. 2016; 4(18):338.
8. World Health Organization. Hepatitis B: Serological and clinical patterns of acute or chronic HBV infections. WHO 2002. http://www.who.int/emcdocuments/hepatitis/docs/whocdscsrlyo20022/disease/serological_clinical_pattern.html
9. Su TH, Chen PJ. *Emerging hepatitis B virus infection in vaccinated populations: a rising concern?* Emerg Microbes Infect. 2012; 1(9):e27.
10. Guthrie R, Susi A. *A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants*. Pediatrics. 1963; 32:338-343.
11. Chace DH, De Jesús VR, Spitzer AR. *Clinical chemistry and dried blood spots: increasing laboratory utilization by improved understanding of quantitative challenges*. Bioanalysis. 2014; 6(21):2791-2794.
12. Smit PW, Elliott I, Peeling RW, Mabey D, Newton PN. *An overview of the clinical use of filter paper in the diagnosis of tropical diseases*. Am J Trop Med Hyg. 2014; 90(2):195-210.
13. Marques B, Brandão C, Silva E, Marques V, Villela-Nogueira C, Do ÓK, de Paula M, Lewis-Ximenez L, Lampe E, Villar L. *Dried blood spot samples: Optimization of commercial EIAs for hepatitis C antibody detection and stability under different storage conditions*. J Med Virol. 2012; 84:1600-1607.
14. Mössner BK, Staugaard B, Jensen J, Lillevang ST, Christensen PB, Holm DK. *Dried blood spots, valid screening for viral hepatitis and human immunodeficiency virus in real-life.* World J Gastroenterol. 2016; 22(33):7604-7612.
15. Krumova S, Golkocheva-Markova E, Pavlova A, Angelova S, Georgieva I, Genova-Kalou. P. *Detection of measles and rubella antibodies in dried blood spots*. Probl Infect Parasit Dis. 2018; 46(2):20-24.
16. Smit PW, Elliott I, Peeling RW, Mabey D, Newton PN. *An overview of the clinical use of filter paper in the diagnosis of tropical diseases.* Am J Trop Med Hyg. 2014; 90(2):195-210.
17. Ross RS, Stambouli O, Grüner N, Marcus U, Cai W, Zhang W, Zimmermann R, Roggendorf M. *Detection of infections with hepatitis B virus, hepatitis C virus, and human immunodeficiency virus by analyses of dried blood spots--performance characteristics of the ARCHITECT system and two commercial assays for nucleic acid amplification*. Virol J. 2013; 10:72.
18. Lim MD. *Dried Blood Spots for Global Health Diagnostics and Surveillance: Opportunities and Challenges*. Am J Trop Med Hyg. 2018; 99(2):256-265.

A CASE OF AN HIV-POSITIVE PATIENT CO-INFECTED WITH MULTIDRUG-RESISTANT TUBERCULOSIS

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ABSTRACT

Tuberculosis has a greater impact on morbidity and mortality in HIV-1-infected individuals than the rest of the opportunistic infections. We report a case of 57-year-old HIV-infected patient co-infected with multidrug- resistant tuberculosis (MDR-TB).

The patient`s leading clinical syndromes were fever, diarrhoea and weight loss. The meticulously performed laboratory investigations revealed severe immune suppression and high HIV viral load. Microbiological and parasitological tests confirmed the presence of two AIDS-defining conditions: disseminated candidiasis and cryptosporidiosis. Sputum smear microscopy for acid-fast bacilli was negative but sputum culture showed positive result for *Mycobacterium tuberculosis*. Drug susceptibility testing determined resistance to isoniazid and rifampicin (MDR-TB). The diagnosis was confirmed with Xpert-MTB/RIF PCR test. Treatment continued with second-line anti-TB drugs, together with antiretroviral therapy. Culture conversion was recorded in the first month. The outcome was reported as „cured" after 16 months` therapy.

This case shows yet again that the clinical manifestation of tuberculosis in HIV-infected patients is very atypical. Multidrug-resistant tuberculosis requires prolonged treatment and represents therapeutic challenge because of the possibility of adverse drug reactions.

KEYWORDS:

HIV, tuberculosis (TB), multidrug-resistant TB (MDR-TB), atypical presentation, therapeutic challenge

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INTRODUCTION

One of the major risk factors for developing both drug-susceptible and drug-resistant tuberculosis (TB) is infection with the human immunodeficiency virus (HIV) (1, 2). It has been estimated that people living with HIV are 16-27 times more likely to develop TB than the general population (1, 2). Approximately 10 million people infected with HIV-1 worldwide are co-infected with *Mycobacterium tuberculosis*. In 2017, an estimated 920,000 people living with HIV worldwide fell ill with TB (1, 2). TB is the primary mortality cause in HIV-positive individuals, and that same year 390,000 deaths from HIV-associated TB were reported (1, 2). Recently, co-infection with the two pathogens has become more noticeable in Eastern Europe, including in Bulgaria (3, 4). There is a growing concern that HIV-1 enhances the spread of multidrug-resistant tuberculosis (MDR-TB) in these regions (3, 4). MDR-TB is 10 times more frequent in Eastern Europe than in Africa (4). Not only does HIV infection increase TB prevalence in general, but it may also be held accountable for the rise in the number of MDR-TB cases. These conditions require regular examination of HIV patients for *Mycobacterium tuberculosis* and monitoring of the patients with latent tuberculosis (5). Since drug-resistant TB is frequently linked to higher mortality rates in people living with HIV, the treatment of patients requires better integration of HIV and TB services in settings with a wide spread of HIV, and in all settings with a high prevalence of the HIV-TB co-infection. (6). The management of drug-resistant TB in

HIV-positive patients requires early diagnosis of the drug-resistant TB and HIV infections, timely commencement of relevant second-line anti-TB medications and antiretroviral treatment (ART), unwavering patient support and strict measures for infection control (6).

CASE PRESENTATION

We present a 57-year-old male who was diagnosed as HIV-positive in March 2017. The patient was hospitalised in the Department of Infectious Diseases at the Multiprofile Hospital for Active Treatment in the city of Burgas with the following complaints and symptoms: watery diarrhoea, fever, night sweats and weight loss. Laboratory investigations revealed anaemia and leucopenia. Microbiological examination of faeces found no bacterial agent. The patient was tested for HIV and the result was positive. He was transferred to the Department of AIDS at the Specialised Hospital for Active Treatment of Infectious and Parasitic Diseases in Sofia. Meticulously performed laboratory tests revealed severe immune suppression (CD4 count of 16 cells/µl; CD8 count of 90 cells/µl; CD4/CD8 ratio of 0.18) and a high HIV viral load of 237,986 copies/µl. Confirmation of HIV diagnosis and quantitative PCR for viral load were performed in the National Reference Laboratory of HIV at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia. The immune status study was performed in the National Reference Laboratory of Immunology at the NCIPD. Microbiological and parasitological examination of stool samples found *Candida albicans* and *Cryptosporidium* spp. Sputum smear microscopy for acid-fast bacilli was negative. Chest X-ray showed evidence of interstitial infiltrates in the right lung (Fig. 1).

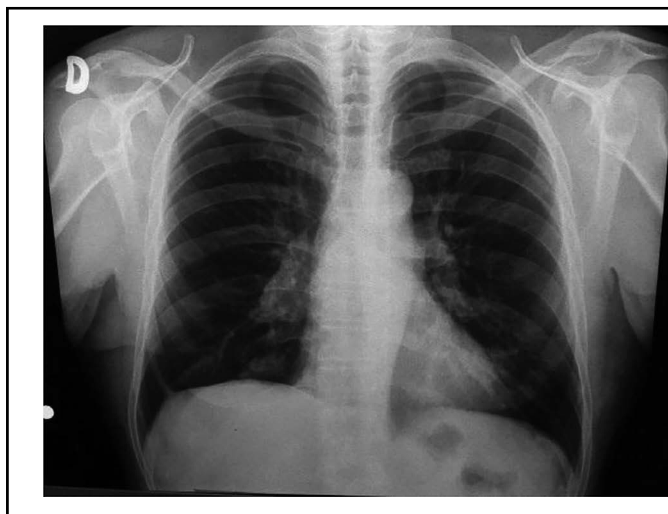

Figure 1. Chest X-ray of the patient on the day of hospitalisation in the Department of AIDS.

The patient was treated effectively for cryptosporidiosis (with azithromycin) and candidiasis (with fluconazole) together with initiation of combined antiretroviral therapy (emtricitabine/tenofovir disoproxil fumarate 445 mg + darunavir/ritonavir 900 mg). However, sputum culture on liquid media (MGIT, Mycobacterial Growth Indicator Tube) showed positive result for *Mycobacterium tuberculosis* after 4 weeks` cultivation.

Standard anti-tuberculosis therapy comprising rifampicin, isoniazid, pyrazinamide and ethambutol was assigned to the patient in the beginning of July 2017, but 2 weeks later phenotypic drug susceptibility testing (DST) by BACTEC MGIT 960 revealed MDR-TB with resistance to both rifampicin and isoniazid, combined with resistance to streptomycin and sensitivity to ethambutol. DST to second-line anti-TB drugs found sensitivity to amikacin (Am), kanamycin (Km), capreomycin (Cm), ofloxacin (Ofx), moxifloxacin (Mfx) and linezolid (Lzd). All DSTs were conducted in the National TB Reference Laboratory (NRL-TB) at the NCIPD. The diagnosis was confirmed by Xpert MTB/RIF (MTB detected, RIF resistance detected).

After receiving the results confirming the presence of MDR-TB, the patient was immediately referred

for hospitalisation in the Hospital for Pulmonary Diseases – Gabrovo, where all confirmed MDR-TB patients are treated and MDR-TB consilium is commenced as inpatient treatment during the intensive phase.

On 14 July 2017 together with ART was started treatment regimen containing second-line anti-TB drugs, including kanamycin (Km) 750 mg, levofloxacin (Lfx) 500 mg, prothionamide (Pto) 500 mg, pyrazinamide (Z) 1500 mg and ethambutol (E) 750 mg, based on body weight (65 kg at the beginning of treatment) and DST results. The baseline audiometry was normal.

Elevated levels of total and conjugated bilirubin, AST and ALT in the blood were registered 24 days after the initiation of treatment, which caused a temporary exclusion of Z, Lfx and Pto, and inclusion of cycloserine (Cs) 500 mg and hepatoprotective therapy. Administration of Lfx was resumed after 10 days with a daily dose increased to 750 mg due to weight gain. On 11 October 2017 bedaquiline (Sirturo) was added in the following dosages: 400 mg daily dose for 14 days and 200 mg 3 times a week for 22 weeks.

After initial presentation of hepatotoxicity, there were no other adverse drug reactions. The drugs were well-tolerated and the patient exhibited good adherence throughout the duration of treatment. All doses received at the hospital were administered under direct observation of therapy (DOT). Clinical assessment was performed daily; sputum smear microscopy and culture, measuring the level of blood cells, total and conjugated bilirubin, AST and ALT, creatinine, sodium, potassium, uric acid, and weight was conducted on a monthly basis at the hospital in Gabrovo to monitor the response to the treatment during the intensive phase. Sputum smear microscopy was negative during the whole treatment course, culture conversion was recorded in the first month and continued to be negative until the end of treatment.

Kanamycin was excluded from the drug regimen on 8 December 2017, 148 days (5 months) after beginning of treatment, with weight gain from 65 to 78 kg.

The patient was discharged from the hospital and referred to continue the treatment in ambulatory settings under monitoring conducted by the regional Hospital for Pulmonary Diseases – Burgas. Drug regimen included levofloxacin 750 mg, prothionamide 750 mg, cycloserine 500 mg and ethambutol 750 mg daily doses. During the continuation phase, DOT was organised by the Regional TB manager and patronage nurses in the hospital in Burgas. In order to encourage adherence to the treatment, food vouchers were handed to the patient throughout the whole therapy course. Drug dosages were modified according to body weight. All drugs for the treatment of MDR-TB and for the management of adverse effects were free of charge. During the ambulatory phase sputum monitoring by smear microscopy and culture was done monthly in the Microbiological laboratory at the Hospital for Pulmonary Diseases – Burgas.

Treatment outcome was reported as “cured” on 14 November 2018, 16 months after the start of MDR-TB therapeutic regimen, with 14 consecutive negative culture results and remarkable clinical progress with improved weight gain, adequate blood levels of total and conjugated bilirubin, AST and ALT. In the case with this patient was achieved optimal viral suppression (undetectable HIV viral load, < 40 copies/μl) and good immunological response (CD4 T cells of 200/μl, CD8 of 652/μl, and CD4/CD8 ratio of 0.30).

DISCUSSION

The diagnosis of tuberculosis in HIV-infected patients with advanced immune deficiency is difficult. In most cases, X-ray findings are not specific and sputum smear microscopy is negative. The leading complaints in our patient were diarrhoea, weight loss and night sweats. The confirmation of two pathogens (*Candida* and *Cryptosporidium*) which can explain the symptoms initially put these agents at the forefront of differential diagnosis. Despite the fact that we did not consider tuberculosis as a possible co-infection, we are providing routine TB testing for all HIV-positive patients. Culture is the golden diagnostic standard but requires a long time to obtain the results – from 4 to 6 weeks. That was the reason for

the delay in diagnosis of tuberculosis in our case. Xpert MTB/RIF is rapid molecular test, which is important especially in cases with drug-resistant TB. Treatment of MDR-TB is prolonged and quite expensive, but timely diagnosis is essential for the patient`s survival and the evolution of the HIV infection.

REFERENCES

1. Cascade data use manual: to identify gaps in HIV and health services for programme improvement. World Health Organization; 2018 [30 August 2018].
2. WHO implementation tool for monitoring the toxicity of new antiretroviral and antiviral medicines in HIV and viral hepatitis programmes. World Health Organization; 2018 [18 July 2018].
3. Van der Werf M, Ködmön C, Zucs P, Hollo V, Amato-Gauci A, Pharris A. *Tuberculosis and HIV coinfection in Europe: looking at one reality from two angles*. AIDS. 2016; 30(18): 2845-2853.
4. Efsen A, Schultze A, Miller R, Panteleev A, Skrahin A, et al. *Management of MDR-TB in HIV-coinfected patients in Eastern Europe: Results from the TB:HIV study*. J Infect.2018; 76(1):44-54.
5. Latent TB Infection : Updated and consolidated guidelines for programmatic management. World Health Organization; 2018 [February 2018].
6. Magis-Escurra C, Günther G, Lange C, Alexandru S, Altet N, et al. *Treatment outcomes of MDR-TB and HIV co-infection in Europe*. Europ Respir J. 2017; 49(6):1602363.

