Indexed by
SCOPUS
from 2008

PROBLEMS
of Infectious and Parasitic Diseases

NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 49, NUMBER 1/2021

ISSN 0204-9155

1504 Sofia; 26, Yanko Sakazov Blvd.
Tel.: +359 2/ 846 83 07, Fax: +359 2/ 943 30 75
e-mail: pipd@ncipd.org

PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 49, NUMBER 1/2021

Preprinting and Printing by Boris Zl. Pavlov & Alliance print

CONTENTS

Manuscript submission
To submit a manuscript, access *PROBLEMS of Infectious and Parasitic Diseases* manuscript submission site https://pipd.ncipd.org/index.php/pipd/about/submissions and follow the directions there make the submission.

Instructions to Authors

Papers should not have been previously published or be currently under consideration for publication.
Manuscripts must be written in English, using British spelling. All manuscripts should be single-spaced, with wide margins and numbered pages. MS Word should be used for word processing, 12-point Times New Roman font.
Named authors must fit the following three criteria:
1. Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data;
2. Drafting the article or revising it critically for important intellectual content; and
3. Final approval of the version to be published.

All people who meet the three criteria should be named as authors. Those who participate in the study but do not meet the requirements of authorship should be acknowledged as contributors.

TITLE PAGE
The title page must contain: 1) title, name and surname of the authors; 2) names of the institution(s) where the research was carried out; 3) the name and full postal address, e-mail address and telephone numbers of the corresponding author; 4) three to five key words; 5) intended category of the manuscript.

ABSTRACT
The abstract should contain about 250 words and must be structured as follows: background, material and methods, results, conclusions. Review Articles should have an informative, unstructured abstract of about 250 words. Brief reports should have a short abstract of no more than 150 words.

TEXT
The text should contain introduction, material and methods, results, discussion and references. No particular format is required for review articles.

ACKNOWLEDGEMENTS
Individuals who supplied facilities, strains or reagents, or gave advice may be acknowledged. Also, supporting grants may be mentioned.

REFERENCES
References should be numbered in order of appearance in the text, in parenthesis, not superscripts, as shown bellow:

Journal articles:
Vellinga A, Cormican M, Hanahoe B, Murphy AW. *Predictive value of antimicrobial susceptibility from previous urinary tract infection in the treatment of re-infection.* Br J Gen Pract. 2010; 60(576):511-513.

Books:
Rosa PA, Hogan D, Margolis N. *Molecular analysis of the major outer surface protein locus from a divergent Borrelia burgdorferi isolate from Europe*. In: Schutzer SE. Lyme borreliosis: Molecular and immunologic aspects. Cold Spring Harbor Laboratory Press, 1992, 95-110.

TABLES
Tables should be incorporated in the manuscript file, not as separate files, MS Word table tool, no wider than 17 cm.

FIGURES
Figures should be provided as separate files, not embedded in MS Word, PC file formats (e.g., MS Excel/PowerPoint). Image files should be submitted without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files.

CONFLICT-OF-INTEREST
Identifying information regarding patients, including patients' names, initials, or hospital numbers, should be omitted unless the information is essential and the patient (or parent or guardian) gives written informed consent for publication.

DO WE KNOW RHINOVIRUSES AND THEIR CLINICAL IMPACT?

(Mini review)

Irina Georgieva, Asya Stoyanova, Svetla Angelova, Savina Stoitzova, Silvia Voleva, Neli Korsun, Lubomira Nikolaeva-Glomb

National Reference Laboratory "Enteroviruses",
Department of Virology,
National Centre of Infectious and Parasitic Disease

ABSTRACT

Acute respiratory infections cause significant morbidity and mortality even before the COVID-19 pandemic. Pandemic restrictions decreased circulation of many respiratory viruses but some less troubling infections such as common cold are still circulating.

One of the most frequent causative agents of common cold are rhinoviruses. The fact that these pathogens have been able to slip through anti-COVID preventive measures raises the question of whether we really know this group of viruses and whether these viruses cause only common cold. The clinical impact of rhinoviruses seems to be underestimated.

In searching of an answer how rhinoviruses have slipped through the anti-COVID precautions we referred to the work of infectious disease specialists, virologists and epidemiologists -much of it conducted decades before the current pandemic. A non-systematic search of the literature is performed. Some of the latest findings on rhinoviruses along with basic knowledge on their biology and clinical impact are summarized in this review.

Keywords: *rhinovirus, common cold, asthma, bronchiolitis*

INTRODUCTION

Rhinoviruses (RVs) are the causative agent for more than a half of the upper respiratory tract infections (1). RVs are widespread and affect all age groups with the highest incidence documented in early childhood (2). Although rhinovirus infections are considered as benign, self-limited and generally mild human diseases, being so common, they have significant economic impact on the health systems and the quality of life (3). The upper respiratory tract is the most common site of the rhinovirus infection, but RVs have been associated with some lower respiratory tract diseases such as bronchitis, bronchiolitis and pneumonia (4).

Rhinovirus infections on top of chronic obstructive pulmonary disease (COPD), asthma, or cystic fibrosis might even become a life-threatening condition (5, 6, 7, 8, 9, 10). Furthermore, RVs have been recognized as a common cause of wheezing in early childhood. Children who experience wheezing during a rhinovirus infection are at a higher risk of asthma development later in life (11, 12). The lack of a specific treatment or vaccines for RVs results in underestimation of their clinical impact. Most often rhinovirus infections are left unobserved and underdiagnosed and hence, the uses of diverse over-the-counter medications or inappropriate and unnecessary antibiotic prescriptions are a common occurrence (13).

BIOLOGY OF RHINOVIRUSES

1. Classification

RVs are extremely heterogeneous group of viruses - members of the *Enterovirus* genus within the *Picornaviridae* family. RVs were discovered in the 1950s and initially were classified into two groups, designated as A and B (RV-A and RV-B) based on their antigenic characteristics and other physical characteristics of the virions (e.g. pH lability). Molecular and genetic characterization of RVs reveals a much greater diversity. More than one-third of the rhinovirus infections are caused by a third group of RVs (RV-C), which do not grow in cells culture and therefore were left undetected until 2006 (14, 15, 16, 17, 18, 19).

To date, 169 RV types have been described. RVs are now assigned to the species *Rhinovirus A* (n=80), *Rhinovirus B* (n=32) and *Rhinovirus C* (n=57): (http://ictv.global/report/picornaviridae/enterovirus/).

Recommendations on the nomenclature of enteroviruses and RVs have recently been published (20). There is a molecular typing system, originally proposed for enteroviruses but then adapted for RVs.

ADDRESS FOR CORRESPONDENCE:
National Reference Laboratory „Enteroviruses",
Department of Virology,
National Centre of Infectious and Parasitic Diseases,
Sofia, Bulgaria, 44A Stoletov Blvd, 1233 Sofia, Bulgaria;
E-mail: georgieva@irrito.com; Tel/Fax: +359 28310042

The accepted threshold for type assignment on the basis of the divergence of the VP1 nucleotide sequences is at least 13% (for RV-A), 12% (RV-B), or 13% (RV-C) nucleotide divergence from all other RV types (17).

Some aspects of the previously used biological classification of RVs are still accepted as far as the groups are partly associated with their genetic relationships. On the basis of the cellular binding sites, RVs are grouped into "major" and "minor" receptor groups.

2. Structure

RVs are small, non-enveloped RNA viruses (21). The virus particle is about 30 nm in size, icosahedral with a pseudo T = 3 (P = 3) type of symmetry. The capsid consists of 60 copies of all four structural proteins referred to as VP1, VP2, VP3 and VP4. On the virion surface there is a centrally located depression called a "canyon" which surrounds the fivefold axis of symmetry of the icosahedron. At the basis of the canyon there is a hydrophobic "pocket", formed by VP1 (22). The canyon frequently serves as the receptor binding site (23). This structure is the target for some antiviral agents (24, 25).

The structural features are common for most of the enteroviruses. Despite the similarities, the capsid of RV-C contains protrusions on its surface which are smoother and spherical. Canyons of RV-C particles are shallower and narrower, and there is no hydrophobic pocket at their floor (26).

3. Genome

The rhinovirus genome is a single positive-stranded RNA of about 7.2 to 7.5 kb in size. The genetic information is coded in a single open reading frame flanked by two untranslated regions (UTRs). Although positive-stranded RNA can serve directly as a messenger RNA for translation, it lacks the typical *cap*-structure at the 5'-end. Instead, there is a small viral protein (VPg) covalently bound to the 5'- end of the genome. The 5' -UTR contains also an internal ribosomal entry site (IRES) allowing translation via *cap*-independent mechanism (27, 28).

4. Replication cycle

RVs utilize several types of cellular receptors: The RVs from the major group, which accounts for about 90% of *Rhinovirus A* and *Rhinovirus B*, utilize intercellular adhesion molecule 1 (ICAM-1) for cell entry (29, 30). The minor receptor group alternatively binds low density lipoprotein receptor (LDLR). RV-C binds a different receptor molecule - human cadherin-related family member 3 (CDHR3) (18, 19). The canyon is the receptor binding site for most of the RVs. For some members of the minor receptor group, however, despite the presence of such a structure in the virion, attachment to the receptor occurs with a star-shaped plateau, located on the fivefold axis of symmetry, which is surrounded by the canyon (31). For some viruses from the major receptor group, heparin sulfate serves as an additional receptor (32).

Viral RNA genome is released into the cytoplasm of the infected cell, where the host-cell translation machinery directly translates positive-sense RNA (33, 34). Translation is initiated by a *cap*-independent mechanism (35), resulting in a single large polyprotein which is further proteolytically processed into ten proteins and several functional intermediates. The replication of the genome takes place on virus-induced membrane structures (36). The process is carried out by a virus specific RNA- dependent RNA polymerase via semi-conservative mechanism (35).

Mature rhinovirus virions exit the host cells without destroying the cell. By analogy with other enteroviruses, a possible spread from cell to cell by microvesicles carrying the virus can be assumed (37, 38).

5. Evolution and genetic diversity

One of the major characteristics of RVs is their vast genetic diversity. Like other RNA viruses this feature arises mostly from the error-prone nature of the viral RNA- dependent RNA polymerase. The frequency of misincorporated nucleotides is 10^{-3} to 10^{-5} per nucleotide site. Fast replication cycle and high mutation frequency result in the existence of mixtures of related, but non-identical viral variants or quasispecies (39). Many of these mutations lead to a variety of amino acids sequences of the capsid proteins, which can explain the existence of many antigenically distinct RV variants (40).

Another possible mechanism for genetic diversity is recombination, and for non-RV enteroviruses recombination has been extensively studied and documented (41, 42, 43). Surprisingly, recombination events in rhinoviruses seem to be rare and are probably limited to ancient events. Evidence for such ancient evolutionary events have been identified as a result of interspecies recombination between RV-A and RV-C in the 5'UTR and 2A sequences (44). Contemporary recombination events among RV circulating strains are believed to occur mainly between the same species and thus would give

rise to recombinants highly related to the parental strains. Such intraspecies recombinations within the coding region have been documented for RV-A (45), but not for RV-B and –C (17, 45).

PATHOGENESIS

1. Transmission

The transmission of rhinoviruses from infected to susceptible individuals occurs via inhalation of viral particles – direct contact, or through a fomite, with self-inoculation into eye or nose in the absence of adequate hand hygiene. RVs are able to survive on hands for several hours, which allow an easy human-to-human transmission through this route, particularly when viral load is higher and secretions are plentiful and difficult to control (46). RVs spread most efficiently within families, school groups, students, and on military bases (47, 48).

2. Target tissues and receptors

The primary site of rhinovirus infection is the nasal mucosa and the airway epithelium. Rhinovirus receptors can be found both in ciliated and non-ciliated epithelium cells of the nasopharynx. Until recently, rhinovirus infection was thought to be restricted to the upper respiratory tract due to temperature sensitivity of the viruses. This was supported by early observations of reduced RV replication at higher temperatures (37°C or 39°C compared to 33°C). Recent studies suggest that rhinovirus replication is reduced by host-defense systems, and particularly interferon-response, because IFN induction is increased at 37°C, compared to 33°C (49). The RV replication is not only effective in lower airway epithelium, but also the difference in replication capacity at lower temperatures is minimal and may be RV type-specific (50, 51).

It was generally accepted that RVs are unable to spread by viremia and to infect other organs. But currently there are multiple studies reporting detection of RV RNA in sinuses (52) or in the middle ear (53). However, infection of these sites is presumed to happen by local extension. The detection of RVs in blood and stools, as well as the great number of different RV types add an extra complexity to the understanding of rhinovirus pathogenesis. It remains unclear if detection of rhinovirus RNA in plasma or stools represents systemic infection (54, 55, 56, 57, 58).

3. Pathogenesis

RVs do not cause epithelial cell destruction by themselves, but as a result of rhinovirus replication the tight junctions between cells are dissociated and hence, the barrier function of the epithelium is compromised. This may increase paracellular permeability and would promote the translocation of the virus and other pathogens like bacteria across the polarized airway epithelial cells, which can result in a complicated disease (59). Some authors suggest similar mechanism for airway inflammation and allergic sensitization and the rhinovirus associated development of asthma (60, 61).

4. Host response

Once rhinovirus infection occurs, the host responds with an impetuous inflammatory response including a variety of antiviral factors, proinflammatory cytokines and chemokines. The concentration of these inflammatory mediators correlates with the severity of symptoms, hereof it is generally accepted that the majority of symptoms are due to the host inflammatory response. There are some indicators that neurogenic reflexes also play a role in the pathogenesis of the infection with parasympathetic nerves controlling the flow of secretions from the nasal seromucous glands (62).