INVESTIGATION OF MURINE RODENTS FOR THE PRESENCE OF LEPTOSPIRA DNA BY NESTED PCR

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ABSTRACT
Leptospirosis is a zooanthroponosis with natural foci. People become infected with leptospires either directly from host animals or by means of certain elements of the external environment. Circulation of leptospires in nature is maintained by reservoirs and supporting hosts. For the first time in Bulgaria we applied nested PCR in examining organs of murine rodents for the presence of *Leptospira* spp. DNA. A total of 109 rodents were investigated after being collected from 4 districts in Southern Bulgaria: Plovdiv, Pazardzhik, Smolyan and Blagoevgrad. The genome of *Leptospira* spp. was found in 5 species of rodents. Results show that *Microtus* spp. is a potential carrier of leptospires, especially in urban areas. The high rate of leptospiral DNA-positive rodents captured in the region of Pazardzhik confirms the activity of the epizootic process in this natural focus, where epidemics of benign leptospirosis have been recorded in the past. The introduced method would help to clarify the epidemiological links more quickly in case of a leptospirosis outbreak in some regions. Stronger measures are needed to combat rats, murine rodents and their entry in warehouses, slaughterhouses and mass caterers, in order to maintain the cleanliness of open-air ponds, water sources and prevent contamination of food products. Future studies in this area would enrich the knowledge on the circulation of leptospires in their reservoirs in more areas of our country.

KEYWORDS:
Leptospira spp.*,* rodents, nested PCR

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INTRODUCTION

Leptospirosis is one of the most widespread bacterial zoonoses in the world. It is caused by over 200 different serovars belonging to several serogroups of the genus *Leptospira* (1). Clinical presentation ranges from mild, flu-like illness to severe symptomatology, including Weil's syndrome with multiple organ failure and often fatal pulmonary haemorrhagic syndrome (13). Contact with stray animals, rodents, poor sanitation, heavy rainfall and flooding are the main risk factors in developing countries, whereas recreational activities, such as freshwater swimming, fishing or sporting events are associated with clinical leptospirosis in developed countries (7, 12). Rodents are the main animal reservoir in urban settings, with *Rattus norvegicus* primarily involved in pathogenic *Leptospira interrogans* serovar Copenhageni transmission (4, 5). Leptospirosis is reported with high prevalence in the rodent population of major cities in developed countries, such as Baltimore in the USA (18), Tokyo in Japan (5) and Copenhagen in Denmark (6).

Human leptospirosis is not a common infection in countries with moderate climate, where Bulgaria also falls, and is therefore often difficult to recognise and treat. A study on pathogenic *Leptospira* in the main reservoirs (rats and mice) would contribute to the faster eradication of the causative agents in their natural outbreaks. In the past, large-scale bacteriological studies have been carried out in Bulgaria with over 1500 animals, mainly rice mice (*Micromys minutus*), water rats (*Arvicola terrestris*), common forest mice (*Apodemus sylvaticus*), yellow-necked mice (*Apodemus flavicollis*) and hedgehogs (*Erinaceus europaeus*) (9).

However, for the last two decades no study has been conducted on detection of leptospires in

rodents. Furthermore, it would be valuable to introduce for the first time a genetic method for detection of leptospiral DNA in rodents.

The purpose of this study is to identify the presence of *Leptospira* spp. DNA in rodent organs for the first time in Bulgaria by using nested PCR.

MATERIAL AND METHODS

One hundred and nine rodents were tested by a nested PCR method. The specimens were collected from districts in southern Bulgaria for the period December 2012-December 2014. Species of the captured rodents were determined by using the identifier of Ts. Peshev (14). Following dissection of the animals, the spleen was taken and examined for the presence of leptospiral DNA. Nine of the rodents were newborn and the remaining 100 were adult specimens. Species distribution of rodents and the areas where they were captured are shown in Table 1.

Before examination organs of the rodents were homogenised with Microtube Homogeniser "Bead Bug" with a power of 40 W for 1 minute. *Leptospira* spp. DNA was extracted and purified by DNeasy Blood & Tissue kit (QIAGEN GmbH, Germany). The obtained DNA products were subjected to amplification. Four oligonucleotide primers (BIONEER, Korea) were used with sequences presented in Table 2 (11). DNA extracted from *L. interrogans* serovars Copenhageni, Pomona and Canicola (18-day cultures) were used as a positive control. The amplified products were analysed by electrophoresis on 1.5% agarose gel.

Table 1. Distribution of rodents tested by nested PCR by species and district in which they were captured.

District / Species of rodent	Pazardjik	Smolyan	Plovdiv	Blagoevgrad	Total	% of the total
Apodemus spp.	39	13	1		53	(48.6)
Microtus arvalis	20				20	(18.4)
Microtus spp.	3		2		5	(4.6)
Myodes glareolus	15	3		1	19	(17.4)
Mus musculus	5				5	(4.6)
Mus macedonicus			4		4	(3.7)
Crocidura suaveolens		1			1	(0.9)
Sorex minutus	1				1	(0.9)
Micromys minutes	1				1	(0.9)
Total	84	17	7	1	109	

Table 2. Primers used in the enzymatic amplification of the test samples from rodents.

Primers	*Nucleotide sequence 5' to 3'*	*Product in bp*
Lepto A	5'-GGCGGCGCGTCTTAAACATG-3'	**331 bp**
Lepto B	5'-TTCCCCCCATTGAGCAAGATT-3'	
Lepto C	5'-CAAGTCAAGCGGAGTAGCAA-3'	**289 bp**
Lepto D	5'-CTTAACCTGCTGCCTCCCGTA-3'	

RESULTS

In the wild, besides rats, the main reservoirs of *Leptospira* are the small murine rodents. The relationship between certain species of rodents with a specific serogroup of leptospires is known. In the past, extensive studies have been carried out in Bulgaria on the distribution of leptospires and their reservoirs (9). However, for the last 30 years there is no current data on the spread of leptospires and their reservoirs in our country.

In our study *Leptospira* spp*.* DNA was detected in 28.44% (31/109) of the rodents by nested PCR. The genome of *Leptospira* spp*.* was found in 5 species of rodents: *Apodemus* spp*., Myodes glareolus, Microtus arvalis, Microtus* spp*.* – belonging to the order *Rodentia,* and *Sorex minutes* – belonging to the order *Soricomorpha.* They originated from 3 of the surveyed areas: Pazardzhik, Smolyan and Plovdiv (Table 3).

Table 3. Distribution of rodents with *Leptospira* spp*.* DNA.

District	Total number of captured rodents	Rodents, carriers of *Leptospira* spp. (%)	Infected rodents from the total (%)
Pazardzhik	**84**	**25/84 (29.8)**	**25/109 (22.94)**
Smolyan	**17**	**5/17 (29.4)**	**5/109 (4.59)**
Plovdiv	**7**	**1/7 (14.3)**	**1/109 (0.09)**
Blagoevgrad	**1**	**0**	**0**
Total	**109**	**31/109**	**31/109 (28.44)**

Species distribution of rodents with *Leptospira* spp. DNA is presented in Fig. 1. The highest percentage of infected rodents was found for *Myodes glareolus* – 42%, followed by *Apodemus* spp. – 33% and *Microtus arvalis* – 30%. In Pazardzhik was captured 1 *Sorex minutus* which gave a positive result.

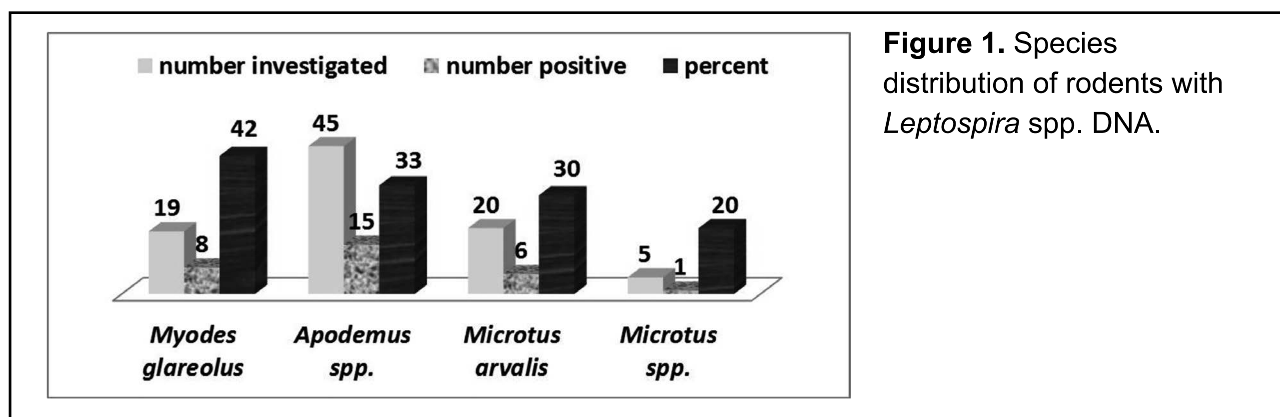

Figure 1. Species distribution of rodents with *Leptospira* spp. DNA.

The lowest percentage of infected rodents was found for *Microtus* spp. – 20%. The percentage of infected rodents by species in the region of Pazardjik is presented in Fig. 2. The highest percentage was found for *Apodemus* spp. – 13% (11/84), followed by *Myodes glareolus* – 8% (7/84) and *Microtus arvalis* – 7% (6/84).

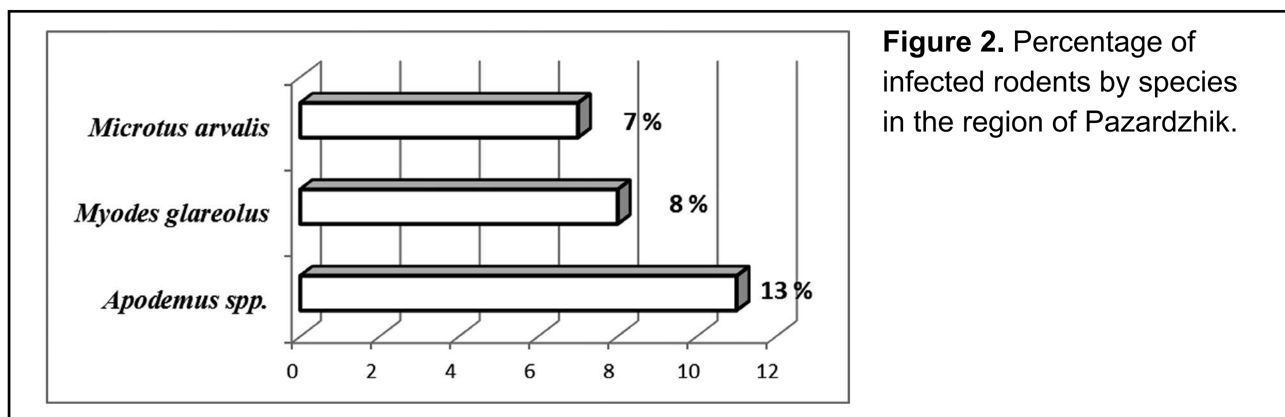

Figure 2. Percentage of infected rodents by species in the region of Pazardzhik.

The percentage of adult specimens infected with leptospiral DNA was 30% (30/100), and of newborns – 11.11% (1/9). The percentage of infected male rodents was 29.79% (14/47), exceeding that of females – 27.42% (17/62).

DISCUSSION

In Bulgaria large-scale studies on natural reservoirs of leptospirosis have been conducted in the past (1958-1980) (9, 10). A relatively high proportion of animals positive for leptospirosis was established in Southwestern Bulgaria. This indicates the presence of active natural outbreaks. In the present study, rodents carrying *Leptospira* spp. DNA were found in 3 areas. The highest percentage of infected rodents was found for the region of Pazardzhik – 22.94%. *Leptospira* spp. genome was not detected in the species *Mus musculus* and *Microtus* spp. In Smolyan region 29.41% (5/17) were infected but the number of captured rodents was quite small (only 17). *Leptospira* spp. DNA was present in the species *Apodemus* spp. and *Myodes glareolus.* In Plovdiv region 1 of the captured *Microtus* spp. was infected, whereas *Mus macedonicus* specimens were not infected. In Pazardzhik region the only captured specimen of *Sorex minutus* was infected. The proportion of captured rodents from this species is significantly low – 0.9%, and therefore, it is not statistically significant (Table 1).

The representatives of genus *Apodemus* are found in wetlands in many regions of the country but with low settlement density. Wild murine rodents of the species *Apodemus agrarius* are a reservoir of serogroup Pomona serovar Mozdok (8). In the past, the highest infection rate was found in Petrich (30.1%) and Srebarna Nature Reserve (18.6%). Because of the low settlement density, *Apodemus* species are not capable of sustaining leptospires from this serogroup for long time. Therefore, the infection rate of this species found in our study is comparatively lower – 33% (Fig. 1). The infection rate of *Myodes glareolus* is higher –42.10%, probably due to the more extensive distribution of this species, especially in wet woodlands. Similar studies have been conducted in a number of European countries. In Croatia was found a high infection rate of rodents from the species *Apodemus* spp. and *Myodes glareolus* with *Leptospira* – 21.5% (16). In Palermo (Italy), the percentage of infected rodents captured near green areas and a small river reached 40%. This was due to the climate change in Sicily from dry and hot to subtropical, with hot wet summer and a sudden storm favouring the spread of pathogenic leptospires (19).