An antibody response to RV infection also occurs with the development of serotype-specific neutralizing serum antibodies and secretory antibodies (IgA) in the airways, detectable usually after one- or two-weeks post inoculation and maintained for at least one year. However, there is large number of RV types, which means repeated infections are common. Moreover, antibody production in natural RV infections occurs on an average only in 50% of patients (63). The resolution of the symptoms and clearance of virus (usually within 7 days) occur before the induction of the antibodies suggesting that clearance involve other mechanism like cellular immune response (64). Taken together immune response against RVs as well as the recovery from rhinovirus infection is still incompletely understood.

5. Clinical syndromes and complications

Human susceptibility to rhinovirus infection is high and depends on age, immune status, and ambient temperature. Risk factors like stress, lack of sleep, tobacco smoke and other air pollutants increase the body susceptibility to RVs (2).

In children, rhinovirus detection in asymptomatic patients ranges from 12% to 40% (65, 66, 67, 68, 69), but only 2% for adults (70). Children are most often the target for rhinovirus infections

and experience up to 12 infections per year. The susceptibility to RV infections decreases with age and an immunocompetent adult may be infected two to three times per year (71) and is more likely to be symptomatic.

The incubation period varies from 1-2 days to 6 days. When symptomatic, the infection has an acute beginning with symptoms peak at 48-72 hours after infection. The duration of the illness is about 7 days on average, but in some cases may be up to 2 weeks (72, 32, 73). The most frequent clinical manifestation is the common cold or acute upper respiratory infection. Symptoms include sore throat, cough, sneezing, nasal congestion, rhinorrhea with clear, muco-watery secretion flows, which later become mucoid or purulent. Low-grade fever, malaise and headache may also be presented. In imunocompetent individuals symptoms spontaneously resolve whithin a week although viral shedding in nasal secretion may continue up to three weeks (73).

Rhinovirus infections are considered as benign, self-limited and generally mild human diseases, but complications are not uncommon.

In children acute otitis media (AOM) is a frequent complication (74) with an abnormal middle ear pressure, swelling and obstruction of the Eustachian tube. AOM may be due to direct viral infection of the middle ear fluid or bacterial co-infection (75).

In adults the frequent complication is acute sinusitis, possibly through the increased pressure during nose blowing, sneezing, and coughing (21). Rhinovirus infection may trigger exacerbation of pre-existing chronic rhinosinusitis, especially in combination with cigarette smoking (76).

It is not uncommon to develop some longer-lasting olfactory disorders after a rhinovirus infection. This complication affects adults, mainly women in a percentage varying from 11% to 40%. In some cases complete recovery may take up to two years (77).

RVs have the ability to infect lower airways and are linked to laryngotracheobronchitis, bronchiolitis and pneumonia. In fact, RVs are the second most common viral causative agent (after respiratory syncytial virus) for children hospitalization due to bronchiolitis and pneumonia (4, 78).

In immunocompromised individuals rhinovirus infections are associated with severe lower respiratory tract disease and fatal pneumonia (79).

The linkage of rhinovirus infection and asthma development and exacerbation has been extensively studied in last decades. Several studies suggest that rhinovirus-induced wheezing in early childhood may be associated with increased risk for recurrent wheezing and subsequent childhood asthma development (11, 12). It is characterized by reversible airflow obstruction, bronchial hyper-responsiveness, and underlying inflammation leading to clinical symptoms (80, 81). RV infection may cause an acute loss of symptoms control or exacerbation. While the association is clear, the mechanisms behind RV-induced asthma exacerbations remain uncertain and many authors suggest that aberrant immune response to RV infection as a possible reason for exacerbation of asthma (reviewed by *Hammond et al.* (81) *and Stone et al.* (82). In addition to asthma, RVs have been associated with more than 40% of acute exacerbations of COPD (21).

DIAGNOSIS

The symptoms of a rhinovirus infection are indistinguishable from those of other viral respiratory pathogens. For that reason etiologic diagnosis rely on laboratory conformation. RVs can be found at the highest titers in nasal secretions, hence nasal secretions and nasal lavage fluids, nasopharyngeal swabs, and combined nose and throat swabs are the most suitable specimen types for diagnostic purposes. Considering the ability of RVs to infect lower respiratory tract, sputum and bronchoalveolar lavage samples also can be used (9). Excreted RVs are at their highest titers during the first days after onset of symptoms (83).

The specific virologic diagnosis is usually done by a molecular assay applying RT-PCR. Although virus isolation is considered as a “golden standard” for identification of viruses, it is a very time-consuming method and hence, is not appropriate for diagnostic purposes. The cytopathic effect, produced by RVs and enteroviruses is quite similar and it cannot be relied on for differentiation. Furthermore, not all RVs grow in cell cultures, like RV-C, in particular. Consequently laboratory confirmation is rarely performed by viral culture methods.

Rapidly advancing molecular methods have led to a better understanding of the burden of diseases associated with RV infection and RT-PCR is proven to be efficient, sensitive and specific for detection of RVs. RT-PCR assays use primers that target a conserved

region in the 5'-UTR of the rhinovirus genome, but still there is the problem of differentiation between rhinoviruses and enteroviruses and rhinovirus typing can be done only by sequencing (62).

TREATMENT

Picornaviruses are one of the most studied virus group and there are plenty of compounds tested for antiviral activity against them. Many compounds alone or in combination exhibit anti-rhinoviral activity *in vitro* (84, 85, 86). However, currently there is no specific antiviral therapeutic agent that is licensed for treatment of rhinovirus infections. A few agents showed modest results in decreasing either symptom severity or viral activity, in clinical trials (62, 21). Currently, the therapy is supportive with the use of over-the-counter products aimed at symptoms relief. These include nonsteroidal anti-inflammatory drugs, antihistamines, decongestants, and anticholinergic nasal solutions. The use of antihistamines is a subject of debates, because the beneficial effect on severity of symptoms is limited and short-term, and they are often associated with side effects like sedation (62, 87).

PREVENTION

Since the transmission of RVs occurs via a direct contact, adequate hand hygiene is the most appropriate and effective preventive strategy. It should be noted that the lack of lipid envelope in the virion makes RVs resistant to ether, chloroform, ethanol and other organic solvents so that ethanol-containing hand rubs should be avoided as a substitute for hand washing with soap and water (88). In the presence of contaminated surfaces, handwashing could be insufficient to prevent transmission. Effective disinfection of environmental surfaces could be applied with the use of bleach, phenol-based and ammonium-based environmental surface disinfectants (89).

The development of vaccines for specific prevention is labored due to the large number of RV types, the lack of common group antigen and large genetic variability in antigenic regions. Moreover, unlike influenza where usually one strain dominates a given flu season and the vaccine can be tailored to match, RVs do not have such pattern and several strains co-circulate simultaneously in a given population at any given time (90). RVs replicate only in higher primates and the lack of suitable small animal model to test vaccine candidate effectiveness add an extra complexity to developing of cross-serotype rhinovirus vaccine. For that reason RV vaccine research was abandoned for more than 20 years. Recent progress in molecular techniques and sequencing of RV genomes (91), including RV-C (19), as well as developments of mouse models may speed-up the process and maybe a vaccine against all rhinovirus serotypes could be possible (92).

EPIDEMIOLOGY

Not much is known about the circulation pattern of rhinoviruses. This is mostly because RV infection is considered as mild and often is not diagnosed. Understanding of RV distribution is also hampered by diagnostic methods used until recently. Molecular epidemiological studies as well as whole-genome sequencing of circulating viruses may contribute to clearly understand the virus's circulation patterns. The seasonality of RV is still not clearly understood as well. In the temperate zone, respiratory infections are traditionally associated with the colder part of the year. Growing number of studies reported RV detections in all seasons with slightly higher incidence rate in the autumn and spring (93, 94). It is only in winter that other infective agents predominate (95). In many parts of the world, including many European countries and Bulgaria rhinovirus infections are left unobserved and therefore, not much is known about their circulation patterns and seasonality.

CONCLUSIONS

Even with the advances of today's medicine and health-care systems RVs constitute a significant burden with associated sociological and economic impact. It is more concerning that in the ongoing COVID-19 pandemic and all the precautions, rhinoviruses are still there. To date, 169 RV types have been described. What we know is that RVs are characterized with vast genetic diversity due to high mutation frequency and recombinantions. This can explain the existence of many antigenically distinct RV variants, but present knowledge still does not provide a strategy for controlling them. Human susceptibility to rhinovirus infection is high and symptoms of a RV infection are indistinguishable from those of other viral respiratory pathogens. The lack of lipid envelope in the virion makes RVs resistant to ethanol-containing hand sanitizers, which are widely

recommended as a precaution against other viruses. In the light of current COVID-19 pandemic, it should be kept in mind that olfactory disorders are not uncommon after a rhinovirus infection.

Although upper respiratory tract is the most common site of the rhinovirus infection, it remains unclear whether RVs are able to cause systemic infection. Moreover, linkage of RV with complicated lower respiratory tract diseases like bronchiolitis and pneumonia underlines the fact that RVs are not such a benign cause of the ordinary common cold. For people living with COPD or asthma mere rhinovirus infections might become a life-threatening condition. The development of vaccines for specific prevention is labored due to the large number of RV types, the lack of common group antigen and the large genetic variability in antigenic regions.

There are still many aspects of rhinovirus pathogenesis, immune response, as well as the recovery from infection that are not fully understood. Design of effective preventive and therapeutic strategies to control RVs will be supported by improved knowledge of their pathogenesis, immune response and transmission.

ACKNOWLEDGEMENTS:

This work was supported by project КП-06-Н23/10 of the Bulgarian National Science Fund, Ministry of Education and Science, Sofia, Bulgaria

REFERENCES

1. Makela MJ, Puhakka T, Ruuskanen O, Leinonen M, Saikku P, Kimpimaki M, Blomqvist S, Hyypia T, Arstila P. *Viruses and Bacteria in the Etiology of the Common Cold*. J Clin Microbiol. 1998; 36: 539–542.
2. Brownlee JW, Turner RB. *New developments in the epidemiology and clinical spectrum of rhinovirus infections*. Curr Opin Pediatr. 2008; 20(1):67-71.
3. Fendrick AM, Monto AS, Nightengale B, Sarnes M. *The Economic Burden of Non–Influenza-Related Viral Respiratory Tract Infection in the United States*. Arch Intern Med. 2003; 163(4):487–494.
4. Hayden FG. *Rhinovirus and the lower respiratory tract.* Rev Med Virol. 2004; 14(1):17-31.
5. Thibaut HJ, Lacroix C, De Palma AM, Franco D, Decramer M, Neyts J. *Toward antiviral therapy/prophylaxis for rhinovirus-induced exacerbations of chronic obstructive pulmonary disease: challenges, opportunities, and strategies*. Rev Med Virol. 2016; 26(1):21–33.
6. Gern JE, Busse WW. *Association of rhinovirus infections with asthma*. Clin Microbiol Rev.1999; 12(1):9–18.
7. McManus TE, Marley AM, Baxter N, Christie SN, O'Neill HJ, Elborn JS, Coyle PV, Kidney JC. *Respiratory viral infection in exacerbations of COPD*. Respir Med. 2008; 102(11):1575-80.
8. Gern JE. *The ABCs of rhinoviruses, wheezing, and asthma*. J Virol. 2010; 84(15):7418–7426.
9. Papadopoulos NG. Bates PJ, Bardin PG, Papi A, Leir S, Fraenkel DJ, Meyer J, Lackie PM, Sanderson G, Holgate ST, Johnston SL. *Rhinoviruses Infect the Lower Airways*. The Journal of Infectious Diseases. 2000; 181(6):1875–1884.
10. Gutman JA, Peck AJ, Kuypers J, Boeckh M. *Rhinovirus as a cause of fatal lower respiratory tract infection in adult stem cell transplantation patients: a report of two cases*. Bone Marrow Transplantation. 2007; 40:809-811.
11. Gavala ML, Bertics PJ, and Gern JE. *Rhinoviruses, allergic inflammation, and asthma*. Immunol Rev 2011; 242(1):69-90.
12. Jackson DJ, Gangnon RE, Evans MD, Roberg KA, Anderson EL, Pappas TE, Printz MC, Lee W-M, Shult PA, Reisdorf E, Carlson-Dakes KT, Salazar LP, DaSilva DF, Tisler CJ, Gern JE, Lemansake Jr RF. *Wheezing Rhinovirus Illnesses in Early Life Predict Asthma Development in High-Risk Children*. Am J Respir Crit Care Med. 2008; 178(7):667-72.
13. Kenealy T, Arroll B. *Antibiotics for the common cold and acute purulent rhinitis*. Cochrane Database Syst Rev. 2013;6: doi:10.1002/14651858.CD000247.pub3.
14. Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. *Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections.* J Med Virol. 2006; 78(9):1232-1240.
15. Kaiser L, Aubert JD, Pache JC, Deffernez C, Rochat T, Garbino J, Wunderli W, Meylan P, Yerly S, Perrin L, Letovanec I, Nicod L, Tapparel C, Soccal PM. *Chronic rhinoviral infection in lung transplant recipients*. Am J Respir Crit Care Med. 2006;174(12):1392–1399.
16. Lee WM, Kiesner C, Pappas T, Lee I, Grindle K, Jartti T, Jakiela B, Lemanske Jr RF, Shult PA, Gern JE. *A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants*. PloS One. 2007; 2(10):e966.
17. McIntyre CL, Knowles NJ, Simmonds P. *Proposals for the classification of human rhinovirus species A, B and C into genotypically assigned types*. J Gen Virol. 2013;94(Pt 8):1791–1806.
18. Bochkov YA, Watters K, Ashraf S, Griggs TF, Devries MK, Jackson DJ, Palmenberg AC, Gern JE. *Cadherin-related family member 3, a childhood asthma susceptibility gene product, mediates rhinovirus C binding and replication*. Proc Natl Acad Sci USA. 2015; 112(17):5485–5490.
19. Bochkov YA, Palmenberg AC, Lee WM, Rathe JA, Amineva SP, Sun X, Pasic TR, Jarjour NN, Liggett SB, Gern JE. *Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C.* Nat Med. 2011;17(5):627-632.
20. Simmonds P, Gorbalenya AE, Harvala H, Hovi T, Knowles NJ, Lindberg AM, Oberste MS, Palmenberg AC, Reuter G, Skern T, Tapparel C, Wolthers KC, Woo PCY, Zell R. *Recommendations for the nomenclature of enteroviruses and rhinoviruses*. Arch Virol. 2020;165(3):793-797.
21. Greenberg SB. *Respiratory consequences of rhinovirus infection*. Arch Intern Med. 2003;163(3);278–284.
22. Hendry E, Hatanaka H, Fry E, Smyth M, Tate J, Stanway G, Santti J, Maaronen M, Hyypiä T, Stuart D. *The crystal structure of coxsackievirus A9: new insights into the uncoating mechanisms of enteroviruses*. Structure (London, England:1993).1999; 7(12):1527–1538.
23. Rossmann MG, He Y and Kuhn RJ. *Picornavirus-receptor interactions*. Trends Microbiol. 2002; 10(7):324–331.
24. Hayden FG, Herrington DT, Coats TL, Kim K, Cooper EC, Villano SA, Liu S, Hudson S, Pevear DC, Collett M, McKinlay M, Pleconaril Respiratory Infection Study Group. *Efficacy and safety of oral pleconaril for treatment of colds due to picornaviruses in adults: results of 2 double-blind, randomized, placebo-controlled trials*. Clin Infect Dis. 2003; 36(12):1523–1532.
25. McKinlay MA. *WIN 51711, a new systematically active broad-spectrum antipicornavirus agent*. J Antimicrob Chemother. 1985;16(3): 284–286.
26. Liu Y, Hill MG, Klose T, Chen Z, Watters K, Bochkov YA, Jiang W, Palmenberg AC, Rossmann MG. *Atomic structure of a rhinovirus C, a virus species linked to severe childhood asthma*. Proc Natl Acad Sci USA. 2016; 113(32):8997-9002.
27. Borman AM, Bailly JL, Girard M, Kean KM. *Picornavirus internal ribosome entry segments: comparison of translation efficiency and the requirements for optimal internal initiation of translation in vitro*. Nucleic Acids Res.1995; 23(18):3656–3663.
28. Paul AV and Wimmer E. *Initiation of protein-primed picornavirus RNA synthesis*. Virus Res. 2015;206:12–26.
29. Uncapher CR, DeWitt CM, Colonno RJ. *The major and minor group receptor families contain all but one human rhinovirus serotype*. Virology. 1991;180(2):814-817.
30. Palmenberg AC, Rathe JA, Liggett SB. *Analysis of the complete genome sequences of human rhinovirus.* J Allergy Clin Immunol. 2010;125(6):1190-1201.
31. Verdaguer N, Fita I, Reithmayer M, Moser R, Blaas D. *X-ray structure of a minor group human rhinovirus bound to a fragment of its cellular receptor protein*. Nat Struct Mol Biol. 2004;11(5):429–434.
32. Jacobs SE, Lamson DM, George KSt, Walsh TJ. *Human rhinoviruses*. Clin Microbiol Rev. 2013; 26(1):135–162.
33. Fuchs R and Blaas D. *Uncoating of human rhinoviruses*. Rev Med Virol. 2010;20(5):281–297.
34. Baggen J, Thibaut HJ, Strating J, van Kuppeveld F. *The life cycle of*

non-polio enteroviruses and how to target it. Nat Rev Microbiol. 2018;16(6): 368–381.
35. Rowlands DJ. *Picornaviruses*. In: eLS, John Wiley & Sons, Ltd (Ed.). 2015, doi:10.1002/9780470015902.a0001080.pub3.
36. van der Schaar HM, Dorobantu CM, Albulescu L, Strating J, van Kuppeveld F. *Fat(al) attraction: Picornaviruses Usurp Lipid Transfer at Membrane Contact Sites to Create Replication Organelles*. Trends Microbiol. 2016; 24(7):535–546.
37. Inal JM, Jorfi S. *Coxsackievirus B transmission and possible new roles for extracellular vesicles*. Biochem Soc Trans. 2013; 41(1):299–302.
38. Lai JK, Sam IC, Chan YF. *The Autophagic Machinery in Enterovirus Infection*. Viruses. 2016; 8(2):32.
39. Domingo E, Sheldon J, Perales C. *Viral quasispecies evolution*. Microbiol Mol Biol Rev. 2012; 76(2):159-216.
40. Royston L, Tapparel C. *Rhinoviruses and Respiratory Enteroviruses: Not as Simple as ABC*. Viruses. 2016; 8(1):16.
41. Lukashev AN, Lashkevich VA, Ivanova OE, Koroleva GA, Hinkkanen AE, Ilonen J. *Recombination in circulating Human enterovirus B: independent evolution of structural and non-structural genome regions*. J Gen Virol. 2005; 86(Pt 12):3281–3290.
42. Santti J, Hyypiä T, Kinnunen L, Salminen M. *Evidence of recombination among enteroviruses*. J Virol, 1999; 73(10): 8741–8749.
43. Simmonds P, Welch J. *Frequency and dynamics of recombination within different species of human enteroviruses*. J Virol. 2006; 80(1):483–493.
44. McIntyre CL, McWilliam Leitch EC, Savolainen-Kopra C, Hovi T, Simmonds P. *Analysis of genetic diversity and sites of recombination in human rhinovirus species C*. J Virol. 2010; 84(19):10297–10310.
45. Tapparel C, Junier T, Gerlach D, Van-Belle S, Turin L, Cordey S, Mühlemann K, Regamey N, Aubert JD, Soccal PM, Eigenmann P, Zdobnov E, Kaiser L. *New respiratory enterovirus and recombinant rhinoviruses among circulating picornaviruses*. Emerg Infect Dis. 2009;15(5):719–726.
46. L'Huillier AG, Tapparel C, Turin L, Boquete-Suter P, Thomas Y, Kaiser L. *Survival of rhinoviruses on human fingers.* Clin Microbiol Infect. 2015; 21(4): 381–385.
47. Fox JP, Cooney MK, Hall CE. *The Seattle virus watch. V. Epidemiologic observations of rhinovirus infections, 1965-1969, in families with young children*. Am J Epidemiol. 1975;101(2):122–143.
48. Fox JP, Cooney MK, Hall CE, Foy HM. *Rhinoviruses in Seattle families, 1975-1979*. Am J Epidemiol. 1985;122(5):830–846.
49. Foxman EF, Storer JA, Fitzgerald ME, Wasik BR, Hou L, Zhao H, Turner PE, Pyle AM, Iwasaki A. *Temperature-dependent innate defense against the common cold virus limits viral replication at warm temperature in mouse airway cells*. Proc Natl Acad Sci USA. 2015;112(3):827–832.
50. Papadopoulos NG, Sanderson G, Hunter J, Johnston SL. *Rhinoviruses replicate effectively at lower airway temperatures*. J Med Virol. 1999; 58(1):100–104.
51. Tapparel C, Sobo K, Constant S, Huang S, Van Belle S, Kaiser L. *Growth and characterization of different human rhinovirus C types in three-dimensional human airway epithelia reconstituted in vitro*. Virology. 2013; 446(1-2):1–8.
52. Pitkäranta A, Arruda E, Malmberg H, Hayden FG. *Detection of rhinovirus in sinus brushings of patients with acute community-acquired sinusitis by reverse transcription-PCR*. J Clin Microbiol, 1997; 35(7): 1791–1793.
53. Chantzi FM, Papadopoulos NG, Bairamis T, Tsiakou M, Bournousouzis N, Constantopoulos AG, Liapi G, Xatzipsalti M, Kafetzis DA. *Human rhinoviruses in otitis media with effusion*. Pediatr Allergy Immunol. 2006; 17(7):514–518.
54. Lupo J, Schuffenecker I, Morel-Baccard C, Bardet J, Payen V, Kaiser L, Constant S, Lobrinus JA, Lin-Marq N, Lina B, Morand P, Tapparel C. *Disseminated rhinovirus C8 infection with infectious virus in blood and fatal outcome in a child with repeated episodes of bronchiolitis*. J Clin Microbiol, 2015; 53(5): 1775–1777.
55. Harvala H, McIntyre CL, McLeish NJ, Kondracka J, Palmer J, Molyneaux P, Gunson R, Bennett S, Templeton K, Simmonds P. *High detection frequency and viral loads of human rhinovirus species A to C in fecal samples; diagnostic and clinical implications*. J Med Virol. 2012; 84(3):536–542.
56. Esposito S, Daleno C, Scala A, Castellazzi L, Terranova L, Sferrazza Papa S, Longo MR, Pelucchi C, Principi N. *Impact of rhinovirus nasopharyngeal viral load and viremia on severity of respiratory infections in children*. Eur J Clin Microbiol Infect Dis. 2014; 33(1):41–48.
57. Urquhart GE, Stott EJ. *Rhinoviraemia*. BMJ. 1970; 4(5726): 28–30.
58. Honkanen H, Oikarinen S, Peltonen P, Simell O, Ilonen J, Veijola R, Knip M, Hyöty H. *Human rhinoviruses including group C are common in stool samples of young Finnish children*. J Clin Virol. 2013; 56(3): 250–254.
59. Sajjan U, Wang Q, Zhao Y, Gruenert DC, Hershenson MB. *Rhinovirus disrupts the barrier function of polarized airway epithelial cells*. Am J Respir Crit Care Med. 2008;178(12):1271–1281.
60. Lambrecht BN, Hammad H. *Allergens and the airway epithelium response: gateway to allergic sensitization*. J Allergy Clin Immunol. 2014; 134(3): 499–507.
61. Lambrecht BN, Hammad H. *Asthma: the importance of dysregulated barrier immunity*. Eur J Immunol. 2013; 43(12): 3125–3137.
62. Turner RB. *177 – Rhinovirus*. In Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition), Elsevier Inc. 2015; 2113-2121.
63. Barclay WS, al-Nakib W, Higgins PG, Tyrrell DA. *The time course of the humoral immune response to rhinovirus infection*. Epidemiology and infection.1989;103(3):659–669.
64. Steinke JW, Liu L, Turner RB, Braciale TJ, Borish L. *Immune surveillance by rhinovirus-specific circulating CD4+ and CD8+ T lymphocytes*. PloS One. 2015;10(1):e0115271.
65. van Benten I, Koopman L, Niesters B, Hop W, van Middelkoop B, de Waal L, van Drunen K, Osterhaus A, Neijens H, Fokkens W. *Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants*. Pediatr Allergy Immunol. 2003; 14(5): 363–370.
66. Jansen RR, Wieringa J, Koekkoek SM, Visser CE, Pajkrt D, Molenkamp R, de Jong MD, Schinkel J. *Frequent detection of respiratory viruses without symptoms: toward defining clinically relevant cutoff values*. J Clin Microbiol. 2011; 49(1):2631–2636.
67. Jartti T, Jartti L, Peltola V, Waris M, Ruuskanen O. *Identification of respiratory viruses in asymptomatic subjects: asymptomatic respiratory viral infections*. Pediatr Infect Dis J. 2008; 27(12):1103–1107.
68. Iwane MK, Prill MM, Lu X, Miller EK, Edwards KM, Hall CB, Griffin MR, Staat MA, Anderson LJ, Williams JV, Weinberg GA, Ali A, Szilagyi PG, Zhu Y, Erdman DD. *Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children*. J Infect Dis. 2011; 204(11):1702–1710.
69. Singleton RJ, Bulkow LR, Miernyk K, DeByle C, Pruitt L, Hummel KB, Bruden D, Englund JA, Anderson LJ, Lucher L, Holman RC, Hennessy TW. *Viral respiratory infections in hospitalized and community control children in Alaska*. J Med Virol. 2010; 82(7):1282–1290.
70. Graat JM, Schouten EG, Heijnen ML, Kok FJ, Pallast EG, de Greeff SC, Dorigo-Zetsma JW. *A prospective, community-based study on virologic assessment among elderly people with and without symptoms of acute respiratory infection*. J Clin Epidemiol. 2003; 56(12):1218–1223.
71. Turner RB. *Epidemiology, pathogenesis, and treatment of the common cold*. Ann Allergy Asthma Immunol. 1997; 78(6):531–540.
72. Pappas DE, Hendley JO, Hayden FG, Winther B. *Symptom profile of common colds in school-aged children*. Pediatr Infect Dis J. 2008; 27(1):8–11.
73. Gwaltney JMJ, Hendley JO, Patrie JT. *Symptom severity patterns in experimental common colds and their usefulness in timing onset of illness in natural colds*. Clin Infect Dis. 2003; 36(6):714–723.
74. Armengol CE, Hendley JO, Winther B. *Occurrence of acute otitis media during colds in children younger than four years*. Pediatr Infect Dis J. 2011; 30(6):518–520.
75. Marom T, Nokso-Koivisto J, Chonmaitree T. *Viral-bacterial interactions in acute otitis media*. Curr Allergy Asthma Rep. 2012; 12(6): 551–558.
76. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, Cohen N, Cervin A, Douglas R, Gevaert P, Georgalas C, Goossens H, Harvey R, Hellings P, Hopkins C, Jones N, Joos G, Kalogjera L, Kern B, Kowalski M, Price D, Riechelmann H, Schlosser R, Senior B, Thomas M, Toskala E, Voegels R, Wang dY, Wormald PJ. *European Position Paper on Rhinosinusitis and Nasal Polyps*. Rhinol Suppl.2012; 23:3–298.
77. Welge-Lüssen A, Wolfensberger M. *Olfactory disorders following upper respiratory tract infections*. Adv Otorhinolaryngol. 2006; 63:125–132.
78. Papadopoulos NG. *Do rhinoviruses cause pneumonia in children?.* Paediatr Respir Rev. 5 Suppl A. 2004; S191–S195.
79. Ghosh S, Champlin R, Couch R, Englund J, Raad I, Malik S, Luna M, Whimbey E. *Rhinovirus infections in myelosuppressed adult blood and marrow transplant recipients*. Clin Infect Dis.1999; 29(3):528–532.
80. National Asthma Education and Prevention Program, *Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report*. 2007. J Allergy Clin Immunol. 2007; 120(5 Suppl), p. S94–S138.
81. Hammond C, Kurten M, Kennedy JL. *Rhinovirus and asthma: a storied history of incompatibility.* Curr Allergy Asthma Rep. 2015; 15(2):502.
82. Stone CA, and Miller EK. *Understanding the Association of Human Rhinovirus with Asthma*. Clin Vaccine Immunol. 2015; 23(1):6–10.
83. Versalovic J, Caroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW.