In our country there are 2 species belonging to the family *Soricidae*: *Crocidura leucodon* and *Crocidura suaveolens*. In previous studies, there were positive serological findings of leptospirosis

in 1 out of 267 specimens of *Crocidura suaveolens* (serogroup Icterohaemorrhagiae) (9). No infected rodents of this species were found in the present study. The overall percentage of infected rodents in our study is close to that observed in France – 34.7% (2), in Poland the rates were between 2 and 40% (17), while in Germany – 21% (15). The high rate of infected rodents demonstrated by this study indicates a risk of high level of soil contamination with urine from these animals. The close interaction between humans, animals, water and soil also determines the possibility of outdoor animal infestation. Considering the significantly high percentage of infected *Microtus* spp. rodents – 50% (Fig.1), although only a small number of specimens were captured, it can be concluded that this species is a potential carrier of *Leptospira*, especially in urban areas. Secondary habitats of *Microtus* spp. are farmland and shallow-sloping terrains, and therefore it should be taken into account their movement from dense forests and meadows to urban grasslands and the potential contamination affecting primarily activities such as gardening.

CONCLUSIONS

The role of small rodents in the epidemiology of leptospirosis is elusive and leaves some aspects unclear. The relationship between reservoirs, people and animals in the epidemiological chain of this infection should be studied further, especially at the molecular biology level. In the future, it would be worthwhile to carry out a more detailed study on detection of *Leptospira* in a larger number of rodents from more areas of the country.

The control of the disease in Bulgaria is limited because our knowledge of the main aspects of epidemiology, including the mode of transmission of *Leptospira* among rat populations, remains incomplete. In our country, after heavy rainfall and floods, there was an increase in the number of cases of leptospirosis in certain regions, which is probably related to an increase in the population of rodents (3). The results we obtained confirm the circulation of *Leptospira* in the reservoirs. The combination of heavy rainfall and the accumulation of waste in deserted buildings and riverbeds are a prerequisite for the populations of rats and murine rodents to grow in urban areas as well. Further research is needed to provide new perspectives for epidemiological surveillance.

REFERENCES

1. Adler B, de la Pena Moctezuma A. *Leptospira and leptospirosis*. Vet Microbiol. 2010; 140:287-296.
2. Aviat F, Blanchard B, Michel V, Blanchet B, Branger C, Hars J, Mansotte F, Brasme L, de Champs C, Bolut P, Mondot P, Faliu J, Rochereau S, Kodjo A, Andre-Fontaine G. *Leptospira exposure in the human environment in France: A survey in feral rodents and in fresh water.* Comp Immunol Microbiol Infect. 2009; 32:463-476.
3. Christova I, Tasseva E. *Human leptospirosis in Bulgaria,* 2010-2014. Probl Infect Parasit Dis. 2016; 44(2): 23-29.
4. Ciceroni L, Stepan E, Pinto A, Pizzocaro P, Dettori G, Franzin L, et al. *Epidemiological trend of human leptospirosis in Italy between 1994 and 1996.* Eur J Epidemiol. 2000; 16:79–86.
5. Koizumi N, Muto M, Tanikawa T, Mizutani H, Sohmura Y, Hayashi E, et al. *Human leptospirosis cases and the prevalence of rats harbouring Leptospira interrogans in urban areas of Tokyo, Japan.* J Med Microbiol. 2009; 58:1227-1230.
6. Krojgaard LH, Villumsen S, Markussen MD, Jensen JS, Leirs H, Heiberg AC. *High prevalence of Leptospira spp. in sewer rats (Rattus norvegicus).* Epidemiol Infect. 2009; 137:1586-1592.
7. Lau CL, Smythe LD, Craig SB, Weinstein P. *Climate change, flooding, urbanization and leptospirosis: fuelling the fire?* Trans R Soc Trop Med Hyg. 2010; 104:631-638.
8. Manev Ch. *Distribution and etiological structure of leptospirosis from serogroup Pomona in Bulgaria*. Habilitated Labor, Sofia, 1976; 1-215.
9. Manev Ch. *Antigenic characteristics and tanks of leptospires in Bulgaria*. Dissertation for Doctor of science, 1986; Sofia, 1-337.
10. Mateev D, Kuyumdzhiev D, Stoyanov D. *Natural reservoirs of leptospiroses in Petrich and Gotse Delchev. C: Natural outbreaks in Petrich and Gotse Delchev*, 1962; BAS, 33-49.
11. Merien F, Amouriauz P, Perolat P, Baranton G and Saint Girons I. *Polymerase chain reaction for detection of Leptospira spp. in clinical samples.* J Clin Microbiol. 1992; 30:2219-2224.
12. Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. *Environmental and behavioural determinants of leptospirosis transmission: a systematicreview*. PLoS Negl Trop Dis. 2015; 9:e0003843.
13. Papa A, Theoharidou D, Antoniadis A. *Pulmonary involvementand leptospirosis*, *Greece*. Emerg Infect Dis. 2009; 15:834-835.
14. Peshev T, Peshev D. *Determination of vertebrates in Bulgaria*; 2000.
15. Runge M, von Keyserlingk M, Braune S, Becker D, Plenge-Bönig A, Freise JF, Pelz HJ, Esther A. *Distribution of rodenticide resistance and zoonotic pathogens in Norway rats in Lower Saxony and Hamburg, Germany.* Pest Manag Sci. 2012; 69:403-408.
16. Tadin A, Tokarz R, Markotić A, Margaletić J, Turk N, Habuš J, Svoboda P, Vucelja M, Desai A, Jain K, Lipkin WI. *Molecular Survey of Zoonotic Agents in Rodents and Other Small Mammals in Croatia.* Am J Trop Med Hyg. 2016; 94(2):466-473.
17. Theuerkauf J, Perez J, Taugamoa A, Niutoua I, Labrousse D, Gula R, Bogdanowicz W, Jourdan H, Goarant C. *Leptospirosis risk increases with changes in species composition of rat populations.* Naturwissenschaften. 2013; 100:385-388.
18. Vinetz JM, Glass GE, Flexner CE, Mueller P, Kaslow DC. *Sporadic urban leptospirosis.* Ann Intern Med. 1996; 125:794-798.
19. Vitale M, Di Bella C, Agnello S, Curro V, Vicari D, Vitale F. *Leptospira interrogans survey by PCR in wild rodents coming from different urban areas of Palermo, Italy.* Rev Cubana Med Trop. 2007; 59(1):59-60.

T CELL EFFECTOR AND REGULATORY SUBSETS, DIFFERENTIATING BETWEEN ACTIVE AND LATENT MTB INFECTION

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ABSTRACT

Last generation IFN gamma – based assays (IGRAs) evaluate bulk CD4 and CD8 T cell responses, and do not discriminate between latent and active *Micobacterium tuberculosis* (MTB) infection. The identification of biomarkers predicting the clinical course and specific therapy effect in latent MTB infection (LTBI) is a major contemporary challenge.

Using multicolor flow cytometry, we compared the levels of circulating CD8 and CD4 effector subsets, in relation to the levels of phenotypically defined regulatory subsets, in two groups of age- and sex-matched MTB-infected individuals: clinically and microbiologically confirmed ATB (n=15), and QFT+ stable LTBI (n=15).

As compared to LTBI subjects, ATB patients are characterized with decreased proportions of CD4 and CD8 CD45RO+CCR7- effectors (14.6% vs. 24%, and 28% vs. 40%, $p < 0.05$ for both), decreased Th1 (10% vs. 16,5 %) and Th1/Th17 (12,5% vs. 21,5%) effector subsets. These changes are accompanied by a significantly increased share of induced (CD39+) FoxP3+CD4Treg (46% vs. 22.6%, $p < 0.05$). The difference affected mostly the Th17-specific (CD39+CCR6+Treg) subset (10.5% vs 4.8%, $p < 0.05$), which correlated inversely with the level of Th1/Th17 effectors ($R = -0.5$, $p < 0.05$).

In conclusion, we describe a clear-cut distinction between the effector/ regulatory T subset balance in ATB and LTBI. The combined evaluation of Th17Treg and Th1/Th17 effectors in peripheral blood can be employed for MTB-infection monitoring.

KEYWORDS:
LTBI, Th1/Th17, CD39+Treg, Th17Treg

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INTRODUCTION

Micobacterium tuberculosis (MTB) infection is still a major healthcare concern worldwide. About one third of world's population is estimated to be infected with MTB, while only 10% would develop active disease [1]. Latent MTB infection (LTBI) represents an enormous reservoir of the pathogen, of high concern in the ageing populations with increased risk of secondary immune deficiencies [2]. The best approach to limit the spread of the infection is to detect latent infections and prevent their activation. The recently developed interferon gamma release assays (IGRA) recommended for diagnosis of LTBI evaluate the production of IFN-γ in response to MTB-specific peptides [3]. In spite of a very high specificity and sensitivity, IGRAs do not discriminate between latent and active TB [4]. Yet, there is no consensus that the quantity of MTB-specific IFN-γ is related to the activity of pathogen and predicts disease progression or therapy effect. This requires additional and handy phenotypic and functional biomarkers discriminating between phases of TB disease [5].

A lot of evidence indicates the critical role of CD8 T cells for MTB control. On this grounds the last generation IGRA (QFT-GIT Plus) evaluates both CD4 and CD8 MTB-specific responses [6-8]. The efficiency of CD8 T cell response depends on the balanced differentiation of effector and memory pathogen-specific cells. This balance is finely regulated by induced CD4+ FoxP3+ T cells (iTreg) with inhibitory potential [9]. We and others have previously shown that in conditions of chronic infection, such as HIV, HCV or HBV, the differentiation, subset composition and function of FoxP3+Treg is also affected, which contributes to the absence of protective immune response [10]. Likewise, CD4 T effector and regulatory subsets (Th1, Th2, Th17, Treg) are of remarkable plasticity, depending on antigen burden and individual cytokine background [11]. The balance between Th effector and regulatory subsets has not been extensively studied during the different phases of MTB infection.

In this study, we compared the levels of CD8 and CD4 effector and memory subsets, in relation to the levels of circulating regulatory subsets, in two well-defined groups of MTB-infected individuals: clinically and microbiologically confirmed ATB, and QFT+ stable LTBI.

MATERIAL AND METHODS

Samples were collected during the routine diagnostic activity of NRLI from 40 men, and 52 women, average (min-max) age 52 (18-76) years. Additional samples of 4 ml peripheral blood were drawn, PMNC were isolated, and frozen after informed consent, approved by the local ethics committee. IGRAs were performed using QFT-GIT-Plus (Qiagen) and T-spot (T.SPOT.TB Oxford Immunotec) tests, according to manufacturer's instructions. ATB status was defined based on a combination of clinical, microbiological and radiological data; LTBI was defined as asymptomatic MTB infection, detected by immunological test, in the absence of clinical, microbiological or radiological data for tuberculosis [3]. Percentage and absolute count (AC) of CD4+CD3+ and CD8+CD3+T cells were determined by direct flow cytometry platform, using MultiTest and TRUCount standard tubes (BD Biosciences). Based on the co-expression of CD45RO and CCR7, naïve (RO-R7+), central-memory (RO-R7-), effector-memory (RO+R7-) and terminally differentiated (RO-R7-) CD4 and CD8 T were defined. Co-expression of CD196 (CXCR6) and CD183 (CXCR3) was used to define Th1 (CXCR3+CCR6-), Th2 (CXCR3-CCR6-), Th1/Th17 (CXCR3+CCR6+), and Th17 (CXCR3-CCR6+) effector subsets. T-regulatory CD4 FoxP3+ cells were defined as CD4+ $CD25^{hi}CD127^{lo}$. Within Treg, the share of CD39+Treg and CD39+CD196+Treg was determined. All flow cytometry analyses were performed with 6 to 8-color combinations and FACSDiva 6.1.2 software (FACSCanto II, BD Biosciences). Statistical analysis was performed with parametric T-test or Spearman's rank correlation test for quantitative data (GraphPad v.6).

RESULTS

1.**Bulk MTB-specific interferon-gamma responses of CD4 and CD8 T cells does not differentiate between ATB and LTBI.**

During the period 01.2018 – 04.2019, 92 consecutive patients were tested for MTB infection with the last generation IGRA QFT-GIT-Plus. ATB cases (n=28) and LTBI cases (n=27) were defined as stated in M&M section. In 8 (27.5%) ATB cases QFT-GIT-Plus results were negative (TB1–Nil< 0.35 and TB2–Nil < 0.35) and 2 were indeterminate (6.9%). The average interferon-gamma secretion (IU/ml) in response to TB1 and TB2 was as follows: 2.29 and 2.40, respectively, in ATB; 3.62 and 3.57 - in LTBI. No significant differences were established between CD4 and CD8 specific responses within each group (paired T-test p>0.05), or between the two groups (non-paired T-test p>0.05), (Fig.1). Further on, no domination of TB1 (8/55) or TB2 (12/55) responses could be attributed to either ATB or LTB. Therefore, we concluded that analyzing separately bulk CD8

and CD4 T cell responses did not contribute to the discrimination between ATB and LTBI.

2. ATB patients are in shortage of CD4 Th1, Th1/Th17 and CD8 effector T cells.

In order to compare the proportions of phenotypically defined CD4 and CD8 effector and memory subsets in ATB and LTBI, we retrospectively selected age- and sex-matched subgroups. The absence of known secondary immune deficiency or autoimmune condition was additional criterion.

A significantly lower proportion of EM CD4 and CD8 T cells was observed in ATB as compared to LTBI (14.6% vs. 24%, and 28% vs. 40%, p <0.05 for both comparisons), **Fig.2A.** While the level of Th17 (CD183-CD196+) cells seemed unaffected (25 vs. 29 % in LTBI), the Th1 (CD183+CD196-) subset was decreased (10% vs. 16,5 % in LTBI, p<0.05). A significantly lower Th1/Th2 ratio (0,34 vs. 0,54) reflected an additional increase of Th2 cells in ATB (49,5% vs. 34% in LTBI). Finally, the Th1/Th17 (CD196+CD183+) subset that was shown to contain most of the MTB-specific effectors [12], was importantly decreased in ATB, as compared to LTBI (12,5% vs. 21,5%, p<0.05), Fig.2B. The changes observed in ATB corresponded to a prominent humoral response and deficient cellular immunity in the presence of the vigorously replicating pathogen.

3. The subsets of CD39+ and CCR6+Treg contribute to the exhaustion of T cell effector functions in ATB

Since the balance between antigen-specific effector and memory subset differentiation depends on iTreg, we further evaluated their proportions and subset composition in the ATB and LTBI subgroups. While the difference between the levels of FoxP3+ CD4 in ATB and LTBI did not reach statistical significance (6,6% vs. 4,9% and 38 vs. 40 cells/μl), ATB was characterized with a domination of CD39+Treg, corresponding to induced Treg with strong inhibitory activity (46% vs. 22.6%, p<0.001), **Fig. 3A, B**. In addition CD196+ CD39+ Treg that were proposed to inhibit specifically Th17 [13] were importantly increased in the settings of ATB as compared to LTBI (10.5% vs 4.8%), **(Fig. 4A,B)** These distinct Treg proportions corroborated with the skewed differentiation of CD4 and CD8 effector cells in ATB. We concluded that the increased bacterial load in ATB drives the differentiation of Treg subsets that affect the balanced differentiation of effector and memory pools, and of Th1 and Th17 responses. Indeed, a significant inverse correlation was established between the level of Th1/Th17 effectors, and the Th17-specific Treg (R=-0.5, p<0.01, **Fig. 4C**. In a limited number of ATB patients (n=5) who had completed a specific therapy course, the proportion of CD39+Treg significantly decreased in successfully treated patients (35% vs. 46%), accompanied with consequent increase of Th17 effectors (43 vs. 25%).

DISCUSSION:

Adequate monitoring and control of LTBI are important to prevent ATB and limit the spread of infection. Our results show that the proportions of Th1/Th17 effectors and the lineage-specific CD196+CD39+Treg (Th17Treg) subset change reciprocally in relation to bacterial burden and may differentiate between stages of MTB-infection.

It is well recognized that adaptive immune response to MTB relies mostly on T cells. MTB antigens are primarily presented to CD4 T cells, and elicit a Th1 response dominated by IFN-γ production. Current immune tests rely exclusively on MTB-specific IFN-γ detection. However, a number of large studies and meta-analyses have suggested that the predictive value of IGRAs for progression to TB disease, and for benefit from specific therapy remains unsatisfactory and not significantly higher than that of the TST. [14-17].

We analyzed results obtained with last generation IGRA (QFT-GIT), containing additional CD8 T cell-stimulating MTB antigens (TB2). Indeed, CD8 T cells can also release IFN-γ after stimulation with MTB antigens, even without

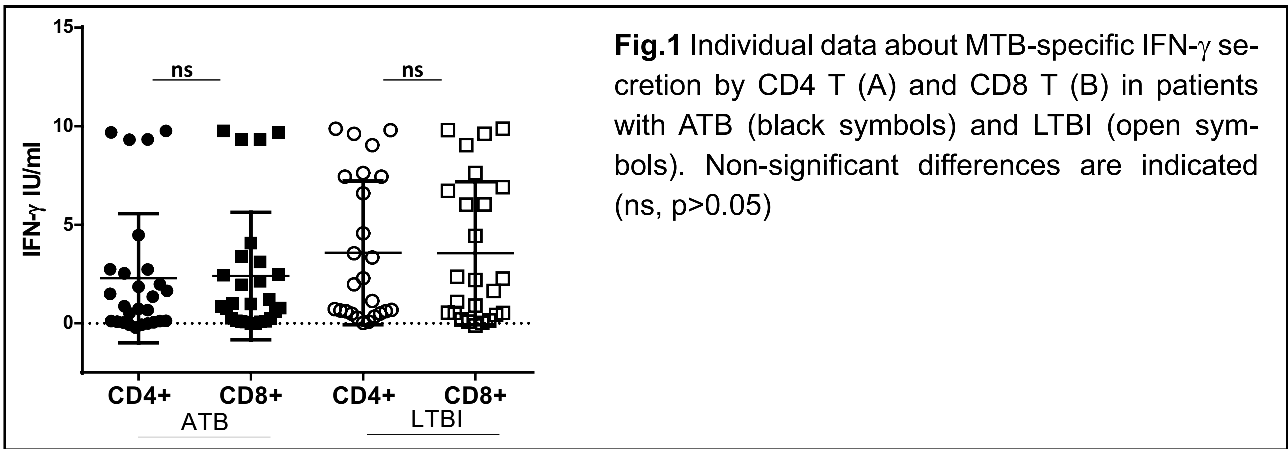

Fig.1 Individual data about MTB-specific IFN-γ secretion by CD4 T (A) and CD8 T (B) in patients with ATB (black symbols) and LTBI (open symbols). Non-significant differences are indicated (ns, $p>0.05$)

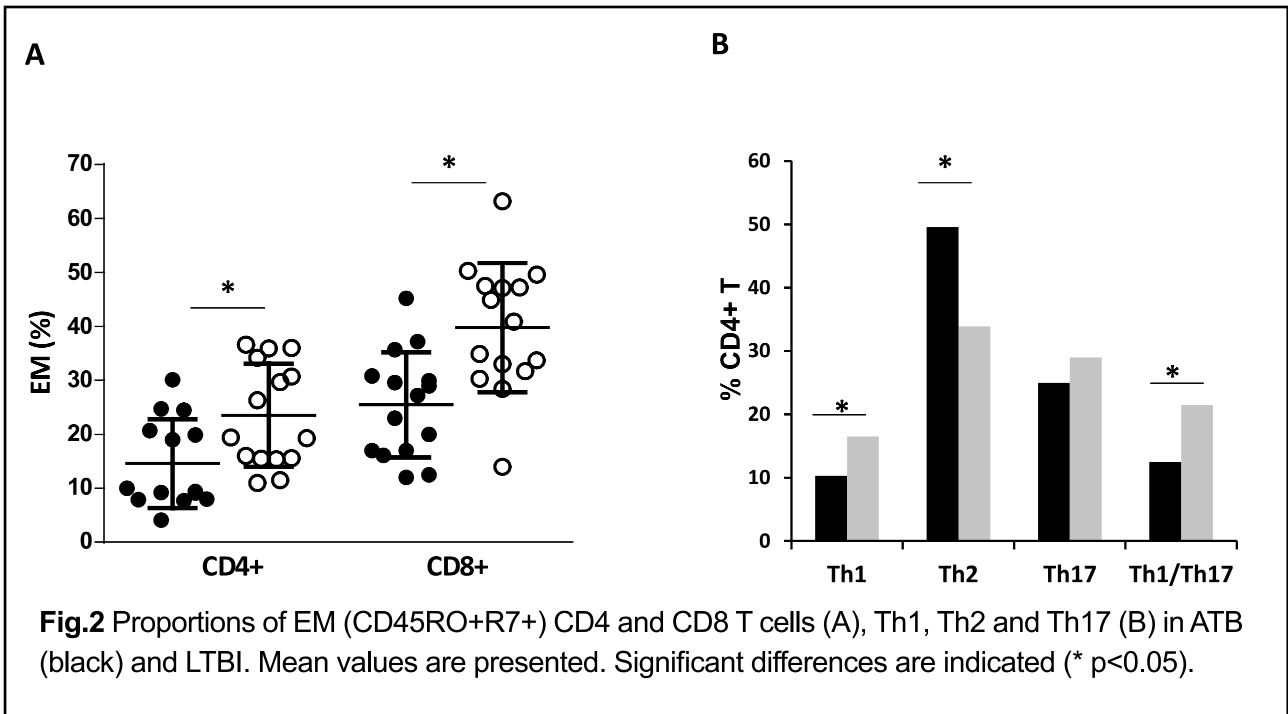

Fig.2 Proportions of EM (CD45RO+R7+) CD4 and CD8 T cells (A), Th1, Th2 and Th17 (B) in ATB (black) and LTBI. Mean values are presented. Significant differences are indicated (* $p<0.05$).

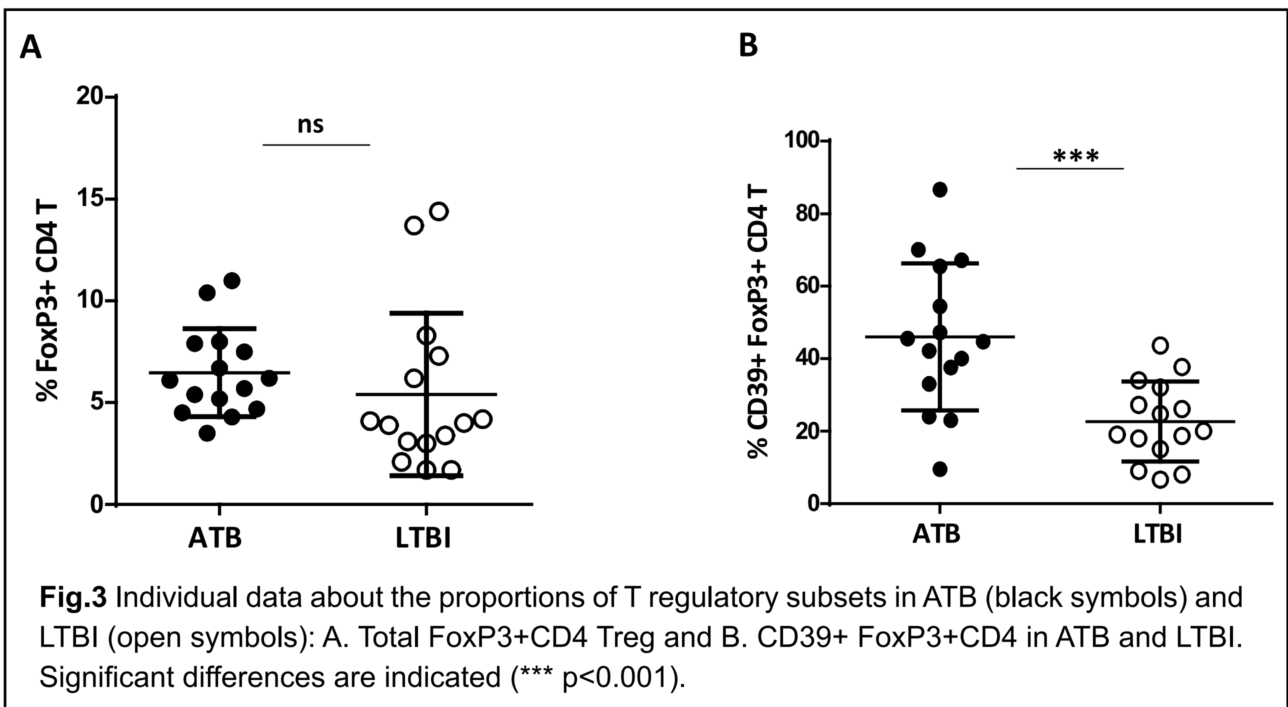

Fig.3 Individual data about the proportions of T regulatory subsets in ATB (black symbols) and LTBI (open symbols): A. Total FoxP3+CD4 Treg and B. CD39+ FoxP3+CD4 in ATB and LTBI. Significant differences are indicated (*** $p<0.001$).

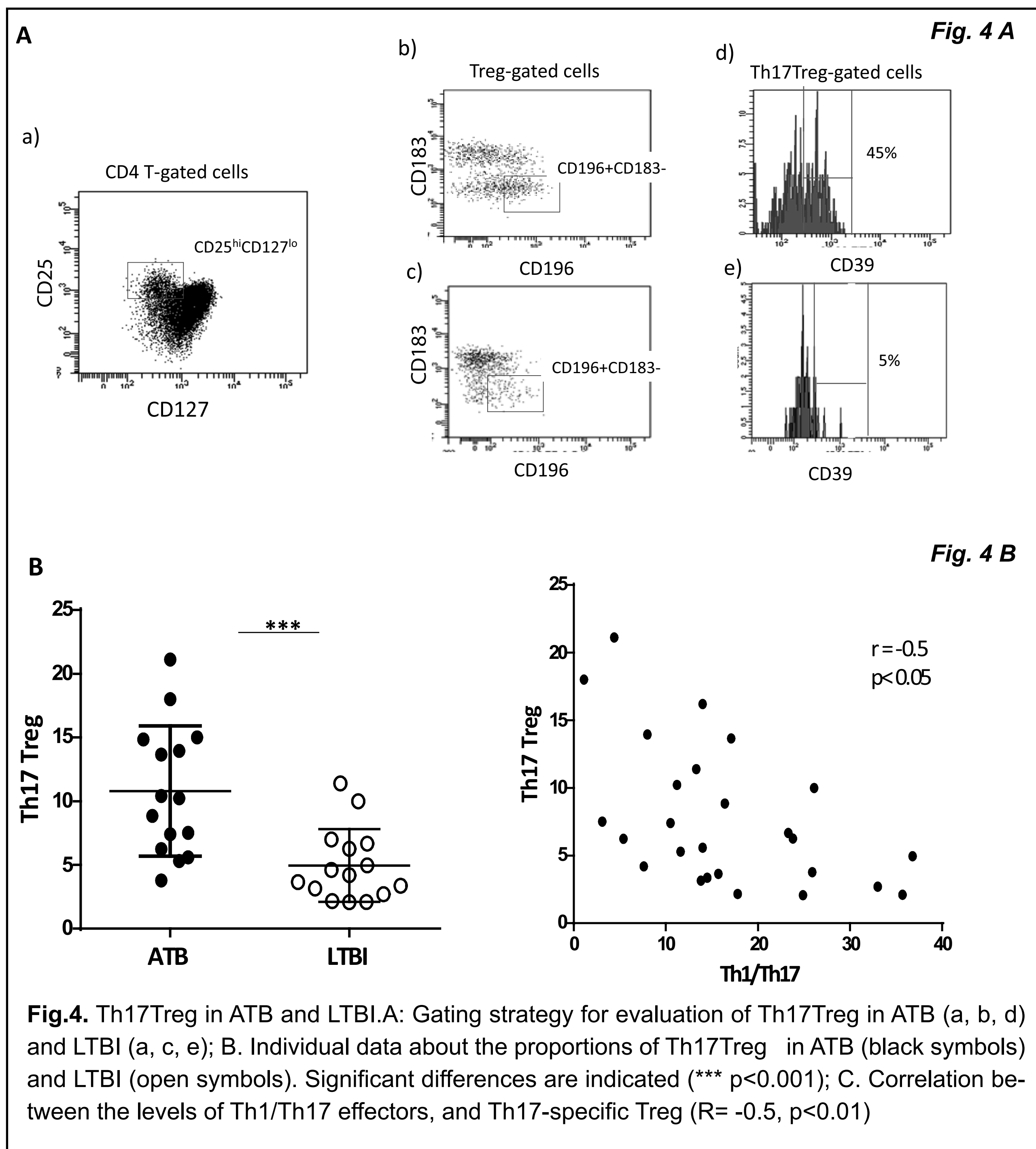

Fig.4. Th17Treg in ATB and LTBI.A: Gating strategy for evaluation of Th17Treg in ATB (a, b, d) and LTBI (a, c, e); B. Individual data about the proportions of Th17Treg in ATB (black symbols) and LTBI (open symbols). Significant differences are indicated (*** $p<0.001$); C. Correlation between the levels of Th1/Th17 effectors, and Th17-specific Treg ($R= -0.5$, $p<0.01$)

help from CD4+ T-cells, preferentially recognize cells from a host heavily infected with MTB, and the magnitude of their response correlates well with the bacterial load and smear-positivity [18 – 21]. Using a different approach (flow cytometry ICS) we have previously compared CD8 T cell responses elicited against RD-1 antigens, and reported differences between patients with active TB, LTBI, and recent MTB infection [22]. Therefore, it may be expected that evaluation of CD8 T response would not only increase the sensitivity of the assay, but also - its ability to differentiate between phases of the latent and active MTB “spectrum”. However, the reports on QFT-GIT-Plus are rather contradictory. Petruccioli et al. found that the majority of LTBI subjects simultaneously responded to both TB1 and TB2 antigens, and that an “only to TB2” response was associated with active TB [7]. Another study among older adults categorized most of those with “only to TB2” response as definite LTBI [8]. Although in a limited number of patients, our results did not establish an association between a predomi-

nant TB1 or TB2 response and the activity of MTB infection. Most probably, specific CD4 and CD8 T cell subsets are associated with the activity and stage of MTB infection.