Manual of clinical microbiology. ASM Press, ©2011, Washington, D.C.
84. Georgieva I, Galabov A. *In Vitro Anti-Rhinovirus Activity of Some Picornavirus Replication Inhibitors*. Acta microbiologica Bulgarica, 2016; 32: 237-242.
85. Mello C, Aguayo E, Rodriguez M, Lee G, Jordan R, Cihlar T, Birkus G. *Multiple classes of antiviral agents exhibit in vitro activity against human rhinovirus type C*. Antimicrob Agents Chemother. 2014;58(3):1546–1555.
86. Choi HJ. *In Vitro Antiviral Activity of Sakuranetin against Human Rhinovirus 3*. Osong Public Health Res Perspect. 2017; 8(6): 415–420.
87. De Sutter AI, Saraswat A, van Driel ML. *Antihistamines for the common cold*. Cochrane Database Syst Rev. 2015; 11:CD009345.
88. Savolainen-Kopra C, Korpela T, Simonen-Tikka ML, Amiryousefi A, Ziegler T, Roivainen M, Hovi T. *Single treatment with ethanol hand rub is ineffective against human rhinovirus--hand washing with soap and water removes the virus efficiently*. J Med Virol. 2012; 84(3):543–547.
89. Sattar SA, Jacobsen H, Springthorpe VS, Cusack TM, Rubino JR. *Chemical disinfection to interrupt transfer of rhinovirus type 14 from environmental surfaces to hands*. Appl Environ Microbiol. 1993; 59(5): 1579–1585.
90. Glanville N, Johnston SL. *Challenges in developing a cross-serotype rhinovirus vaccine*. Curr Opin Virol. 2015;11:83–88.
91. Palmenberg AC, Spiro D, Kuzmickas R, Wang S, Djikeng A, Rathe JA, Fraser-Liggett CM, Liggett SB. *Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution*. Science . 2009; 324(5923):55–59.
92. McLean GR. *Developing a vaccine for human rhinoviruses*. Journal of vaccines & immunization. 2014; 2(3): 16–20.
93. L'Huillier AG, Kaiser L, Petty TJ, Kilowoko M, Kyungu E, Hongoa P, et al. *Molecular epidemiology of human rhinoviruses and enteroviruses highlights their diversity in sub-Saharan Africa*. Viruses. 2015;7:6412-23
94. Panda S, Mohakud NK, Panda S, Kumar S. *Epidemiology and phylogenetic analysis of human rhinovirus/Enterovirus in Odisha, Eastern India*. Indian J Med Microbiol. 2019; 37:569-73
95. Monto AS. *The seasonality of rhinovirus infections and its implications for clinical recognition.* Clinical Therapeutics. 2002; 24(12):1987-1997.

CORRELATION BETWEEN THE ANTIBODY RESPONSE TOWARD SPECIFIC HCV PROTEINS AND HCV VIRAL LOAD

Chiydem Ismailova, Viliana Yontcheva, Tencho Tenev, Elitsa Golkocheva-Markova

NRL "Hepatitis viruses", NCIPD

ABSTARCT

Background: Hepatitis C virus (HCV) is an RNA virus causing acute or chronic infection and affecting more than 2% of population worldwide. The first-line tests for diagnosis of HCV infection are 3rd or 4th generation enzyme immunoassays - ELISA and CIA. They indicate the presence of antibodies against HCV in serum. These tests are characterized by high sensitivity and specificity, but they cannot distinguish past, acute or chronic infection, and sometimes produce false positive results. Confirmatory tests, such as recombinant immunoblot-line immune assay (LIA), and quantitative PCR, are used to validate the positive antibody response. The recombinant immunoblot assay can be used to determine the specificity of antibody to HCV. The aim of the present study is to determine the correlation between the anti-HCV response in confirmatory immunoblot assay and the HCV viral load, measured by PCR.

Materials and methods: Twenty-nine anti-HCV positive sera were included in the study. Third generation ELISA assay was used for anti-HCV screening of the samples and for detection of anti-HCV antibodies against specific HCV proteins. Third generation line immunoassay INNO-LIA HCV Score, based on the principle of an enzyme immunoassay, was used as a confirmatory test. The HCV viral load was measured by quantitative PCR method – Abbott Real Time HCV (Abbott Molecular Inc., USA) with linear sensitivity range from 1.08 Log_{10} IU/ml (12 [IU/ml]) to 8.00 Log_{10} IU/ml (100 000 000 [IU/ml]).

Results: HCV RNA was quantified in all studied samples. Ten of 29 serum samples (34%, Group I) were HCV RNA negative. The rest of the samples were HCV RNA positive as follows: 3 serums were with minimal viral load from < 12 to 10 000 IU/ml (10%, Group II); 3 serum samples –between 10 000 and 100 000 IU/ml (10%, Group III); 10 serum samples – between 100 000 and 1 000 000 IU/ml (34%, Group IV) and in 3 serum samples HCV RNA concentration was over 1 000 000 IU/ml (10%, Group V).

Conclusion: HCV screening strategies involving anti-HCV detection by ELISA combined with recombinant immunoblot assay can be the method of choice in laboratories with limited equipment and finances.

Keywords: *Hepatitis C virus, recombinant immunoblot assay, antibodies*

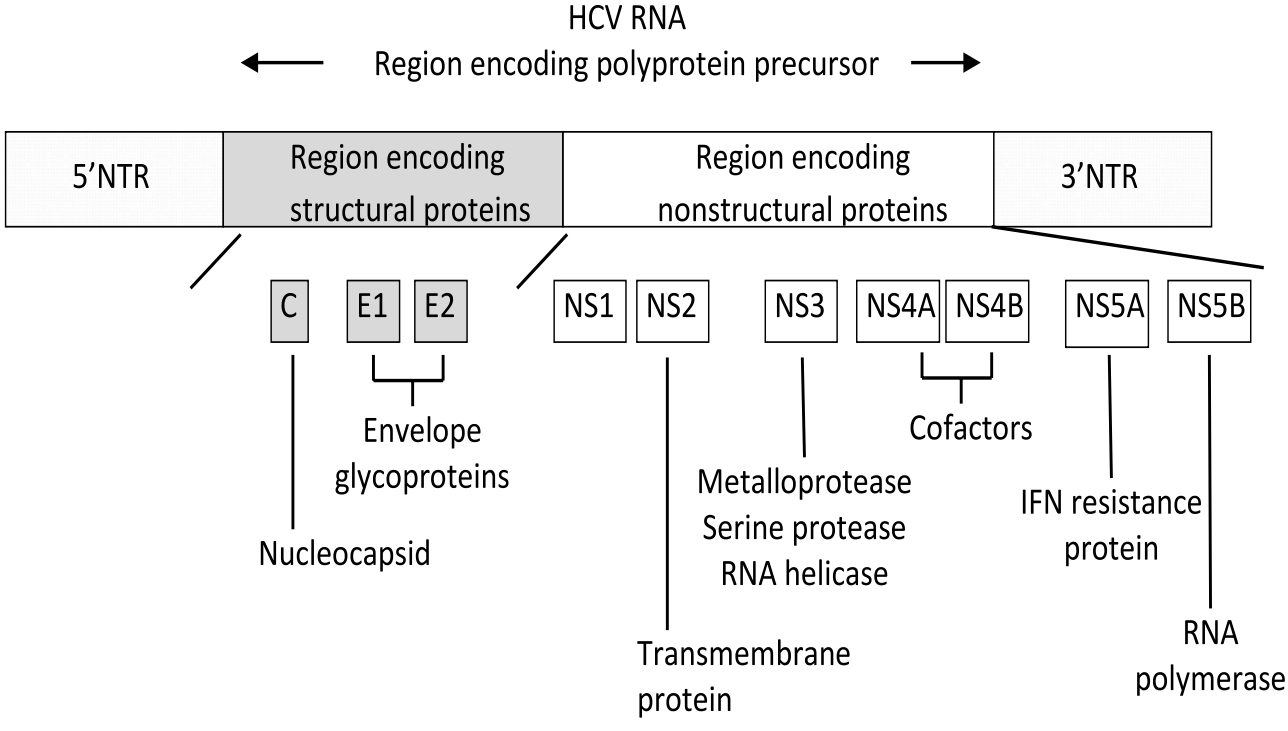
ADDRESS FOR CORRESPONDENCE:
Chiydem Ismailova, NRL "Hepatitis viruses",
Departament of Virology, NCIPD, 44A Stoletov BLVD.
1233 Sofia, Bulgaria
Tel: +359 2 8329 118;
e-mail: chiydem.ismailova@abv.bg

INTRODUCION

Hepatitis C virus (HCV) is an RNA virus that belongs to the *Flaviviridae* family. HCV causes acute or chronic infection and affects more than 2% of the individuals worldwide. The virus is transmitted by blood transfusion or organ transplantations, injection drug use, vertically during pregnancy, or sexually. Because of its modes of transmission, current recommendations for HCV screening include the following categories: pregnant women; blood donors; healthcare workers; hemodialysis patients; patients undergoing surgery or chemotherapy; people living with HIV, sex workers. The time of appearance of serological and virological markersfor HCV infection is not exactly defined yet. At present, the natural course of acute HCV infection is described as follows: initial eclipse phase when no serological and virological markers of HCV infection can be detected; appearance of HCV RNA, followed by the appearance of HCV core antigen in the absence of an antibody response, and finally development appearance of anti-HCV antibody response leading to HCV core antigen disappearance [WHO 2017]. Usually, the acute HCV infection is clinically silent and in up to 45% of the infected, spontaneous clearance occurs. In addition, in most patients, HCV infection progresses slowly and is diagnosed after the development of co-morbidity [Bruno S., C. Facciotto. 2008]. The diagnosis of HCV infection is initialized by the detection of anti-HCV antibodies in serum, as a marker of past or present infection.

At present, the first-line tests for diagnosis of HCV infection are 3rd or 4th generation serological assays based on immunoassay principles. They are available as rapid diagnostic tests (RDTs) or laboratory-based enzyme immunoassays (EIAs), chemiluminescence immunoassays (CLIAs) and electrochemiluminescence immunoassays (ECLs), and indicate the presence of antibodies against HCV (anti-HCV) in serum. These tests are characterized by high sensitivity and specificity, but they cannot distinguish past, acute or chronic infection, and sometimes produce false positive results. Anti-HCV assays have a lower positive predictive value in low-prevalence populations [Kamili S, et.al. 2012] In this case a second anti-HCV test can be used to confirm the first positive test result. Therefore, the Center for Disease Control and Prevention (CDC) recommended that a positive result from an initial anti-HCV screening test should be followed by confirmatory tests such as recombinant immunoblot and quantitative PCR method [Kodani M, et. al., 2019]. The latter methods are used to validate the positive antibody response. Recombinant immunoblot assay allows tracking of the antibody response against specific HCV (structural and non-structural) proteins (Figure 1). He viral proteins encoded by HCV genome, and used in INNO-LIA HCV test are as follows: core protein (C), NS4A, NS4B, E-protein and NS5A. The two envelope glycoproteins, E1 and E2, play pivotal role at different steps of HCV replicative cycle. They are essential for the host-cell entry by binding to receptors and inducing fusion with the host-cell membrane. The latter proteins are not genetically stable and their application in tests might be controversial. On the other hand, the core protein is an RNA– binding phosphoprotein that forms the viral nucleocapsid. It is genetically stable protein so it is used in many techniques for HCV detection. The nonstructural protein NS3 is a multifunctional enzyme with serine protease, NTPase, and RNA helicase activities. It is an essential replicative component of HCV. NS3 exhibits NTPase and helicase activity from its C-terminal helicase domain and serine protease activity from its N-terminal protease domain when it is bound by the HCV NS4A cofactor. NS4 protein is recruitment of other viral proteins and NS5 plays role in viral replication, modulation of cell signaling pathways and interferon response [Warkad SD, et.al., 2019].
The present study examines the correlation between the anti-HCV response in confirmatory immunoblot assay and the HCV viral load.