In a simplified scheme, antigen-specific T-cell response leads to differentiation of both effector cells, eliminating antigen burden, and long-living memory cells, preventing new infections. This process is strictly regulated by FoxP3+ T cells with inhibitory potential, induced simultaneously with the effectors. The balance memory/effector/regulatory cells is crucial for a protective immune response [10]. In chronic infections, this balance is skewed, either towards accumulation of immature and poorly functional effectors, [23, 24] or - in excessive differentiation and exhaustion of the effector potential [25].

According to our results, ATB was distinguished by decreased shares of EM (CD45RA-CCR7-) CD4 and CD8 T cells. In addition, ATB was associated with accumulation of Th2-like effectors at the expense of Th1, and Th17 cells.

We have previously described accumulation of CD45RA-CD27+ CD8 T cells in stable LTBI as compared to ATB [26]. Our present results are not contradictory, since the CD45RA-CD27+ subset is contained within the CD45RA/CCR7-defined EM population. Phenotypically defined T cell differentiation stages correlate with certain functional capacities. The EM subset contains mostly IFN-γ and IL-2 producing cells, while TE (CD45RA+CCR7-) cells have decreased IFN-γ and increased perforin and TNF- α secretion potential [27]. In line with our results, Sutherland et al. reported a significantly higher proportion of cells single-positive for TNF-α, but a lower proportion of cells producing IL-2 alone in TB cases compared with contacts, for both CD4(+)and CD8(+) T cells [28].

Th1, Th2, and Th17 are of major importance for protection against MTB [29]. The role of Th1-type cytokines, including IL-12, IFN-γ, IL-2, and TNF-α in MTB-specific response is well recognized. Genetic defects leading to IL-12/IL-12R deficiency are associated with a high susceptibility to MTB infection [30]. Multi-functional (IFNγ+ TNFα+ IL-2+) MTB-specific CD4+ T cells have been defined as a correlate of protective immune response [28, 31]. The role of Th17-related cytokines is not so evident. They were shown to contribute to immune protection against primary MTB infection: suppressing IL-17A production increases TB susceptibility, since IL-17A is involved in the formation and stability of granulomas; IL-17 expression levels were found decreased in TB patients by different studies [32, 33]. At the same time, Th17 responses in TB may also participate in pathology through recruitment of neutrophils and promotion of inflammatory responses, and some studies find significantly higher percentages of Th17 in active TB patients than in latent TB or control groups [34, 35].

The Th1/Th17 (CXCR3+CCR6+) population producing both IL-17 and IFN-γ was only recently identified. Th1/Th17 cells were shown to include more than 80% of the MTB-reactive memory CD4 T cell response, and were reported as the main source of IL-17 in MTB infection, regulated by IFN-γ [11, 36, 37]. According to our results, the most prominent difference regarding T cell effector subsets in LTBI and ATB subjects was a decreased level of Th1/Th17 cells. Data about the dynamics of this subset during the phases of MTB infection are scarce. Unlike us, Jurado et al. reported markedly augmented levels of IL-17 in ATB patients and concluded that the ratio of antigen-expanded CD4+IFN-γ+IL-17+ lymphocytes correlated directly with disease severity [36]. However, the comparison was with BCG-vaccinated healthy donors and not with LTBI. These contradictory results only underline the critical importance of Th1/Th17 cytokine balance in MTB infection, provided by Treg and the necessity to evaluate effector and regulatory subsets in parallel.

Our study shows that the changes in effector subsets during ATB are accompanied with significant increase in particular Treg subsets, and these changes are transient after treatment.

The physiological role of Treg is to fine-tune the memory-effector cell balance. In condi-

tions of high antigen load Treg would prevent the extreme differentiation of effectors and eventual loss of important specificities, and also prevent collateral damage [22]. A number of studies reported elevated levels of circulating iTreg in the settings of chronic infection, including MTB. Expansion of Treg has been observed both at organ-specific sites and in blood in active TB. [34, 36, 38] Feruglio et al. demonstrated a transient increase in ex-vivo Treg levels during therapy [38], while decreasing levels of Tregs have been reported in response to TB treatment by others [35, 39]. High Treg levels are also seen in response to preventive treatment for latent TB [40]. However, all these studies have compared ATB with healthy controls, and not with LTBI patients. We did not find a significant difference between the levels of the entire FoxP3+ population in LTB and ATB. Therefore, we supposed that specific subsets as CD39/Treg and CCR6+Treg might be significantly associated with infection activity.

CD39 is the dominant immune system ectonucleotidase that hydrolyses extracellular ATP and ADP, and in concert with CD73 generates highly suppressive adenosine monophosphate at the sites of immune activation. In humans, CD39 expression is highly variable and restricted to a subset of Foxp3+ Treg [41]. Increased CD39+Treg subset has been reported in chronic HIV,HBV and HCV infection, as a direct sequence of pathogen-induced response. Therefore, it is legitimate to consider CD39+Treg as iTreg. In the long term, CD39-mediated inhibition of T cell proliferation might exert an adverse effect on the immediate generation of T-cell immune responses, thus contributing to disease progression. Most available data about CD39+Treg in MTB infection are based on *in vitro* stimulation studies. While Feruglio documented a decline in CD39 expression after MTB antigen stimulation [38], Chiacchio et al. showed that the fraction of CD39+ Treg in active TB increased after in-vitro stimulation and that depletion of CD39 improved TB-specific responses [42]. Aggrawal found a markedly higher expression for CD39 (in terms of MFI) when compared between ATB and healthy controls. Higher frequencies of CD39+Treg were observed in [TST+, QTF+] individuals when compared with [TST-, QTF-] control group [40, 43]. In our hands, CD39+T-cells were markedly increased in ATB as compared to LTBI, and readily decreased upon treatment. In line with our results, Kim et al. concluded that MTB-reactive CD39+CD4+ T cells were more abundant in active TB than LTBI [39].

Recently, a new subset of RORγt+Foxp3+ Treg (Tr17) cells was described, generated in an antigen-specific manner through Stat3 signaling. These effector Treg express CCR6, and hence can be recruited within the inflammatory milieu and regulate Th17-dependent immune responses [44, 45]. In fact, only activated CD39+Tregs co-expressing Th17-associated markers CCR6 and IL-23R, phosphorylate Stat3, and not the CD39-negative ones. Thus the CD39+CCR6+Treg subset is the first lineage-specific Treg subset in human [46]. Our results show that Th17-specific Treg are the regulatory subset that differs most significantly between ATB and LTBI, correlates best with the level of Th1/Th17 effectors, and may be used to monitor the course of infection.

The balance Th1/Th17/Treg determines the clinical course of MTB infection. This balance is based on the possibility of Th1, Th17 and Treg cells to transdifferentiate, one into each other, including the Th1/Th17 stage, and depends on shared differentiation pathways, the characteristics of antigen burden, and cytokine background [33]. Overall, Th17 responses fit well with the situation of stable LTBI, whereby the stability of granulomas is important, while the prevalence of Th1 over Th1/Th17 lymphocytes, is the basis of protection against replicating MTB. However, overproduction of IL-17, accompanied by increase of IL-10-producing Treg in LTBI was associated with a high risk of reactivation [47]. Similarly, too strong proinflammatory Th1 reponse, leading to too strong

Treg-mediated inhibition may be detrimental for efficient antigen-specific response.

In conclusion, we describe a clear-cut distinction between the effector/regulatory T subset balance in ATB, and LTBI, and propose the combination of Th17 Treg and Th1/Th17 subset as accessible markers differentiating between latency and disease.

REFERENCES

1. GLOBAL TUBERCULOSIS REPORT 2018© World Health Organization 2018
2. Panajotov S, Velev V, Kostov T, Kantarjiev T, Levterova V. *79-year old man with wrist-joint tuberculosis – case report.* Probl Infect Parasit Dis. 2003 31 (2):22.
3. Методическо указание за насочване, диагноза, проследяване и лечение на лицата с латентна туберкулозна инфекция. Министерство на здравеопазването, 2016. Утвърдено със Заповед № РД-01-74/10.03.2016 г. Налично на: http://www.mh.government.bg/
4. Andersen P, Doherty TM, Pai M, Weldingh K. *The prognosis of latent tuberculosis: can disease be predicted?* Trends Mol Med. 2007 May;13(5):175-82.
5. Pai M., Denkinger C., Kik S. et al *Gamma Interferon Release Assays for Detection of Mycobacterium tuberculosis Infection.* Clinical Microbiology Reviews. 2014; 27 (1): 3–20
6. Yi L., Sasaki Y, Nagai H, et al. *Evaluation of QuantiFERON-TB Gold Plus for Detection of Mycobacterium tuberculosis infection in Japan* Scientific Reports. 2016; 6: Article number: 30617
7. Petruccioli E, Vanini V, Chiacchio T, et al A*nalytical evaluation of QuantiFERON-Plus and QuantiFERON-Gold In-Tube assays in subjects with or without tuberculosis*. Tuberculosis (Edinb) 2017; 106:38 – 43.
8. Chien J, Chiang H, Lu M et al. *QuantiFERON-TB Gold Plus Is a More Sensitive Screening Toolthan QuantiFERON-TB Gold In-Tube for Latent TuberculosisInfection among Older Adults in Long-Term Care Facilities* Journalof Clinical Microbiology. 2018; 56: 8 e00427-18
9. Nikolova M, Lelievre JD, Carriere M, et al. *Regulatory T cells differentially modulate the maturation and apoptosis of human CD8+ T-cell subsets*, Blood 2009 113:4556-4565;
10. Nikolova M, Wiedemann A, Lacabaratz C, Lévy Y. *Deficiency of HIV-specific T cell responses: are the Treg guilty?* Med Sci (Paris). 2017 Aug-Sep;33 (8-9):723-726.
11. Caza T, Landas S. *Functional and Phenotypic Plasticity of CD4+ T Cell Subsets* Biomed Res Int. 2015; 2015: 521957.
12. Lindestam Arlehamn CS, Gerasimova A, Mele F,et al. *Memory T Cells in Latent Mycobacterium tuberculosis Infection Are Directed against Three Antigenic Islands and Largely Contained in a CXCR3⁺CCR6⁺ Th1 Subset.* PLoS Pathog. 2013 9(1): e1003130
13. Magid-Bernstein JR and Rohowsky-Kochan CM. *Human CD39+ Treg Cells Express Th17-Associated Surface Markers and Suppress IL-17 via a Stat3-Dependent Mechanism* J Interferon Cytokine Res. 2017 Apr 1; 37(4): 153–164
14. Rangaka MX, Gideon HP, Wilkinson KA, et al. *Interferon release does not add discriminatory value to smear-negative HIV-tuberculosis algorithms*. Eur. Respir. J. 2012. 39:163–171.
15. Metcalfe JZ, Cattamanchi A, Vittinghoff E, et al. *Evaluation of quantitative IFN-gamma response for risk stratification of active tuberculosis suspects.*. Am. J. Respir. Crit. Care Med. 2010.181:87–93.
16. Ling DI, Pai M, Davids V, et al. *Are interferon-gamma release assays useful for diagnosing active tuberculosis in a high-burden setting?* Eur. Respir. J. 2011.38:649–656.
17. Ling DI, Nicol M, Pai M, et al. *Incremental value of T-SPOT.TB for diagnosis of active pulmonary tuberculosis in children in a high-burden setting: a multivariable analysis.* Thorax 2013. 68:860–866.
18. Lewinsohn DA, Heinzel AS, Gardner JM, et al. *Mycobacterium tuberculosis–specific CD8+ T cells preferentially recognize heavily infected cells*. Am J Respir Crit Care Med 2003; 168: 1346–1352.
19. Day CL, Abrahams DA, Lerumo L, et al. *Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load.* J Immunol 2011; 187: 2222–2232
20. Rozot V, Vigano S, Mazza-Stalder J, et al. *Mycobacterium tuberculosis-specific CD8+ T cells are functionally and phenotypically different between latent infection and active disease.* Eur J Immunol 2013; 43: 1568–1577.
21. Barcellini L., Borroni E., Brown J., et al. *First evaluation of QuantiFERON-TB Gold Plus performance in contact screening*. European Respiratory Journal 2016 48: 1411-1419;
22. Nikolova M, Markova R, Drenska R, et al. *Antigen-specific CD4- and CD8-positive signatures in different phases of Mycobacterium tuberculosis infection*. Diagn Microbiol Infect Dis 2013; 75: 277–281.Pollock KM et al. J Infect Dis. 2013;208(6):952-68 32.
23. Champagne P, Ogg GS, King AS, et al. *Skewed maturation of memory HIV-specific CD8 T lymphocytes.* Nature. 2001; 410:106–111. [PubMed] [Google Scholar]
24. Appay V, Nixon DF, Donahoe SM, et al. *HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function*. J Exp Med. 2000; 192:63–75.
25. Sacre K, Carcelain G, Cassoux N, et al. *Repertoire, diversity, and differentiation of specific CD8 T cells are associated with immune protection against human cytomegalovirus disease*. J Exp Med. 2005;201:1999–2010
26. Nikolova M., Muhtarova M., Drenska R., et al *Peripheral blood CD8 T cell responsein different phases of MTB infection*. Comptes rendus de l'Acad´emie bulgare des Sciences 2013; 66, No 4
27. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. *The who's who of T-cell differentiation: human memory T-cell subsets*. Eur J Immunol. 2013 Nov;43(11):2797-809. doi: 10.1002/eji.
28. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MO. *Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease*. Eur J Immunol. 2009 Mar;39(3):723-9. doi: 10.1002/eji.200838693.
29. Zhu J, Yamane H, Paul WE. *Differentiation of effector CD4 T cell populations*. Annu Rev Immunol (2010) 28:445–89.
30. Palamaro L, Giardino G, Santamaria F, et al. *Interleukin 12 receptor deficiency in a child with recurrent bronchopneumonia and very high IgE levels* Ital J Pediatr. 2012; 38: 46.
31. Lewinsohn DA, Lewinsohn DM, Scriba TJ. *Polyfunctional CD4(+) T cells as targets for tuberculosis vaccination*. Front Immunol. (2017) 8:1262.
32. Yoshida YO, Umemura M, Yahagi A, et al. *Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung*. J Immunol 2010; 184:4414–4422.
33. Shen H, Chen ZW. *The crucial roles of Th17-related cytokines/signal pathways in M. tuberculosis infection.* Cell Mol Immunol. 2018 Mar; 15(3):216-225.
34. Luo, J, Zhang, Mingxu, Yan, Baosong, Zhang, Kejun, Chen, Ming, & Deng, Shaoli. *Imbalance of Th17 and Treg in peripheral blood mononuclear cells of active tuberculosis patients. Brazilian Journal of Infectious Diseases*, 2017; *21*(2), 155-161
35. García JRE, Serrano CJ, Enciso Moreno JA, et al.. *Analysis of Th1, Th17 and regulatory T cells in tuberculosis case contact*s Cell Immunol. 2014 May-Jun;289(1-2):167-73
36. Gosselin A, Monteiro P, Chomont N, *Peripheral blood CCR4+CCR6+ and CXCR3+CCR6+CD4+ T cells are highly permissive to HIV-1 infection*. J Immunol. 2010

1;184(3):1604-16. doi: 10.4049/jimmunol.0903058

37. Jurado, O., Pasquinelli, V., Alvarez, I et al *IL17 and IFNγ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease.* Journal of Leukocyte Biology. 2012; 91: 991-1002. doi:10.1189/jlb.1211619
38. Feruglio S., Tonby K., Kvale D., et al *Early dynamics of T helper cell cytokines and T regulatory cells in response to treatment of active Mycobacterium tuberculosis infection.* Clin Exp Immunol. 2015;179(3): 454–465.
39. Kim K, Perera R, Tan DB, et al, *Circulating mycobacterial-reactive CD4+ T cells with an immunosuppressive phenotype are higher in active tuberculosis than latent tuberculosis infection*. Tuberculosis (Edinb). 2014 Sep;94(5):494-501. doi: 10.1016/
40. Agrawal S, Parkash O, Palaniappan AN, et al. Efficacy of T Regulatory Cells, Th17 Cells and the Associated Markers in Monitoring Tuberculosis Treatment Response. *Front Immunol*. 2018;9:157. Published 2018 Feb 5. doi:10.3389/fimmu.2018.00157.
41. Nikolova M., Carriere M., Jenabian MA., et al. *CD39/Adenosine Pathway Is Involved in AIDS Progression* PLoS Pathogens 2011. 7 : e1002110
42. Chiacchio T, Casetti R, Butera O et al. *Characterization of regulatory T cells identified as CD4+CD25highCD39+ in patients with active tuberculosis*. Clin Exp Immunol. 2009; 156:463-70
43. Serrano CJ, Castañeda-Delgado JE, Trujillo-Ochoa JL. *Regulatory T-cell subsets in response to specific Mycobacterium tuberculosis antigens in vitro distinguish among individuals with different QTF and TST reactivity*. Clin Immunol. 2015 ;157(2):145-55. doi: 10.1016/j.clim.2015.02.008...
44. Kim BS, Lu H, Ichiyama K, et al. *Generation of RORγt+ antigen-specific T regulatory 17 (Tr17) cells from Foxp3+ precursors in autoimmunity* Cell Rep. 2017 Oct 3; 21(1): 195–207.
45. Lee JJ, Kao KC, Chiu YL, et al. *Enrichment of Human CCR6+ Regulatory T Cells with Superior Suppressive Activity in Oral Cancer* J Immunol. 2017 Jul 15;199(2):467-476.
46. Kluger MA, Melderis S, Nosko A, et al. *Treg17 cells are programmed by Stat3 to suppress Th17 responses in systemic lupus.* Kidney Int. 2016 Jan;89(1):158-66. doi: 10.1038/ki.2015.296.
47. Lin PL, Maiello P, Gideon HPet al. *PET CT identifies reactivation risk in cynomolgus macaques with latent M. tuberculosis*. PLoS Pathog 2016; 12: e1005739.

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TOXOCARIASIS - WHAT DO WE KNOW? A LITERATURE REVIEW

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ABSTRACT

Toxocariasis is a helminthic zoonosis caused by the presence and migration of animal nematode larvae in human tissue – mostly *Toxocara canis* and *Toxocara cati*. The term visceral larva migrans syndrome was used for the first time in 1952 by Beaver et al. who described the typical clinical presentation. There are difficulties in the diagnosis of toxocariasis because of the variety of symptoms depending on the larva localisation in different tissues and organs. Currently, the most commonly used serological methods are ELISA and Western blot. The disease is characterised by diverse clinical picture and thus toxocariasis is very rarely identified and most patients remain undiagnosed, which requires in-depth study of this widespread but still problematic zoonosis.

KEYWORDS:

Toxocariasis, diagnosis, VLM, OLM

HISTORY OF HUMAN TOXOCARIASIS

The nematode parasites *Toxocara canis* and *Toxocara cati* were described for the first time by Werner in 1782 who initially named the dog parasite *Lumbricus canis,* and Schrank in 1788 naming the cat parasite *Ascaris cati*. In 1947 Perlingiero and György reported the first case of toxocariasis in 2-year-old boy presenting with typical symptoms – liver involvement, anaemia and fever (1). Two years later, in 1949, Zuelzer and Apt investigated 8 similar cases and described the syndrome observed in young children and characterised by pica, pulmonary involvement with fever, enlarged liver, eosinophilic granuloma, chronic blood eosinophilia, anaemia and hyperglobulinaemia (2). The aetiology of the disease was still unknown until Mercer et al. discovered in 1950 the aetiological agent – ascarid larvae in liver biopsy samples from a child with specific syndrome manifestation (3). Human toxocariasis was first described in 1950 by Wilder C. when he discovered the nematode larvae and their residual hyaline capsules and published a report on ocular granuloma in patients with endophthalmitis (4). The larva was identified later in 1956 by Nichols who performed histological examination of Wilder's samples and determined it as *Toxocara* spp. (5). Two years later, in 1952, Beaver et al. described the clinical manifestation of the disease in children characterised by significant chronic eosinophilia, hepatomegaly, lung infiltrates, fever, cough, hyperglobulinaemia and presence of second-stage larvae of *T. canis* in liver biopsy samples (6). The authors established the term "visceral larva migrans" (VLM) referring to the migration of the larvae through tissues of infected persons and the clinical symptoms caused by their presence in tissues and organs (6). Three decades later Taylor et al. defined the third syndrome of human toxocariasis – covert toxocariasis with non-specific symptoms and signs, associated with increased levels of anti-*Toxocara* antibodies and observed in cases which are not categorised as ocular larva migrans (OLM) or classic VLM syndrome (7).

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AETIOLOGY

The causative agents of toxocariasis are classified in kingdom Animalia, phylum Nematoda, class Secernentea, order Ascaridida, superfamily Ascaridoidea, family Toxocaridae, genus *Toxocara*, species

Toxocara canis (Werner, 1782) and *Toxocara cati* (Schrank, 1788). The family Toxocaridae includes 21 species but the most important causative agents of human disease are *T. canis* and *T. cati*. The intestinal parasite in cattle and buffaloes – *T. vitulorum* (Goeze, 1782) is believed to infect children in the tropics, and *T. pteropodis* (Baylis, 1936), a fruit bat nematode, is considered the causative agent of hepatitis outbreak in Australia. Two new species were recently identified – *T. malayasiensis* (8) in domestic cats and *T. lyncus* (9) in wildcats, but their zoonotic potential is not yet proven. *Toxocara canis* and *T. cati* are ascarid nematodes whose adult forms inhabit the intestinal lumen of dogs, cats and wild carnivores. They are large roundworms with size of 6-18cm for *T. canis* female species and 4-10cm for male species. The size of *T. cati* nematodes is 4-12cm for female and 3-6cm for male species (10). The infectious larvae of *T. canis* and *T.cati* are 400µm long and 15-21µm in diameter. These two species are almost morphologically identical but the diameter of the larva of *T. cati* is smaller (*T. canis* – 18-21µm, *T. cati* – 15-17µm). The eggs of the two species are undistinguishable under light microscopy – they are almost spherical, with thick, rough shell and black granular content. The eggs of *T. canis* (75x90µm) are sometimes larger than those of *T. cati* (65x75µm) (11).

LIFE CYCLE

Definitive hosts and source of infection are dogs, cats, foxes, coyotes etc., spreading the parasite's eggs in the external environment. The adult parasites live inside the host for about 4 months and after that they are eliminated spontaneously. Female forms produce more than 200 000 eggs which can remain vital in the soil for a long period of time. The eggs are unembryonated and non-infectious when they are excreted. Special conditions of the environment are required for embryonation. In optimal conditions, such as temperature between 25-30°C and humidity of 85-95%, the larval stage development inside the egg takes 9-15 days (12). During the incubation period the larvae transform from first-stage to second-stage larvae. The duration of the development may vary from 3-6 weeks to several months and the infectious stage may remain viable inside the egg up to 1 year (13). The endurance of *Toxocara* egg coat makes it highly resistant to many disinfectants and only extremely high temperature, dryness and sunlight can kill the parasite. The infection of dogs and cats occurs through ingestion of eggs, larvae transmission from the mother to her offspring or through ingestion of a paratenic host. After ingestion of the eggs the second-stage larvae hatch in the host's intestines, migrate via the liver to the heart and lungs and again enter the gut where the parasite reaches sexual maturity (tracheal migration). Some of the larvae reach the lungs and disseminate to different organs and tissues through the blood vessels (somatic migration) without any further development (hypobiosis). Actually, in older dogs and cats the parasite does not complete its life cycle and the larvae encyst in tissues while migrating throughout the body. The life cycle is usually completed in females and their offspring and larval reactivation occurs only in pregnant or lactating cats and dogs. In pregnant dogs larvae could be activated under hormonal stimulation and transmitted to the foetus transplacentally (14), and therefore newborn dogs are often infected. Transplacental transmission is not observed in cats and the primary infection of kittens is through transmammary transmission which is less significant in dogs (15). In paratenic hosts, larvae encysted in the intestine following ingestion, penetrate the mucosa and migrate to the organs (lungs, liver, eyes, brain) but cannot develop to adult forms and encyst in the tissues. These larvae are potentially infectious to the host's predators. When a cat or dog ingests the infected paratenic host, the larvae complete their development in the intestinal tract after being released from the tissues during the digestive process (10). This transmission type is called

paratenesis. Rodents, lambs, pigs and birds could be *Toxocara* paratenic hosts.

Humans can also be paratenic hosts. Infection occurs after ingestion of eggs from dirty hands, soil, food, water, contact with infected animal or ingestion of larvae after consumption of uncooked meat from domestic paratenic animals. Infection may also ensue after consumption of raw or uncooked chicken, pork, beef or lamb organs containing larvae (16). Different invertebrates such as flies, cockroaches (17) and earthworms (18) can be mechanical carriers of *Toxocara* spp. Toxocariasis is a zoonosis and there is no data on human-to-human direct transmission. Galactogenic transmission of *T. canis* is not mentioned in the literature.

TOXOCARIASIS IN HUMANS

After ingestion of embryonated *Toxocara* eggs, second-stage larvae hatch in the intestines, enter the blood and lymphatic systems from where they reach different organs. In the tissues larvae encapsulate and become surrounded by eosinophilic granulomas. They cause persistent infections and different pathological and clinical disorders. The larvae can remain viable in human tissues for one or more years. Inactive larvae can be reactivated anytime and start to migrate (19). The disease pathology is mainly a result of tissue damage caused by inflammatory response to the presence of larvae and the toxic products they produce. The larvae do not grow in tissues but they are metabolically active and secrete huge amount of enzymes, waste products and cuticle components which cause tissue damage, necrosis and significant inflammatory reaction. Eosinophilia is the main manifestation of this reaction (20) and it is considered that eosinophils produce toxic proteins contributing to toxocariasis pathology and symptomatology (21). *Toxocara* larvae products have high immunogenicity and allergenicity which explains the rate of allergic symptoms in patients with toxocariasis (22). The larvae are found in liver, lungs, heart, eyes and brain (23) where they form migratory tracks characterised by haemorrhage, necrosis and inflammation. The inflammatory response in the eye may lead to partial or total retinal damage with loss of vision (24). Pathology is related to the death of the larva which triggers the development of early or late manifestation of hypersensitivity (25).

Clinical forms of the disease

The ingestion of *Toxocara* larvae by humans may not lead to development of disease or could cause systemic illness affecting different organs. In the literature, systemic toxocariasis is observed in 15.5% of all diagnosed cases and most of the infections caused by *Toxocara* spp. are asymptomatic (26). The severity of the disease depends on the affected tissue, the number of migrated larvae, the immune response and age of the host (25). Due to its variety of symptoms and signs, toxocariasis is divided into three main forms – visceral, ocular and covert (7). According to a newly suggested classification, human toxocariasis is classified in one of the following forms – classical systemic form, asymptomatic, covert and compartmentalised (ocular and neurological). The last two forms are deemed as separate because the eyes and brain are the target organs of larvae migration (27).