Figure1. *HCV genome organization, structural and nonstructural viral proteins and their function (Usman A Ashfaq 2011)*



MATERIALS AND METHODS:

Samples. The sera of twenty-nineanti-HCV positive patients were included in the study. The samples were grouped, according to the detected HCV viral load as follows: Group I - negative for HCV RNA; Group II – from 12 [IU/ml] to 10 000 [IU/ml]; Group III - from 10 000 [IU/ml] to 100 000 [IU/ml]; Group IV - from 100 000 [IU/ml] to 1 000 000 [IU/ml]; and Group V > 1 000 000 [IU/ml].

Detection of HCV antibodies (anti-HCV) by ELISA. Third generation ELISA assay was used for anti-HCV screening of serum samples (DiaPro, Italy). Samples were considered anti-HCV positive if the ratio between sample and calibrator optical density (OD) was greater than 1.1. Samples with a ratio less than 0.8 were considered negative.

Detection of HCV antibodies (anti-HCV) by INNO-LIA HCV Score. For detection of anti-HCV against specific HCV proteins the 3rd generation line immunoassay INNO-LIA HCV Score, was used. Coated HCV antigens were derived from core region, E2 hypervariable region, NS3 helicase region, NS4A, NS4B and NS5A regions. The presence of anti-HCV was associated with the appearance of colored bands corresponding to coated HCV specific antigens. A reactivity rating was made separately for each sample using a reading card. The iIntensity of lines was proportional to the amount of captured HCV-specific antibodies from the sample and was rated as negative (-),weak positive (±) and moderate to strong positive 1+ to 4+ (from). Identification of the lines was done by alignment of the 3+ control line on the developed strip with the corresponding 3+ control line on the reading card. Results were reported as anti-HCV negative, when all HCV antigen lines were not reactive or if only one antigen line except for NS3 line was weak positive,; and as anti-HCV positive if at least two HCV antigen lines had a weak ± or higher reactivity .

Detection and quantification of serum HCV RNA. HCV viral load was measured by quantitative PCR method – Abbott Real Time HCV (Abbott Molecular Inc.,USA) with linear sensitivity from 1.08 Log_{10} IU/ml (12 [IU/ml]) to 8.00 Log_{10} IU/ml. Samples with lower (< 1.08 Log_{10} IU/ml) or higher (> 8.00 Log_{10} IU/ml) viral load were considered positive or. Only samples reported as "not detected" were consider negative.

Results: HCV RNA was quantified in all studied samples (Table 1). Ten out of 29 serum samples (34%, Group I) were HCV RNA negative. The rest of the samples were classified according to HCV RNA viral load as follows: 3 were with minimal viral load to up 10 000 IU/ml (10%, Group II); 3 –between 10 000 and 100 000IU/ml (10%, Group III); 10 – between 100 000 and 1 000 000 IU/ml (34%, Group IV) and 3 over 1 000 000 IU/ml (10%, Group V).

By INNO-LIA assay 11 (38%) of 29 tested samples were negative for the presence of HCV antibodies and 18 (62%) were positive (Table1). Reactivity with different intensity was detected against HCV antigens. The highest frequency of HCV antibody response to all HCV antigens was found in samples from Group IV, while the weakest antibody response was detected in samples from Group II. For the samples of Group I, 6 (60%) serum samples out of the 10, show positive INNO-LIA result. All samples of Group II were negative by INNO-LIA results interpretation. The number of positive samples increased concomitantly with HCV viral load – two (67%) of 3 positive samples in Group III, and 9 (90%) of 10 samples of Group IV.

Table 1. *Group distribution of the tested samples and INNO-LIA reactivity interpretation according to HCV viral load groups*

Groups	HCV RNA [IU/ml]		samples via group		Negative samples		Positive samples	
	≥	<	N	%	N	%	N	%
I	0		10	34%	4	40%	6	60%
II	< 12	10 000	3	10%	3	100%	-	
III	10 000	100 000	3	10%	1	33%	2	67%
IV	100 000	1 000 000	10	34%	1	10%	9	90%
V	> 1 000 000		3	10%	2	67%	1	33%
Total			**29**		**11**	**38%**	**18**	**62%**

According to the presence of specific HCV antibodies the INNO-LIA results were as follows (Table 2). In Group I samples were detected HCV antibodies against all structural and non-structural HCV proteins – C1, C2, E2, NS3, NS4 and NS5, and the intensity of lines varied from weak positive (±) to moderate (1+), except for one sample where the bands intensity was from moderate to strong positive (3+). The highest frequency was established against the nonstructural NS3. As mentioned above, samples from Group II generated the weakest responses, and specific antibodies were detected only against two HCV core proteins – C1 and C3, with a weak intensity of the reacting bands. In samples from Groups III, IV and V, specific antibodies were detected against all HCV antigens. In Group III samples, the highest frequency was established for antibodies against the structural HCV E2, and the nonstructural NS3 antigens. In Group IV, HCV specific antibodies were detected against all antigens - C1, C2, E2, NS3, NS4 and NS5. The intensity of reacting bans ranged from moderate to strong positive and only one sample reacted with a weak positive antibody response against NS3. Finally, in Group V, decreased frequency of specific antibodies was established against all INNO-LIA comprised HCV antigens. Only one sample reacted against all HCV antigens and the intensity of the bands varied from weak to strong positive.

Table 2. *Frequency of specific anti-HCV, detected by INNO-LIA assay*

HCV Ag	I group (N=10)		II group (N=3)		III group (N=3)		IV group (N=10)		V group (N=3)	
	N	%	N	%	N	%	N	%	N	%
C1	4	40%	1	33%	1	33%	8	80%	1	33%
C2	4	40%	1	33%	1	33%	8	80%	1	33%
E2	1	10%	0	-	2	67%	7	70%	1	33%
NS3	5	50%	0	-	2	67%	9	90%	1	33%
NS4	4	40%	0	-	1	33%	8	80%	1	33%
NS5	4	40%	0	-	1	33%	7	70%	1	33%

DISCUSSION

The prevalence of chronic HCV infection in Bulgaria is 0,9%, according to the cross-sectional study in adults (anti-HCV and RNA positive), conducted in 2018 [Sperle, I., et.al., 2020]. The morbidity due to acute HCV infection is 1,26%$_{000}$ [Vladimirova N, et.al]. According to the World Health Organization Guidelines on Hepatitis B and C Testing, a serological assay for initial detection of evidence of past or present infection is recommended prior to supplementary nucleic acid testing (NAT) for evidence of viraemic infection [WHO 2017]. The Guidelines Development Group has recommended the use of secondary confirmatory serological assay instead HCV NAT technology in low HCV prevalence settings (< 0.4%), due to high percent of false positive anti-HCV results, and cost effectiveness,. INNO-LIA HCV Score is a 3th generation immunoblot assay for detection of specific antibodies against six HCV antigens derived from the core region, the E2 hypervariable region (HVR), the NS3 helicase region, the NS4A, NS4B, and NS5A regions.

Diagnosis of HCV infection is based on the detection of specific HCV antibodies in combination with detectible HCV RNA in cases of acute infection, and - on detection of antibodies in the absence of HCV RNA. In cases of resolved infection= Antibodies to HCV appear during the acute infection and persist up to 20 years after recovery [Takaki A, et.al., 2000]. The kinetics of HCV antibodies response is characterized initially with appearance of antibodies against viral capsid and non-structural NS3 proteins, followed by antibodies against NS4 and enveloped glycoproteins - E1 and E2, the last one acting as neutralizing antibodies [Santos, et.al., 2019]. It has been also established that human and murine humoral immune responses to HCV NS3 protein are serologically reactive during the early phase of HCV infection and therefore are routinely used in HCV antibody immunoassays [Bian Y, et.al., 2013]. In our study, the most of reacting serums were positive for antibodies against non-structural NS3 protein and two capsid antigens–C1 and C2. The weak antibody reactivity was measured in

HCV RNA negative samples, which is in agreement with previously conducted cross-sectional studies demonstrating that indeterminate and weak antibody reactivity predicted the absence of HCV viremia [Strasak AM, et.al., 2011]. The anti-HCV positivity with no detectable serum HCV RNA can be due to past or occult HCV infection [De Marco L, et al., 2012]. It is worth mentioning, that 30% of people infected with HCV spontaneously clear the infection by a strong immune response [Grebely J, et al. 2012]. At the same time, for the samples of Group II, negative INNO-LIA results were determined, which can be explained with the early stage of HCV infection. The Increased frequency and HCV antibodies' band insensity were detected in Groups III and IV, with HCV increasing viral load. Group V, with the highest HCV viral load, was characterized by 67% INNO-LIA negative results. The only positive sample responded strongly against all INNO-LIA HCV antigens. It can be hypothesized that the absence of HCV antibodies was due to the diagnostic window period during which specific antibodies are not detected, but the HCV RNA has already reached peak serum levels between 10^5 IU/ml to 10^7 IU/ml [Bruno S., C. Facciotto. 2008]. Tested samples were anti-HCV positive by ELISA where the multiple-target response is measured [Warkad SD, et.al., 2019]. Hence, the result could be explained with the weak antibody response, which cannot be detected against each specific antigen individually, but this should be further studied. Another explanation could be a possible immunosuppression, when the indeterminate immunoblot reaction can be observed occasionally in HCV RNA positive subjects [Makuria AT, et.al. 2012]. Our study had a number of limitations. First of all, the number of tested samples is insufficient for drawing general conclusions about the correlation. Second, patients were with confirmed HCV status, but clinical and biochemical information about the course of the HCV infection was missing.

In conclusion, the outcome of this study will provide highlights for future studies on HCV diagnostic algorithms. HCV screening strategies involving anti-HCV detection by ELISA combined with recombinant immunoblot assay can be the method of choice in laboratories with limited equipment and finances.

Acknowledgments: The devices used in the study were purchased thanks to the European Fund for regional development through OP Science and Education for Smart Growth 2014-2020, Grant BG05M2OP001-1.002-0001-C04 "Fundamental Translational and Clinical Investigations on Infections and Immunity".

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. WHO guidelines on hepatitis B and C testing. Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO
2. Bruno S, Facciotto C. The natural course of HCV infection and the need for treatment. Annals Hepatol., 2008; 7 (2): 114-119
3. Kamili S, Drobeniuc J, Araujo AC, Hayden TM. Laboratory diagnostics for hepatitis C virus infection. Clin Infect Dis. 2012 Jul;55 Suppl 1:S43-8. doi: 10.1093/cid/cis368. PMID: 22715213
4. Kodani M, Martin M, de Castro VL, Drobeniuc J, Kamili S. An Automated Immunoblot Method for Detection of IgG Antibodies to Hepatitis C Virus: a Potential Supplemental Antibody Confirmatory Assay. J Clin Microbiol. 2019;57(3):e01567-18. Published 2019 Feb 27. doi:10.1128/JCM.01567-18
5. Warkad SD, Song KS, Pal D, Nimse SB. Developments in the HCV Screening Technologies Based on the Detection of Antigens and Antibodies. *Sensors (Basel)*. 2019;19(19):4257. Published 2019 Sep 30. doi:10.3390/s19194257
6. Ashfaq, U.A., Javed, T., Rehman, S.*et al.*An overview of HCV molecular biology, replication and immune responses. *VirolJ***8,**161(2011). https://doi.org/10.1186/1743-422X-8-161
7. Sperle I, Nielsen S, Gassowski M et al. Prevalence of hepatitis C in the adult population of Bulgaria: a pilot study. BMC Res Notes 13, 326 (2020). https://doi.org/10.1186/s13104-020-05158-3
8. Vladimirova N, Kamenov G, Stoitsova S, Minkova A, Parmakova K, Acute infectious diseases in Bulgaria in 2019 (Main epidemiological indicators). Epidemiology analysis 2019, NCIPD
9. Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Rehermann B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med. 2000 May;6(5):578-82. doi: 10.1038/75063. PMID: 10802716.
10. Santos, Vanessa Costa dos, Schinoni, Maria Isabel, Oliveira, Isabela Silva de, Atta, Maria Luiza Sousa, & Atta, Ajax Mercês. (2019). IgG1 and IgG4 antibodies against Core and NS3 antigens of hepatitis C virus. Revista da Sociedade Brasileira de Medicina Tropical, 52, e20180491. Epub March 07, 2019.https://doi.org/10.1590/0037-8682-0491-2018
11. Bian Y, Zhao S, Zhu S, Zeng J, Li T, Fu Y et al. (2013) Significance of Monoclonal Antibodies against the Conserved Epitopes within Non-Structural Protein 3 Helicase of Hepatitis C Virus. PLoS ONE 8(7): e70214. https://doi.org/10.1371/journal.pone.0070214
12. Strasak AM, Kim AY, Lauer GM, et al. Antibody dynamics and spontaneous viral clearance in patients with acute hepatitis C infection in Rio de Janeiro, Brazil. BMC Infect Dis. 2011; 11: 15. doi:10.1186/1471-2334-11-15
13. De Marco L, Manzini P, Trevisan M, Gillio-Tos A, Danielle F, Balloco C, et al. (2012) Prevalence and Follow-Up of Occult HCV Infection in an Italian Population Free of Clinically Detectable Infectious Liver Disease. PLoS ONE 7(8): e43541. https://doi.org/10.1371/journal.pone.0043541
14. Grebely J, Prins M, Hellard M, et al. Hepatitis C virus clearance, reinfection, and persistence, with insights from studies of injecting drug users: towards a vaccine. Lancet Infect Dis. 2012;12(5):408-414. doi:10.1016/S1473-3099(12)70010-5
15. Rando-Segura A, Buti M, Quer J, López-Martínez R, Rodríguez-