Visceral larva migrans (VLM)

Symptoms of classical systemic VLM include periods of fever, cough, wheezing, anaemia, eosinophilia, hepatomegaly and positive serological tests. Eosinophilia up to 30% or more is considered significant for the clinical diagnosis in children with a history of eating soil (28). The clinical picture progresses mainly to leucocytosis (29, 30), eosinophilia (6, 31), lung and liver dysfunction, neurological disorders. Lung symptoms caused by larvae invasion are observed at the beginning of the disease. These symptoms may be preceded by eosinophilia and hepatomegaly and could be accompanied by fever. Iron-deficiency anaemia is commonly observed. There is also a considerable increase in isohemagglutinin

titres due to antigenic similarity between parasites and human erythrocytes (32). Lung involvement is associated with cough, dyspnoea, infiltrates and mimics bronchitis or pneumonia (33) with eosinophilic pleural effusions (34) and acute (35) or chronic eosinophilic pneumonia (36). When liver is affected the condition usually progresses to granulomatous hepatitis (37). Heart damage is observed rarely with myocarditis (23, 38) or pericarditis (39). Larvae can reach the brain where they cause neurotoxocariasis. The damage of the central nervous system (CNS) manifests as meningitis, encephalitis (40), myelitis (41) or cerebral vasculitis (42). Patients complain of headache, fever, light sensitivity, weakness, confusion, fatigue and visual disorders. Dementia, depression or behavioural disorders are also reported (43). It is considered that some types of epilepsy are related to brain lesions caused by larvae migration (44, 45, 46). Damage of the peripheral nervous system is rare presenting as radiculitis (47), cranial nerve damage or musculoskeletal dysfunction (48). Toxocariasis may be associated with some less common manifestations, such as generalised lymphadenopathy (49) and idiopathic urticaria (50). Some authors (51) reported occurrence of asthma in patients with toxocariasis and described a correlation between the two diseases (52). It can be concluded that VLM damages many organ systems and the clinical presentation can mimic a lot of diseases (53). The syndrome is usually benign, self-limiting and the prognosis is favourable. Significant damages to lungs, liver, CNS and even a fatal outcome could occur if there is delay in diagnosis and treatment.

Ocular larva migrans (OLM)

Conditions in which the visual system is affected are called ocular larva migrans. *Toxocara* larvae have affinity for the retina and their migration through the tissues causes haemorrhage, necrosis, ocular inflammation and lesions which often lead to loss of vision in the infected eye (54). The inflammatory reaction against larvae and their antigens can cause local or general damage of the retina or other eye structures. Usually, the infection is unilateral and most common in children but sometimes both eyes could be involved. Typical features of ocular toxocariasis include nematode endophthalmitis (4), retinal granuloma (55, 56), reduced vision, leukocoria, red eye and strabismus (57). Often there is no eosinophilia (58) and the diagnosis is usually based on the presence of chorioretinal granuloma, focal lesions in the posterior ocular segment and positive serology. According to the literature, the distribution of OLM is from 0% to 10% (59).

Covert toxocariasis is characterised by non-specific signs and symptoms which are not classified in the classic VLM, OLM or neurological toxocariasis (NLM). The term is introduced by Taylor who described the most common clinical characteristics – stomach ache, hepatomegaly, anorexia, nausea, vomiting, sleep and behaviour disorders, pneumonia, cough, rales, pharyngitis, cervical adenitis and limb pain (7). Lung involvement (asthma, acute bronchitis, pneumonia) (60), skin problems such as chronic urticaria or eczema (61), lymphadenopathy, myositis, pseudorheumatoid syndrome (62) are also observed. Covert toxocariasis is often diagnosed when the symptoms disappear after treatment (19).

Asymptomatic toxocariasis has no symptoms and diagnosis is based only on positive serological test. It is observed as minor or old infections and could be accompanied by eosinophilia (63). The main problem with this form, especially in children, is the risk of progression to OLM or NLM. Bass et al. reported 7% to 44.4% distribution of asymptomatic toxocariasis (63).

Diagnosis of human toxocariasis

Parasitological diagnosis of toxocariasis is difficult because the parasite does not reach sexual maturity or produce eggs. The only way to make a precise diagnosis is based on

observation of the larvae in biopsy tissues (4, 5). However, identification is extremely difficult due to their small size and the procedure posing a risk to the patient. Typical changes in the tissues are the formation of eosinophilic abscess and granuloma with fibrinoid necrosis and larva in the centre often surrounded by thick hyaline capsule. Liver biopsy is performed in patients with VLM. Motile larva can be directly observed below the retina in the ocular form (64). Therefore, immunology methods determining the presence of specific IgG antibodies against the parasite's antigens are the main instruments in the diagnosis of toxocariasis. In 1979 de Savigny developed ELISA methodology based on *T. canis* antigen obtained from *in vitro* cultivation of second-stage larvae, and reported that the assay is sensitive for diagnosis of toxocariasis (65). Later on, researchers confirmed the method and described it as sensitive, specific and easy to perform (66). Nowadays, this is the most commonly used screening tool for diagnosis and sero-epidemic investigations. Cross-reactions (66), difficult interpretation of borderline results and low sensitivity in cases of ocular form are often reported as disadvantages of the method (67). In order to avoid these drawbacks, in 1991 Magnaval et al. developed Western blot assay (68) using excretory-secretory antigens of *Toxocara* larvae. The presence of toxocariasis is associated with formation of a first cluster of low-molecular-weight bands (LMW) of 24, 28, 30 and 35 kDa and a second cluster consisting of three high-molecular-weight bands (HMW) of 132, 147 and 200 kDa, which are observed more often in examination of patients with different helminthiases. According to Magnaval, the Western blot method correlates with ELISA and he suggested to be used as a confirmatory tool of all positive ELISA results (68). Because of the good sensitivity and specificity, nowadays the Western blot method is recognised as a confirmative test for *Toxocara* serology (69).

Immunological reactions of intraocular liquids (aqueous and vitreous humour) (70) are recommended in OLM diagnosis and significantly increase the specificity of ELISA, but such specimens are usually not available for examination.

Serodiagnostic tests have few disadvantages. The occurrence of cross-reactions with other parasites (most often with nematodes) often shows false positive reactions. The stage of disease and treatment success cannot be determined because of the long retention time of specific antibodies (71). Difficulties in the diagnosis of *T. canis* active infection are serious problem and for this reason additional tests are further developed – measurement of circulating antigens (72), determination of total serum IgE level (73, 74, 75) and eosinophil cationic protein (ECP) (76, 77). Other complementary tests include determination of specific IgG avidity (78), anti-*Toxocara* IgE (79) and specific IgG subclass antibodies (80, 81), in order to support the main diagnosis and determine the activity of the disease.

Lesions caused by larvae can be identified with different medical imaging techniques such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) (82, 83, 84).

Genetic methods (PCR) are used for detection of ascarid larvae in animal tissues (cats, dogs and foxes) and species differentiation between *T. canis*, *T. cati* and *T. leonine* (85). Reliable genetic markers for identification and differentiation of *Toxocara* species and related nematodes are the first and second internal transcribed spacers (ITS-1 and ITS-2) of the nuclear rDNA sequence (85, 86, 87). In 2013, Pinelli E. determined *Toxocara* spp. DNA in bronchoalveolar lavage fluid of experimentally infected mice and suggested this method for improving the diagnosis, especially in patients with lung symptoms (88). Mitochondrial genetic markers (mDNA) are used for investigation of *Toxocara* spp. taxonomy and population genetics (89). The main focus of genetic studies is on *T. canis* and the identification of dominant and surface molecules secreted during the infectious larva stage, which have a key role in the immune

invasion (90). The application of modern genetic and bioinformation technologies is of great importance for understanding the biology of *T. canis* and will lead to development of new diagnostic, therapeutic and control strategies (91).

Toxocariasis in Bulgaria

In our country R. Zheleva investigated *T. canis* antigen structure and contributed to the development of different diagnostic techniques (92). I. Raynova performed extensive studies of the disease incidence in Bulgaria and standardisation of modern serological tests (ELISA and Western blot) (93).

Toxocariasis is one of the most widespread helminthic infections in the world. Investigation of the distribution in Western countries reveals 2% to 5% in healthy residents of cities, 14% to 37% in adults living in the countryside (94) and up to 63% in the tropical countries (95). Studies in Bulgaria show 8.6% distribution in healthy people (96). There are several reasons for the high levels of soil contamination with *Toxocara* eggs, such as the wide spread and fertility of adult *Toxocara* spp. parasites and increase in the number of pet animals. Geography and poor personal hygiene increase the risk of *Toxocara* infection, especially in children. Due to difficulties in diagnosis and the variety of clinical forms, the disease often remains undiagnosed. The increase in awareness of this illness will lead to early diagnosis and proper treatment.

REFERENCES

1. Perlingiero J, György P. *Chronic eosinophilia; Report of a case with necrosis of the liver, pulmonary infiltration, anemia and ascaris infestation*. Am J Dis Child. 1947; 73(1):34-43.
2. Zuelzer W, Apt L. *Disseminated visceral lesions associated with extreme eosinophilia; pathologic and clinical observations on a syndrome of young children*. Am J Dis Child. 1949; 78(2):153-181.
3. Mercer R, Lund H, Bloomfield M, Calwell F. *Larval ascariasis as a cause of chronic eosinophilia with visceral manifestations.* Am J Dis Child. 1950; 80(1):46-58.
4. Wilder HC. *Nematode endophthalmitis*. Trans Am Acad Ophthalmol Otolaryngol. 1950; 55:99-109.
5. Nichols R. *The etiology of visceral larva migrans.* J Parasitol. 1956; 42(4):349-362.
6. Beaver P, Snyder C, Carrera G, Dent J, Lafferty J. *Chronic eosinophilia due to visceral larva migrans: report of three cases.* Pediatrics. 1952; 9(1):7-19.
7. Taylor M, Keane C, O'Connor P, Mulvihill E, Holland C. *The expanded spectrum of toxocaral disease.* Lancet. 1988; 1(8587):692-695.
8. Gibbons L, Jacobs D, Sani R. *Toxocara malaysiensis n. sp. (Nematoda: Ascaridoidea) from domestic cat (Felis catus Linnaeus, 1758).* J Parasitol. 2001; 87(3):660-665.
9. Macchioni G. *A new species Toxocara lyncis in the caracal* (*Lynx caracal*). Parasitology. 1999; 41(4):529-532.
10. Glickman L, Schantz P. *Epidemiology of zoonotic toxocariasis*. Epidemiol Rev. 1981; 3:230-250.
11. Thienpoint D, Rochette F, Vanparijs O. *Diagnosing Helminthiasis through Coprological Examination*. Janssen Research Foundation: Beerse, Belgium. 1979; 110-121.
12. Schacher J. *A contribution to the life history and larval morphology of Toxocara canis*. J Parasitol. 1957; 43(6):599-610.
13. Overgaauw P. *Aspects of Toxocara epidemiology: toxocarosis in dogs and cats.* Crit Rev Microbiol. 1997; 23(3):233-251.
14. Dubey J. *Patent Toxocara canis infection in ascarid naïve dogs*. J Parasitol. 1978; 64(6):1021-1023.
15. Burke T, Roberson E. *Prenatal and lactational transmission of Toxocara canis and Ancylostomum canium: experimental infection of the bitch before pregnancy*. Int J Parasitol. 1985; 15(1):71-75.
16. Taira K, Saeed I, Permin A, Kapel C. *Zoonotic risk of Toxocara canis infection through consumption of pig or poultry viscera*. Vet Parasitol. 2004; 121(1-2):115-124.
17. Takahashi J, Uga S, Matsumura T. *Cockroach as a possible transmitter of Toxocara canis*. Jap J Parasitol. 1990; 39:551-556.
18. Pahari T, Sasmal N. *Experimental infection of Japanese quail with Toxocara canis larvae through earthworms*. Vet Parasitol. 1991; 39:337-340.
19. Pawlowski Z. *Toxocariasis in humans: clinical expression and treatment dilemma.* J Helminthol. 2001; 75(4):299-305.
20. Xinou E, Lefkopoulos A, Gelagoti M, Drevelagas A, Diakon A, Milonas I, Dimitriadis A. *CT and MR imaging findings in cerebral toxocaral disease*. Am J Neuroradiol 2003; 24(4):714-718.
21. Kayes S. *Human toxocarosis and the visceral larva migrans syndrome: correlative immunopathology*. In: Freedman D., ed. Immunopathogenetic aspects of disease induced by helminth parasites: chemical immunology. Basel, Karger, 1997; V. 66, p. 99-124.
22. Glickman L, Shofer F. *Zoonotic visceral and ocular larva migrans*. Vet Clin N Am. 1987; 17(1):39-53.
23. Dent J, Nichols R, Beaver P, Carrera G, Staggers R. *Visceral larva migrans*. Am J Pathol. 1956; 32(4):777-803.
24. Neafie R, Connor D. In Pathology of tropical and extraordinary diseases, Binford C, Connor D (eds). Armed Forces Institute of Pathology, Washington DC, USA. 1976. pp. 433-436.
25. Despommier D. *Toxocariasis: clinical aspects, epidemiology, medical ecology, and molecular aspects*. Clin Microbiol Rev. 2003; 16(2):265-272.
26. Loez L, Martin G, Chamorro C, Mario J. *Toxocariossis en ninos de una region subtropical.* Medicina (B Aires). 2005; 65(3):226-230.
27. Carvalho E, Rocha R. *Toxocariasis: visceral larva migrans in children*. J Pediatria. 2011; 87(2):100-110.
28. Snyder C. *Visceral larva migrans*. Paediatrics. 1961; 28:85-91.
29. Aguiar-Santos A, Andrade L, Medeiros Z, Chieffi P, Lescano S, Perez E. *Human toxocariasis: frequency of anti-Toxocara antibodies in children and adolescents from an outratient clinic for lymphatic filariasis in Recife, Northeast Brazil*. Rev Inst Med Trop Sao Paulo. 2004; 46(2):81-85.
30. Luzna-Lyskov A. *Toxocarosis in children living in a highly contaminated area. An epidemiological and clinical study*. Acta Parasitol. 2000; 45(1):40-42.
31. Guerra A, Navarro C, de Guevara C. *Seroprevalence of toxocariasis in children and a case of VLM*. Eur J Epidemiol. 1995; 11(6):701-702.
32. Jacob C, Pastorino A, Peres B, Mello E, Okay Y, Oleska G. *Clinical and laboratorial features of visceral toxocariasis in infancy.* Rev Inst Med Trop Sao Paulo. 1994; 36(1):19-26.
33. Bartelink A, Kortbeek L, Huidekoper H, Meulenbelt J, van