Frías F. Viral Load Measurements in Individuals with Hepatitis C Virus Infection: on the European Association for the Study of the Liver Recommendations on Treatment of Hepatitis C 2018. J Clin Microbiol. 2019;58(1):e01379-19. Published 2019 Dec 23. doi:10.1128/JCM.01379-19

16. Pereira, Felicidade Mota, Zarife, Maria Alice Sant'ana, Reis, Eliana Almeida Gomes, & G. Reis, Mitermayer. (2014). Indeterminate RIBA results were associated with the absence of hepatitis C virus RNA (HCV-RNA) in blood donors. *Revista da Sociedade Brasileira de Medicina Tropical*, *47*(1), 12-17. https://doi.org/10.1590/0037-8682-0222-2013
17. Makuria AT, Raghuraman S, Burbelo PD, Cantilena CC, Allison RD, Gibble J, Rehermann B, Alter HJ. The clinical relevance of persistent recombinant immunoblot assay-indeterminate reactions: insights into the natural history of hepatitis C virus infection and implications for donor counseling. Transfusion. 2012 Sep;52(9):1940-8. doi: 10.1111/j.1537-2995.2011.03524.x. Epub 2012 Feb 5. PMID: 22304422; PMCID: PMC3346857.
18. Zhang K, Wang L, Lin G, Li J: Is Anti-Hepatitis C Virus Antibody Level an Appropriate Marker to Preclude the Need for Supplemental Testing? Intervirology 2015;58:310-317. doi: 10.1159/000441474
19. Rafik M, Bakr S, Soliman D et al. Characterization of differential antibody production against hepatitis C virus in different HCV infection status. Virol J 13, 116 (2016). https://doi.org/10.1186/s12985-016-0572-9

IS THERE AN OUTBREAK OF TICK-BORNE ENCEPHALITIS IN PERNIK DISTRICT, BULGARIA? FOUR CASES REGISTERED FOR A PERIOD OF FOUR YEARS – CLINICAL MANIFESTATIONS AND EPIDEMIOLOGICAL RELATIONS

Evgeniya Taseva[1], Iva. Christova[1], Elitsa Panayotova[1], Diana Ilieva[2], Valentina Pavlova[2]

[1]National reference laboratory of vector-borne infections, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
[2] Department of Infectious Diseases, Rahila Angelova Hospital, Pernik, Bulgaria

ABSTRACT

In Europe, the number of tick-borne encephalitis (TBE) cases has been increasing in the last decade, and the number of endemic areas has also been increasing. Bulgaria, located in southeastern Europe, is not in the TBE endemic area. However, sporadic TBE cases have been occasionally detected. The disease has a natural-focal nature, pronounced seasonality, with a vector- *Ixodes* ticks that transmit the infection from animals to humans. TBE is severe infection with specific lesions of the central nervous system (CNS), with residual phenomena and high lethality. The first cases of TBE in Pernik district were registered in 2015. The aim of the study is to consider clinical cases of TBE in Pernik district during period 2017-2020, to look for an epidemiological link between them and to monitor the severity of the infection. We present briefly the most characteristic clinical and laboratory features of four laboratory confirmed cases of tick-borne encephalitis in the last 4 years. The mean age of patients was 56. All four cases were observed in May, June, and July. They proceeded relatively smoothly with a favourable outcome, without paresis or paralysis of the limbs, without seizures or loss of consciousness. In the first case, no pathological changes in the brain were observed by computed tomography, while in the other three cases multiinfarction encephalopathy, evidence of initial cerebral edema and two porencephalic foci were found. Two of the patients had meningoradicular irritation with positive symptoms of Kerning, Brudzinski and Babinski, while in the other two patients these symptoms were absent. In all cases there was a classic change in the hematgramme: moderate leukocytosis with granulocytosis, and the study of cerebrospinal fluid revealed a slight increase in total protein, moderate pleiocytosis and normal values of sugar and chloride. Only one of the patients was reported to be bitten by a tick, and the other three were most likely infected through food (raw goat's milk). Although isolated , onfirmed cases of TBE in Pernik district , indicate circulation of TBE virus in this region. This is facilitated by a number of factors: climatic changes, activity of the epizootic process in the tick population, different vertebrate species of s in natural foci; presence of a large number of infected goats. Comprehensive measures such as: raising the awareness of clinicians, considering the infection in patients with viral meningitis, intensified screening of raw milk by the Bulgarian Food Safety Agency, future studies of ticks and farm animals for the presence of TBE in this region are needed. This study is a first step in this direction.

Keywords: *tick-borne encephalitis, endemic outbreak, clinical symptoms*

ADDRESS FOR CORRESPONDENCE:
Evgeniya Taseva, PhD
Department of Microbiology
National Centre of Infectous and Parasitic Diseases
44A, Gen. N. Stoletov Blvd.
1233 Sofia, Bulgaria,
e- mail: evgenia_taseva@yahoo.com,
Tel. +359 2832 91 12

INTRODUCTION

Tick-borne encephalitis (TBE) is a zoonosis, caused by TBE virus (TBEV), a member of the genus *Flavivirus* within the family *Flaviviridae*, that causes fatal encephalitis with severe sequelae in humans (22). TBEV is prevalent over a wide area of Europe and the number of reported TBE cases has been increasing in the past years despite of increased use of TBEV-vaccine not subsidized by the healthcare system across the Europe (20,22). The three main subtypes of TBEV are European, Siberian and Far Eastern (11). They are closely related both genetically and antigenically. Tick-borne encephalitis is the most common arboviral infection in Europe. In Europe alone, 5000-6000 cases have been registered annually in the recent years (5, 21). Most autochthonous cases have been registered in the territories of Lithuania, Estonia and the Czech Republic. In 2016, the Netherlands reported the first two autochthonous cases of tick-borne encephalitis. Two years ago, the number of cases in Switzerland by 20% has been seen.

TBE is a focal infection. The sick person is not a source of infection. Different species of vertebrates, mainly rodents (*Apodemus, Myodes, Micrototus, etc.*) serve as natural reservoir of the virus in nature and *Ixodes* ticks are vectors. The main tick vector in Europe is *Ixodes ricinus*, and for Russia and the Far East – *Ixodes persulcatus* (13). The virus is transmitted to humans by the bite of an infected tick or by consuming most often of raw goat's milk (3).

Global climate changes affect the epidemiology of vector-transmitted infections. The increased average temperatures in winter have expanded the northern boundary of the range of vectors distribution to the north. Bulgaria is not an endemic region for tick-borne encephalitis. Climatic changes in our country have contributed to the spread of the main TBE vectors *Ixodes ricinus* ticks, and created real opportunities for contracting the infection. In Bulgaria, single TBE cases have been described since the first one in 1953 (23). Individual cases in humans were described in 1961 (1), 1966 (2) and 2006 (15). The disease is almost unknown to us. It is associated with the consumption of raw goat's milk. In 2003, P. Manolov and G. Katsarov detected a case of TBE in a 57-year-old man in Varna district after consumption of raw goat's milk (15). Between 2009 and 2012, five more cases of TBE were described, one of which was fatal (7,17).

Laboratory diagnosis of TBE, based on serological complement fixation assay, was introduced in Bulgaria in the 1970s. Since then, single case reports of presumed TBE have been reported, but these lack reliable microbiological confirmation. Modernization of the laboratory diagnostic approach allowed targeted detection of patients with tick-borne encephalitis in Bulgaria. Beginning in 2009, the National Reference Laboratory of Vector-Borne infections has introduced reliable laboratory diagnosis methods for TBE, based on polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) (9).

TBE virus infections are mostly asymptomatic. Only in 2015, the first cases of TBE in Pernik district were registered (a 51-year-old man and a 64-year-old woman with neurological manifestations, with detected specific anti-TBEV antibodies of IgM and IgG classes by ELISA). A link has been established between age and severity of the infection (12) and both patients were diagnosed with viral encephalitis. Both cases originated from Pernik region – near border with Serbia and near the districts of Kyustendil and Sofia, where we found relatively high levels of TBEV seroprevalence among healthy individuals - 2% and 3.03%, respectively (8,17).

The two patients lived in the same household. They had no data of tick bites. We assume that of the infection was associated with consumption of raw milk and / or other dairy products. The occurrence of two cases of severe TBE, within half a year, among the total of 225 studied patients (or 0.9%) again suggested that the virus is clearly not so rare in the country, and is a cause of some neuroinfections in Bulgaria (8).

The aim of the study is to consider the clinical cases of TBE in Pernik district during the last 4 years, to look for an epidemiological link between them and to monitor the severity of the infection.

MATERIAL AND METHODS

Four patients with tick-borne encephalitis from Pernik district were observed. Methods for epidemiological observation and follow-up, clinical-laboratory, diagnostic imaging, virological and serological tests were were employed

ELISA commercial kits (Euroimmun, Germany) were used to detect TBEV specific IgM and IgG antibodies. Serum samples were diluted 1: 100 in serum diluent. Peroxidase-labeled anti-human IgM or IgG goat conjugate from was used. Optical density of each well was read on an ELISA rider BIOTEK Elx 800 at a wavelength of 450 nm. The results were calculated and interpreted according to the instructions:

< 0.9 u / ml - negative result

0.9 - 1.1 u / ml - limit result

> 1.1 u / ml - positive result.

Serum samples from the four patients with clinical evidence of tick-borne encephalitis were investigated. The samples were drawn on day 7 of the disease. Three of the patients lived in the town of Pernik and one in the town of Breznik, Pernik district.

RESULTS

We briefly present the most characteristic clinical and laboratory features of the four laboratory confirmed cases of tick-borne encephalitis in the last 4 years.

First case *Patient with viral encephalitis*. A 26-year-old man was admitted to the Clinic of Infectious Diseases of "Rahila Angelova" Hospital in Pernik on June 5, 2017 with complaints of fever, headache, noise and light-provoked irritation, nausea, vomiting, unstable gait, dating from 5-6 days and general fatigue. Physical examination found that the patient was afebrile, adequate, dehydrated and intoxicated . Neurological status showed strong neck rigidity and positive symptoms of Kernig, Brudzinski and of Babinski on the right side. The headache persisted for 4 days. The patient did not report a tick bite. Diagnosis at admission was unspecified viral encephalitis.

Laboratory tests showed leukocytosis - up to 12.0 x 10^9 g/l with an increase of granulocytes (88.4%). CSF examination showed in total protein increase - 0.584 g/l (slightly elevated), and increased number of cells (42). There were no pathological findings

from radiography of the lungs and ultrasound of the abdominal organs. Diffuse and focal changes in the brain parenchyma were not detected by computed tomography examination of the head.

Candida albicans was isolated by microbiological cultivation. ELISA for TBEV antibodies to detected 5.7 IU IgM and 1.6 IU IgG antibodies in the first serum sample of the patient (norm up to 1.1 IU).

Treatment with ceftriaxone and dexamethasone was started. The patient was discharged after complete clinical recovery, with persisting astheno-adynamic syndrome. At the control clinical examination 30-days later, no abnormalities in neurological status were found.

Second case *Viral meningoencephalitis in a patient with hypertensive heart*. An 86-year-old man was admitted in the infectious ward of "Rahila Angelova" Hospital in Pernik on June 17, 2019. He complained of headache, fever with chills, slow speech and difficult moving. The body temperature has been increased for 5 days, dizziness and nausea appeared. The patient reported repeated tick bites in a village where ticks were abundant. He was admitted to the ward in impaired general condition, fever - 38^0, adequate and dehydrated. Neurological status revealed unstable Romberg symptom, positive symptoms of Kernig and Brudzinski bilaterally, and positive symptom of Babinski on the left.

Laboratory tests showed mild anemia - hemoglobin 128 g/l, leukocytosis - up to $11.1x10^9$ g/l with increased level of polymorphonuclear leukocytes and lymphopenia (Lym -12.9%). A lumbar puncture was performed on 17 June. Cerebrospinal fluid examination showed normal of total protein and glucose values, erythrocytes - 4-5, Pandi reaction - opalescence. Microbiological examinations of throat and nasal secretions, blood culture and uroculture did not give growth.

The X-ray showed a reinforced picture of the lung on the left paracardial area. Against this background, an inflammatory-infiltrative process could not be ruled out. Computed tomography of the head showed demyelination of the white matter of vascular type in the area of corona radiata and centrum semiovale. Signs of multiinfarction encephalopathy were present.

Serological testing showed antibodies of significant values against tick-borne virus encephalitis - IgG -2.05 IU and IgM - 7.96 IU (positive results above 1.1 IU). After treatment with ceftriaxone, cyprinol and dexamethasone, the patient's condition gradually improved, he became afebrile, the toxo-infectious and astheno-adynamic syndromes and meningo-radicular reactions disappeared.

Third case *A case of unspecified viral encephalitis*. It concerns a 41-year-old man. He was admitted for treatment in the infectious ward at "Rahila Angelova" Hospital in Pernik on May 23, 2020. For 3 days he had headache, high fever (39^0 C) with chills, nausea, vomiting and general weakness.

At admission, he was in a damaged general condition, febrile (37.8^0 C), adequate, dehydrated, with acute vesicular respiration. Neurological status revealed an unstable symptom of Romberg.

Laboratory tests found leukocytosis - up to $14.8x10^9$ /l with lymphopenia (Lym-8.5%) and granulocytosis (granulocytes-87.6%). On May 27, lumbar function was performed. Examination of the cerebrospinal fluid showed a significant increase of total protein (0.912 g/l), erythrocytes - 3-4 $x10^{12}$/l, reactions of Pandi, Rivalta and Pavlovich - opalescence, 84 cells. Microbiological examination of secretions from the throat, nasopharynx and ears did not give growth.

Computed tomography examination of the head revealed no evidence of focal changes in the brain parenchyma and that part of the arachnoid spaces of the hemispheres were not clearly visualized. Abdominal ultrasound showed no changes in the abdominal organs. An ophthalmological examination revealed initial hypertensive angiopathy. Computed tomography showed data of initial cerebral oedema and ophthalmoscopy showed data of papilledema.

On May 28, a cerebrospinal fluid test for enteroviruses was performed giving a negative result. On June 1, anti-TBEV antibodies were established by a serological test IgG -1.98 IU and IgM - 5.76 IU (positive results above 1.1 IU).

After treatment with ceftriaxone, dexamethasone and tavanic, the patient was discharged with improvement. Toxoinfectious and meningo-radicular symptoms resolved, but astheno-adynamic syndrome was persisting.

Fourth case *Febrile disease with mild neurological symptoms*. It was about a 72-year-old man who fell ill on July 17, 2020 with a body temperature of 37^0 C, general fatigue, headache, dizziness and unstable walk. After treatment with ciprofloxacin, he did not improve and on July 20 was admitted in the infectious ward of Rahila Angelova Hospital in Pernik.

The examination revealed that the patient was afebrile, in a moderately settled general condition, relaxed, dehydrated. Auscultation revealed bilaterally vesicular respiration with dry wheezing. Neurological status showed an unstable symptom of Romberg and missing symptoms of Kernig and Brudzinski. The patient did not report a tick bite.

Clinical laboratory tests showed lymphopenia (Lym 10.6%) and granulocytosis (granulocytes 84.9%). Microbiological examinations of throat and nasal secretions, blood culture and uroculture were negative. Lumbar puncture performed on 21 July 2020 showed no increase of the total protein in CSF,

no xanthochromia and erythrocytes, the reactions of Pavlovich, Pandi and Rivalta were negative. In the following days, normalization of the values of lymphocytes and granulocytes was observed.

Computed tomography examination of the head in the left cerebral sphere showed two temporal and periventricular porencephalic foci. No diffuse demarcation changes were found in the brain of the parenchyma. No changes were observed in the brain parenchyma, part of the arachnoid spaces of the hemispheres was not visualized clear. Computed tomography of the brain showed lacunar strokes. A small pleural effusion was observed by computed tomography of the abdomen.

A serological test on July 23 detected anti- TBEV antibodies - IgG -2.01 IU and IgM - 4.16 IU (positive results above 1.1 IU). Therapy was performed with ceftriaxone, dexamethasone and cyprinol. The patient responded to the applied therapy and was discharged with improvement.

The summarized results from medical history and from radiography of internal organs and computed tomography of the head in the four cases of TBE are presented in Table 1.

Table 1. *Comparative data from the medical history and diagnostic imaging in the four cases of TBE*

patient, gender, age	L.S., male, 26 old	I.D., male, 86 old	A.M., male, 41 old	V.S., male, 72 old
medical history data				
date of illness	June 5, 2017	June 17, 2019	May 23, 2020	July 20, 2020
fever /how many days/	**+**	**+ /**5 days/	**+/**3 days/	**+**
headache	**+** / had in May	**+**	**+**	**+**
chills	-	**+**	**+**	-
speech delay	-	**+**	-	-
sensitivity to light	**+**	-	-	-
nausea, vomiting	**+**	**+**	**+**	-
unstable gait	**+**	**+**	-	-
tick bite	-	**+**	-	-
consumption of raw milk	**+**	-	**+**	**+**
concomitant diseases	-	hypertonic heart	-	-
objectively				
contact, adequacy	**+**	**+**	**+**	**+**
breathing	vesicular without wheezing	wet wheezing	vesicular without wheezing	dry wheezing
neck rigidity	+	-	-	-
symptoms of Kernig, Brudzinski	+ / bilaterally/	+ / bilaterally/	-	-
symptom of Babinski	**+** / in right/	+ / in left/	-	-
symptom of Romberg	-	unstable	unstable	unstable
ultrasound diagnosis of internal organs				
abdominal ultrasound	normal status	pyelonephritis, aortic calcification	normal status	pyelonephritis, globus vesicalis
computed tomography of head				
data	changes in the brain parenchyma were not detected	demyelination of the white matter of vascular type	changes in the brain parenchyma were not detected	two temporal and periventricular porencephalic foci
conclusion	without pathological changes	multiinfarction encephalopathy	initial cerebral edema	lacunar strokes

The summarized results from laboratory tests in the four cases of TBE are presented in Table 2.

Table 2 *Comparative data from laboratory results in the four cases of TBE*

patient, gender, age	L.S., male, 26 old	I.D., male, 86 old	A.M., male, 41 old	V.S., male, 72 old
results from a clinical laboratory /blood/				
haemoglobin	146 g/l /normal/	128 g/l /mild anemia/	153 g/l/normal/	134 g/l /slightly lowered/
leukocytes	12.0 x 10^9 g/l /high/	11.6 x 10^9 g/l / / slightly elevated/	14.8x10^9 g/l /high/	8.7 8x10^9 g/l / normal/
lymphocytes	8.4% /low/	12.9% /low/	8.5% /low/	10.6% /low/
granulocytes	88.4% /high/	76.3% /slightly elevated/	87.6% /high/	84.9% /high/
results from a clinical laboratory / cerebrospinal fluid/				
total protein	0.584 g/l / slightly elevated/	0.330 g/l /normal/	0.912 g/l /high/	0.530 g/l / slightly elevated/
reactions of Pavlovich, Pandi and Rivalta	Pandi opalescens	Pandi opalescens	Pavlovich, Pandi and Rivalta - opalescens	negative
cells in cubic ml	42 /elevated/	4 /normal/	84 /elevated/	2 /normal/
sugar	3.02 mmol/l / normal/	3 mmol/l /normal/	5 mmol/l /slightly elevated/	3.44 mmol/l / normal/
chlorides	114 mmol/l / normal/	115.6 mmol/l / normal/	120 mmol/l /normal/	115 mmol/l / normal/
erythrocytes	6-7	4-5	3-4	-
microbiological results				
serological test ELISA for TBE	IgM -5.7 IU /+/ IgG - 1.6 IU /+/	IgG -2.05 IU /+/ IgM - 7.96 IU /+/	IgG -1.98 IU /+/ IgM - 5.76 IU /+/	IgG -2.01 IU /+/ IgM - 4.16 IU /+/
cerebrospinal fluid test for enteroviruses	-	-	negative	-
microbiological examinations of throat and nasal secretions	*Candida albicans*	without growth	without growth	without growth
blood culture	-	-	-	without growth
uroculture	-	-	-	without growth

DISCUSSION

According to data published in Europe, 2/3 of TBE cases are caused by an European viral subtype (4). In the four cases described in this paper, the classic two-phase course of the disease was observedThe average age of the patients was 56years. There are studies on the relationship between age and severity of the disease. The symptoms of TBE in children are milder than in adults, with meningitis in 97% of cases. In adults, meningoencephalitis and meningoencephalomyelitis account for 49.26% of cases. Nausea and vomiting are common in children, while neurological manifestations are common in adults (14).

In our study, only in the first case no pathological brain changes were detected. In the remaining three cases, computed tomography revealed: multiinfarction encephalopathy, evidence of initial cerebral edema and two porencephalic foci. In the first officially reported TBE case in Bulgaria for the last 10 years (in 2009), atypical course was observed without evidence of meningitis or encephalitis (6).

Cases of TBE occur usually in the warm months - between April and November. All four cases were observed in May, June and July.

All four cases proceeded relatively smoothly with a favourable outcome, without paresis or paralysis of the limbs, without seizures and loss of consciousness.

A recent study in Serbia reported that 50% of patients had meningoencephalitis, that presents with varying degrees of disorder of consciousness and with other neurological disorders: ataxia (100%), paralysis of the limbs (60%), speech impairment and tremor (60%) (19). Except for tick bites, infection is possible by consuming contaminated unpasteurized milk and dairy products from infected domestic animals, especially goats (16).

In most of the cases, between 70 and 98%, TBEV infections are asymptomatic (4). The incubation period varies from 2 to 28 days, usually 7-14 days. As a rule, in the case of alimentary infection, the incubation period is short, usually 3-4 days (10). In this study, only one of the patients reported a tick bite, and the rest were most likely infected through food, as symptoms developed rapidly after 3-4 days. All four cases occur with initial toxoinfectious syndrome, severe headache and adynamy.

Two of the patients had positive symptoms of Kerning, Brudinski and Babinski, while the other two did not have these symptoms but an unstable Romberg symptom was found. Three of the patients developed meningeal form, while in the fourth, such data was not detected by the computed tomography (probably because he was the youngest one and without concomitant diseases).

All four cases followed a classic change in the hematogramme: moderate leukocytosis with granulocytosis. Examination of CSF revealed clear fluid and slightly increased total protein, moderate pleocytosis, and normal sugar and chlorides values.

The diagnosis of all four cases was confirmed by serological examination and detection of significantly increased anti-TBEV antibody values. Because of the possibility of another etiology of CNS infection and especially due to the seasonal character of other viruses, infections with other pathogens such as West Nile Virus (WNV), Herpes Simplex Virus 1 (HSV), and Zoster Virus (VZV) were excluded by serological analysis of the blood.

The district of Pernik is characterized by extremely mountainous terrain, which favors breeding of goats. On the territory of Pernik, there are numerous farms for goat milk production, which predetermines its intensified consumption. Increased control by the Bulgarian Food Safety Agency (BFSA) is needed to prevent the consumption of infected raw milk.

The confirmed TBE cases in Bulgaria during these 4 years are only in Pernik district. Although single, these cases showed TBE virus circulation in this region. The fact that there are two cases in 2020 that have not been confirmed in other areas, indicates the presence of some source of infection. The reasons for this can be complex: climate change, activity of the epizootic process in the tick population, different species of vertebrates in natural foci; presence of a large number of goats infected with the virus and increased consumption of raw, unpasteurized goat's milk. In addition, the Pernik Valley with an altitude between 700 m and 850 m is a prerequisite for the formation of high-risk biotopes with high humidity and moderate temperatures favourable for development of *Ixodes* ticks.

A more in-depth study of the area, examination of a representative number of ticks for the presence of TBEV, and sampling of animals is needed. Given the fact that patients who develop neurological manifestations represent a small proportion of those infected, it can be predicted that the number of people infected with TBEV in Bulgaria is many times higher. It is important to develop clinicians' clinical thinking, as the risk of infection is often underestimated and the outcome can be fatal to the patient if misdiagnosed. TBE should be sought in patients with various manifestations of CNS infection.

CONCLUSIONS

Although TBE cases are reported only sporadically due to lack of sufficient testing and / or reporting, TBEV circulates in Pernik region, causing human infections by tick bites or consumption of unpasteurized milk.

In our country, Tick-borne encephalitis remains still underecognized among the cases of viral encephalitis. Active clinical thinking about a possible CNS infection caused by the tick-borne encephalitis virus, supported by optimized laboratory diagnostics, would help to clarify the actual state of the problem in our country.

ACKNOWLEDGEMENTS

The study was supported by the National Science Fund, Bulgaria, Project № KP-06-M33/3 /13.12.2019.

REFERENCES

1. Andonov P., Rusakiev M., Hristova T., Prodromov A., Petkov P., Penchev S., Grancharov K., Naturalhearth from the group of ticks encephalitis in the Eastern Old Mountain, Hygiene, 1961, 6: 23-26.