Knapen F. *Acute respiratory failure due to Toxocara infection*. Lancet. 1993; 342(8881):1234.
34. Ashwath M, Robinson D, Katner H. *A presumptive case of toxocariasis associated with eosinophilic pleural effusion: case report and literature review*. Am J Trop Med Hyg. 2004; 71(4):764.
35. Roig J, Texido A, Domingo C, Morera J. *Acute eosinophilic pneumonia due to toxocariasis with bronchoalveolar lavage findings*. Chest. 1992; 102(1):294-296.
36. Inoue K, Inoue Y, Arai T, Nawa Y, Kashima Y, Yamamoto S, Sakatani M. *Chronic eosinophilic pneumonia due to visceral larva migrans*. Intern Med. 2002; 41(6):478-482.
37. Zibaei M, Sadjjadi S, Geramizadeh B, Firoozeh F. *Hepatic Toxocariasis in a child: a case report from Shiraz, Southern Iran.* Hepatitis monthly. 2008; 8(4):310-312.
38. Vargo T, Singer D, Gillatte P, Fernbach D. *Myocarditis due to visceral larva migrans.* J Pediatr. 1977; 90(2):322-323.
39. Kuenzli E, Neumayr A, Chaney M, Blum J. *Toxocariasis – associated cardiac diseases – a systematic review of the literature.* Acta Tropica. 2016; 154:107-120.
40. Moreira-Silva S, Rodrigues M, Pimenta J, Gomes C, Freire L, Pereira F. *Toxocariasis of the central nervous system: with repott of two cases.* Rev Soc Bras Med Trop. 2004; 37(2):169-174.
41. Osoegawa M. *Diagnosis and treatment of CNS parasite infection with special reference to parasitic myelitis*. Rinsho Shinkeigaku . 2004; 44(11):961-964.
42. Dousset V, Sibon I, Menegon P. *Case no 6. Cerebral vasculitis due to Toxocara canis (or catis) origin*. J Radiol. 2003; 84(1):89-91.
43. Richartz E, Buchkremer G. *Cerebral toxocariasis: a rare cause of cognitive disorders. A contribution to differential dementia diagnosis*. Nervenarzt. 2002; 73(5):458-462.
44. Critchley E, Vakil S, Hutchinson D, Taylor P. *Toxoplasma, Toxocara, and epilepsy.* Epilepsia. 1982; 23:315-323.
45. Woodruff A, Bisseru B, Bowe J. *Infection with animal helminths as a factor in causing poliomyelitis and epilepsy.* Brit Med J. 1966; 1(5503):1576-1579.
46. Glickman L, Cypess R. *Toxocara infection and epilepsy in children*. J Pediatr. 1979; 94(1):75-78.
47. Robinson A, Tannier C, Magnaval J. *Toxocara canis meningoradiculitis*. Rev Neurol. 2002; 158:351-353.
48. Rayes A, Nobre V, Teixeira D, Serufo Y, Filho G, Antunes C, Lambertucci Y. *Tropical pyomyositis and human toxocariasis: a clinical and experimental study.* Am J Med. 2000; 109(5):422-425.
49. Amir J, Harel L, Eidlitz-Markus T, Varsano I. *Lymphedema as a presenting sign of toxocariasis.* Infection. 1995; 23(6):389-390.
50. Qualizza R, Incorvaia C, Grande R. *Chronic idiopathic urticarial and neglected Toxocara infection*. World Allergy Organ J. 2012; 5(Suppl 2):s202.
51. Taylor M, Keane C, O’Connor P, Girdwood R, Smith H. *Clinical features of covert toxocariasis.* Scand J Infect Dis. 1987; 19(6):693-696.
52. Figueiredo S, Taddei J, Menezes J, Novo N, Silva E, Cristóvão H, Curi M. *Estudo clínico-epidemiológico da toxocaríase em população infantil.* J Pediatr (Rio J). 2005; 81(2):126-132.
53. Bachmeyer C, Lamarque G, Morariu R, Molina T, Bouree P, Delmer A. *Visceral larva migrans mimicking Lymphoma*. Chest. 2003; 123(4):1296-1297.
54. Steward J, Cubillan L, Cunning E. *Prevalence, clinical features and causes of vision loss among patients with ocular toxocariasis*. Retina. 2005; 25(8):1005-1013.
55. Ashton N. *Larval granulomatosis of the retina due to Toxocara*. Brit J Ophthal. 1960; 44(3):129 -148.
56. Ahn S., Woo S, Jin Y, Chang Y, Kim T, Ahn Y, Heo Y, Yu H, Chung H, Park K, Hong S. *Clinical features and course of ocular toxocariasis in adults*. PLOS Neglected Trop Dis. 2014; 8(6):e 2938.
57. Pollard Z, Jarrett W, Hagler W. *ELISA for diagnosis of ocular toxocariasis.* Ophthalmology. 1979; 86:743-749.
58. Magnaval J, Glickman L, Dorchies P, Morassin B. *Highlights of human toxocariasis*. Korean J Parasitol. 2001; 39(1):1-11.
59. Good B, Holland C, Taylor M, Larragy J, Moriarty P, O’ Regan M. *Ocular toxocariasis in schoolchildren.* Clin Inffect Dis. 2004; 39(2):173-178.
60. Feldman G, Parker H. *Visceral larva migrans associated with the hypereosinophilic syndrome and the onset of severe asthma*. Ann Intern Med. 1992; 116(10):838-840.
61. Wolfrom E, Chene G, Boisseau H, Beylot C, Geniaux M, Taieb A. *Chronic urticarial and Toxocara canis*. Lancet. 1995; 345(8943):196.
62. Le Luyer B, Menager V, Andebert C, Le Ropux P, Briguet M, Boulloche J. *Inflamatory joint disease as a manifestation of Toxocara canis larva migrans*. Ann Pediatr. 1990; 37:445-448.
63. Bass J, Mehta K, Glickman L, Blocker R, Eppes B. *Asymptomatic toxocariasis in children. A prospective study and treatment trial.* Clin Pediatr. 1987; 26(9):441-446.
64. Meyer-Riemann W, Petersen J, Vogel M. *Extraktionsversuch einer intraretinalen Nematode im papillomakularen Bundel*. Klin Monatsbl Augenheilkd. 1999; 214:116-119.
65. De Savigny D, Voller A, Woodruff A. *Toxocariasis: serological diagnosis by enzume immunoassay.* J Clin Pathol. 1979; 32(3):284-288.
66. Jacquier P, Gottstein B, Stingelin Y, Eckert J. *Immunodiagnosis of toxocarosis in humans: evaluation of a new enzyme-linked immunosorbent assay kit.* J Clin Microbiol. 1991; 29(9):1831-1835.
67. Glickman L, Schantz P, Grieve R. *Toxocariasis*. In immunodiagnosis of Parasitic Diseases, volume 1: Helmintic diseases, Walls K., Schantz P. (eds.). NewYork: Academic Press, NYC, USA. 1986; 201-231.
68. Magnaval J, Fabre R, Maurieres P, Charlet J, de Larrard B. *Application of the Western blotting procedure for the immunodiagnosis of human toxocariasis*. Parasitol Res. 1991; 77(8):697-702.
69. Buijs J, Borsboom G, Renting M, Hilgersom W, van Wieringen J, Jansen G, Neijens Y. *Relationship between allergic manifestations and Toxocara seropositivity: A cross-sectional study among elementary school children*. Eur Respir J. 1997; 10:1467-1475.
70. Petithory J, Chaumeil C, Liotet S, Rosseau M, Bisognani C. *Immunological studies on ocular larva migrans.* In Lewis J., Maizels R. (eds), Toxocara and Toxocariasis. London: Institute of Biology and the British Society for Parasitology.1993; 81-89.
71. Gill D, Dunne K, Kenny V. *Toxocariasis in children*. Lancet i. 1998; 1172.
72. Robertson B, Burkot T, Gillespie S, Kennedy M, Wambai Z. *Detection of circulating parasite antigen and specific antibody in Toxocara canis infection*. Clin Exp Immunol. 1988; 74(2):236-241.
73. Hogarth-Scott R, Johansson S, Bennich H. *Antibodies to Toxocara in the sera of Visceral larva migrans patients: the significance of raised levels of IgE*. Clin Exp Immunol. 1969; 5:619-625.
74. Elsheikha H, El-Beshbishi S, El-Shazly A, Hafez A, Morsy T. *Kinetics of eosinophilia and IgE production in experimental murine toxocariasis*. J Egypt Soc Parasitol. 2008; 38 (1):53-64.
75. Dattoli V, Freire S, Mendonca L, Santos P, Meyer R, Alcantara-Neves N. *Toxocara canis infection is associated with eosinophilia and total IgE in blood donors from a large Brazilian centre.* Trop Med Int Health. 2011; 16(4):514-517.
76. Magnaval J, Berry A, Fabre R, Morassin B. *Eosinophil cationic protein as a possible marker of active human Toxocara infection*. Allergy. 2001; 56(11):1096-1099.
77. Niedworok M, Sordyl B, Borecka A, Gawor J, Malecka-Panas E. *Estimation of eosinophilia, immunoglobulin E and eosinophilic cationic protein concentration during the treatment of toxocariasis*. Wiad Parazytol. 2008; 54(3):225-230.
78. Hubner J, Uhlikova M, Leissova M. *Diagnosis of the early phase of larval toxocariasis using IgG avidity*. Epidemiol Mikrobiol Imunol. 2001; 50(2):67-70.
79. Magnaval J-F, Fabre R, Maurieres P, Charlet J, de Larrard B. *Evaluation of an immunoenzymatic assay detecting specific anti-Toxocara immunoglobulin E for diagnosis and posttreatment follow-up of human toxocariasis.* J Clin Microbiol.

1992; 30(9):2269-2274.

80. Obwaller A, Jensen-Jarolim E, Auer H, Huber A, Kraft D, Aspöck H. *Toxocara infestations in humans: symptomatic course of toxocarosis correlates significantly with levels of IgE/anti-IgE immune complexes*. Parasite Immunol. 1998; 20(7):311-317.
81. Watthanakulpanich D, Smith H, Hobbs G, Whalley A, Billington D. *Application of Toxocara canis excretory-secretory antigens and IgG subclass antibodies (IgG1-4) in serodiagnostic assays of human toxocariasis.* Acta Tropica. 2008; 106(2):90-95.
82. Dupas B, Barrier J, Barre P. *Detection of Toxocara by computed tomography*. Br J Radiol. 1986; 59(701):518-519.
83. Ruttinger P, Hadidi H. *MRI in cerebral toxocaral disease*. J Neurol Neurosurg Psychiatr. 1991; 54:361-362.
84. Baldisserotto M, Conchin C, Da Soares M, Araujo M, Kramer B. *Ultrasound findings in children with toxocariasis: report on 18 cases*. Pediatr Radiol. 1999; 29:316-319.
85. Jacobs D, Zhu X, Gasser R, Chilton N. *PCR-based methods for identification of potentially zoonotic ascaridoid parasites of the dog, fox and cat*. Acta Tropica. 1997; 68(2):191-200.
86. Zhu X, Chilton N, Jacobs D, Boes J, Gasser R. *Characterisation of Ascaris from human and pig hosts by nuclear ribosomal DNA sequences*. Int J Parasitol. 1999; 29 (3):469-478.
87. Li M, Zhu X, Gasser R, Lin R, Sani R, Lun Z, Jacobs D. *The occurrence of Toxocara malaysiensis in cats in China, confirmed by sequence-based analyses of ribosomal DNA.* Parasitol Res. 2006; 99:554-557.
88. Pinelli E, Roelfsema J, Brandes S, Kortbeek T. *Detection and identification of Toxocara canis DNA in bronchoalveolar lavage of infected mice using a novel real-time PCR.* Vet Parasitol. 2013; 193(4):337-341.
89. Jex A, Waeschenbach A, Littlewood D, Hu M, Gasser R. *The mitochondrial genome of Toxocara canis*. PLoS Negl Trop Dis. 2008; 2 e273.
90. Maizels R, Tetteh K, Loukas A. *Toxocara canis7 genes expressed by the arrested infective larval stage of a parasitic nematode*. Int J Parasitol. 2000; 30(4):495-508.
91. Chen J, Zhou D, Nisbet A, Xy M, Huang S, Li M, Wang C, Zhu X. *Advances in molecular identification, taxonomy, genetic variation and diagnosis of Toxocara spp*. Infect Gen Evol. 2012; 12(7):1344-1348.
92. Zheleva R. *Evaluation of the effectiveness of the indirect hemagglutination test with Ascaris and Toxocara antigens in diagnosis of larva migrans symptom complex in man.* Med Parazitol. 1976; 45(2):160-165.
93. Raynova Y. *Development and characterization of ELISA and Western blot with Toxocara canis excretory / secretory antigens for diagnosis and investigation of the spread of toxocarosis in humans in our country.* PhD Thesis, NCIPD, Sofia 2006.
94. Magnaval J-F, Glickman I, Dorchies P. *La toxocarose, une zoonose helminthique majeure*. Rev Med Vet. 1994; 145:611-627.
95. Chomel B, Kasten R, Adams C, Lambillotte D, Theis Y, Goldsmith R, Koss Y, Chioino C, Widjana P, Sutisna P. *Serosurvey of some major zoonotic infections in children and teenagers in Bali, Indonesia*. Southeast Asian J Trop Med Public Health. 1993; 24(2)v:321-326.
96. Raynova I. *Study of Toxocara antibody bearing among healthy people in Bulgaria*. X European Multicolloquium of Parasitology-EMOP 10. 2008.

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