(in bulgarian).
2. Andonov P., Rusakiev M. Natural hearth of ticks encephalitis from a sick child, Epidem., microb., and inf. bol., 1966, 3: 128-138. (in bulgarian).
3. Balogh Z, Ferenczi E, Szeles K, Stefanoff P, Gut W, Szomor KN, Takacs M, Berencsi G. Tick-borne encephalitis outbreak in Hungary due to consumption of raw goat milk. J Virol Methods, 2010, 163,; 481–485.
4. Bogovic P., Franc Strle Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management World J Clin Cases, 2015, 16; 3(5): 430-441.
5. Christie L. The global burden of bacterial and viral zoonotic infections. Clin Microb Inf 17, 2011; 326–330.
6. Christova I, L. Andonova, M. Slavova, E. Tasseva, T. Gladnishka, I. Trifonova, V. Ivanova, Atipical case of tick-borne encephalitis, Probl. Inf. Parasit. Dis, 37, 2009, 2, 13-14.
7. Christova I., I. Dikov, L. Andonova, E. Taseva, N. Kalvachev, Sh. Popal, R. Mikhailova, M. Pishmisheva, I. Baltadzhiev, M. Slavova, K. Plochev, Four cases of tick-borne encephalitis, Science Infectious Diseases and Parasitology, 2011, 1, 43-45. (in bulgarian).
8. Christova I., Panayotova I., Tchakarova S., Taseva E., Trifonova I., Gladnishka T., A nationwide seroprevalence screening for West Nile virus and Tick-borne encephalitis virus in the population of Bulgaria, Journal of Medical Virology, 2017; 89(10):1875-1878.
9. Christova I. TBE in Bulgaria. Chapter 12b. In: Dobler G, Erber W, Bröker M, Schmitt HJ, eds. The TBE Book. 3rd ed. Singapore: Global Health Press; 2020.
10. Dumpis U, Crook D, Oksi J. Tick-borne encephalitis. Clin Infect Dis, 1999; 28:882-890.
11. Ecker, M., Allison, S. L., Meixner, T. & Heinz, F. X. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. J Gen Virol, 1999, 80, 179–185.
12. Kaiser R: Presented at 7th annual meeting of ISW-TBE in Vienna, 2005.
13. Kaiser R. Tick-borne encephalitis. Infect Dis Clin North Am, 2008; 22: 561-575.
14. Krawczuk K., Czupryna P., Pancewicz S., Ołdak E., Moniuszko-Malinowska A., Comparison of tick-borne encephalitis between children and adults—analysis of 669 patients, J of NeuroVirology, 2020; 26(4):565-571.
15. Manolov P., Katsarov B. A case of tick-borne encephalitis after consummate of raw goat's milk. Infectology, 2006, 2: 37-38. (in bulgarian).
16. Mansfield K., Johnson N., Phipps L., Stephenson J., Fooks A., and Solomon T., Tick-borne encephalitis virus – a review of an emerging zoonosis. J. Gen. Virol. 2009, 90:1781–1794.
17. Mohareb E. , I Christova , A Soliman , R Younan , T Kantardjiev, Tick-borne encephalitis in Bulgaria, 2009 to 2012, Eurosurveillance, 2013, 18, (46), 1-4.
18. Panayotova E., Study of the distribution of some Bunya and Flaviviruses in Bulgaria, dissertation, 2016. (in bulgarian).
19. Poluga J., Barac., Katanic N., Rubino S. at all, Tick-borne encephalitis in Serbia: A case series, J Infect Dev Ctries, 2019; 13(6):510-515.
20. Sormunen JJ, Klemola T, Hänninen J, Mäkelä S, Vuorinen I, Penttinen R, Sääksjärvi IE, Vesterinen EJ The importance of study duration and spatial scale in pathogen detection-evidence from a tick-infested island. Emerg Microbes Infect, 2018, 7: 189.
21. Suss J. Tick-borne encephalitis in Europe and beyond – the epidemiological situation as of 2007. Eurosurveillance 13, 2008; pii 8916.
22. Yoshii K. Epidemiology and pathological mechanisms of tick-borne encephalitis. J Vet Med Sci, 2019, 81: 343-347.
23. Vaptsarov I., Tarpomanov A., Spasov Z., Nikov D., Dragiev M. Reversible (two-wave) meningoencephalitis in Southern Bulgaria, Modern. Medic.,1964, 2: 86-103.(in bulgarian).

COMPARATIVE ANALYSIS OF CLINICAL AND LABORATORY PARAMETERS BETWEEN VIRAL AND BACTERIAL NEUROINFECTIONS

Pekova L[1], Halacheva K[2], Dochev I[3]

[1] Dept. of Infectious Diseases, Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[2] Dept. of Molecular biology, Immunology and Medical genetics, Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[3] Dept.of Ophthalmology, University hospital, Stara Zagora, Bulgaria

ABSTRACT

Neuroinfections are acute inflammatory diseases of the central and peripheral nervous system that can lead to serious consequences, and even death. Recently, viruses have played a leading role in the emergence of neuroinfections. Rapid identification of the etiological agents is an important prerequisite for proper therapy and a good outcome of the disease. The aim of this study is to determine the role of the cytokines IL-6 and IFN-γ in the cerebrospinal fluid and serum of patients with viral and bacterial neuroinfections in relation to their diagnosis and prognosis. Materials and methods: From 2012-2018, 91 patients were included, aged from 2 months to 82 years. They were divided into 3 groups: 57 with viral neuroinfections , 24 - with bacterial and 10 - control group with cerebral edema. Clinical, epidemiological, laboratory, microbiological, serological and molecular tests were performed in all patients, and in some of them imaging techniques (CT and MRI) had been performed. Cytokines IL-6 and IFN-γ in serum and cerebrospinal fluid were determined by immunological tests. Conclusion: Viral neuroinfections are more common than bacterial ones, they had a milder clinical course and a more favorable outcome. Cytokine levels in the cerebrospinal fluid are a better indicator of inflammatory process in terms of severity than those in the serum. IL-6 levels in the cerebrospinal fluid of viral neuroinfections were higher than IFN-γ. A proportional relationship was established between leukocytes and IL-6 in the cerebrospinal fluid of patients with aseptic meningitis .

Keywords: *neuroinfections, IL-6, IFN-γ, prognosis*

ADDRESS FOR CORRESPONDENCE:
Professor Liliya Pekova, MD, PhD
Section of Infectious diseases
Dept. of Hygiene, Epidemiology, Microbiology, Parasitology and Infectious Diseases, Medical Faculty, Trakia University, 11, Armeyska Str.
6000 Stara Zagora, Bulgaria
e-mail: dr.pekova@abv.bg

INTRODUCTION

Neuroinfections are acute inflammatory diseases of the central and peripheral nervous system (CNS and PNS) that can be severe thus leading to serious consequences even death. They are caused by a wide group of etiological agents: bacteria, viruses, rickettsia, mycoplasmas, chlamydia, fungi, and parasites. Recently, viruses have played a leading role in the emergence of neuroinfections. Regardless of the etiological agent, neuroinfections are clinically similar. It is difficult to distinguish between them without specific microbiological, virological and serological tests.

The aim of the studywas to determine the diagnostic and prognostic role of the cytokines Interleukin-6 (IL-6) and Interferon- γ (IFN-γ) in the cerebrospinal fluid (csf) and serum (ser) of patients with viral and bacterial neuroinfections.

Materials and methods: From 2012-2018 91 patients, 43 women and 48 men, aged from 2 months to 82 years ($\overline{x}$ 19.8 ± 23.4)were involved..

The diagnostic criteria for meningitis/encephalitis included clinical manifestations as well as laboratory changes in the csf of all patients. The clinical findings observed varied including:

-sudden onset
-headache
-fever
-nausea and/or vomiting
- neurological signs of meningeal irritation: neck stiffness, Kernig's sign, upper and lower Brudzinski's sign
-possible paralysis of the cranial and peripheral nerves
- pathological reflexes
- impaired consciousness

According to the relevant laboratory biochemical parameters in csf, the patients were divided into 3 groups:

Group 1. Patients with aseptic (viral) neuroinfections. This group consisted of 57 patients, from 11 months to 82 years ($\bar{x}$ 15.74 ± 19.32). The group was differentiated by the presence of aseptic meningitis, encephalitis or meningoencephalitisdetermined by:
-increase in albumin level usually below 1,0 g/L
- pleocytosis up to 1000.10^6/L
- prevalence of lymphomononuclear cells
- slightly positive Pandy's and Nonne-Apelt's tests
- serological and/or molecular-based evidence of a virus as an etiological agent
- negative microbiological test for bacterial flora.

Group 2. Patients with purulent (bacterial) neuroinfections. This group consisted of 24 patients aged between 1 month and 76 years ($\bar{x}$ 17.07 ± 11.04). Laboratory criteria included:
- albumin above 1.0 g/L
- increased cell count more than 1000.10^6/L
- neutrophil proliferation
- strong positive tests of Pandy and Nonne-Apelt
- glucose level below 2,22 mmol/L
- positive microbiological and/or serological and molecular-based data of a bacteria agent

Group 3. Controls. The control group involved 10 patients aged from 1 to 56 years ($\bar{x}$ 15.29 ± 17.14). They were admitted based on clinical symptoms and history of cerebral edema. The following CSF parameters were within the reference values:
Clinical, epidemiological, laboratory, microbiological, serological and molecular based tests were performed in all patients. Computed tomography (CT) was used in all and magnetic resonance imaging (MRI) - only in selected patients. . Concentrations of IFN-γ and IL-6 in csf and ser were examined in all patients. Cytokine levels were measured by enzyme linked immunosorbent assay (ELISA) using commercially available kits (Bender MedSystems GmbH (eBioscience), Vienna, Austria) according to the manufacturer's instructions. The sensitivity of the assay was set at 0.92 pg / ml for Il-6 and 0.99 pg / ml for IFN γ. The data was processed using the statistical program IBM SPSS Statistics v.19. All data was analyzed and included in the study after obtaining of informed consent from all particiapants.

Results: The neuroinfections in the studied group of patients were caused by arious etiological agents, as indicated in **Fig 1**.

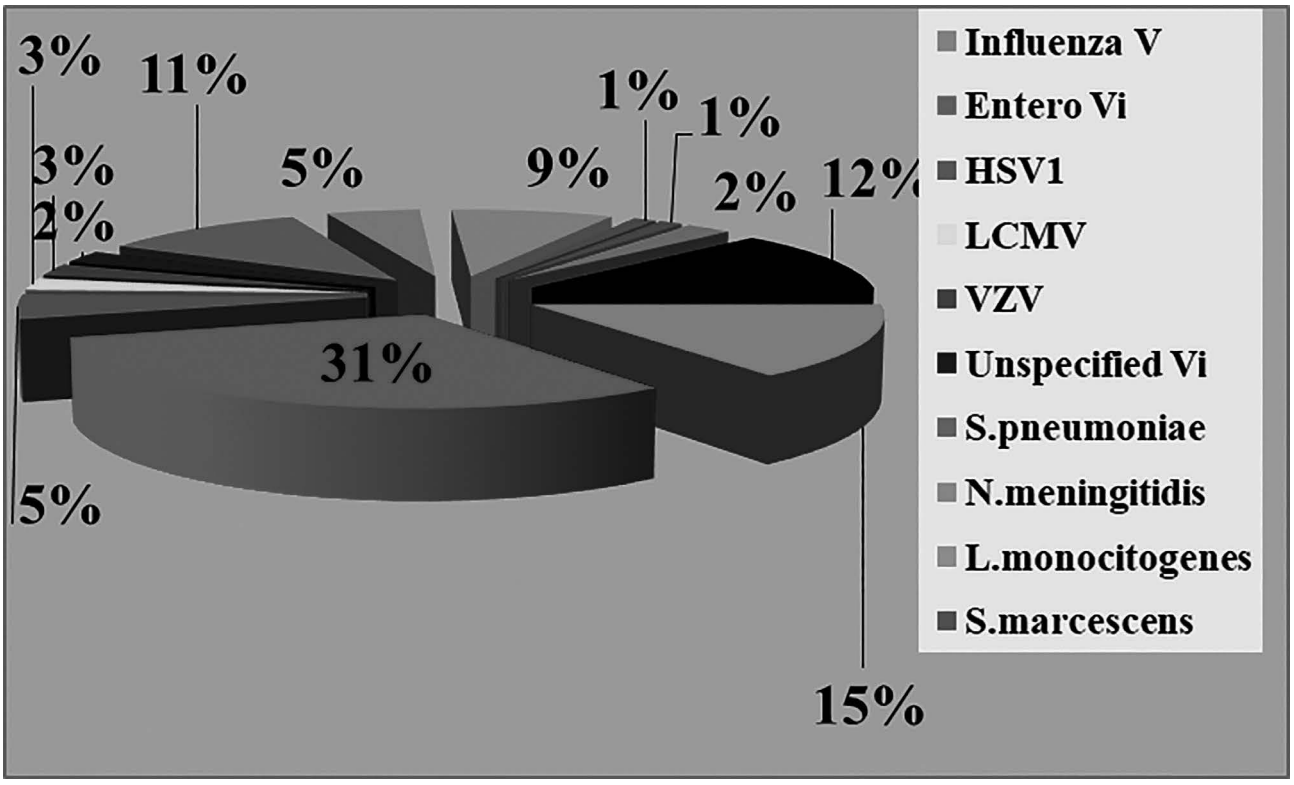


Figure 1. The etiological structure of neuroinfections - Influenza virus (IV), Enterovirus (EV), Hereps simplex virus 1 (HSV1), Lymphocytic Choriomeningitis virus (LCMV), Varicella-Zoster virus (VZV), Unspecified virus (UV), *Streptococcus pneumoniae* (*S. pneumoniae*), Neisseria meningitides (*N.meningitides*), Listeria monocitogenes (*L. monocitogenes*), Serratia marcescens (*S. marcescens*)

Group 1. The majority showed an acute onset of disease with headache, fever and vomiting. Fever was seen in all patients from 37.5 to 40.5° C ($\bar{x}$ 38.4 ± 2.5) with a duration of 2 to 8 days ($\bar{x}$ 3.32 ± 3.19). Headache was reported in 48 patients (84.21%). Vomiting was shown as a common sign in 46 (80.70%) patients with viral neuroinfections. Abdominal pain was observed in 37 (64.91%) patients and diarrhea was seen in 32 (56.14%). In most of these patients, EV was mostly found, followed by IV. Catarrhal manifestations such as , sore throat, runny nose and cough were more common in IV neuroinfections. There were 53 patients with severe asthenia upon hospitalization. Meningeal syndrome was found in 49 patients (78.76%).

Group2. Complaints also began acutely within 1–3 days with headache, vomiting, and temperature of 37.6 to 40.1° C ($\bar{x}$ 38.8 ± 0.72). Neurological symptoms of meningeal syndrome were observed in all patients. Changes in consciousness were significantly more frequent as compared to the other two groups,. Rash was commonly found in meningococcal and pneumococcal meningitis.

Group3. These patients were in the control group diagnosed with cerebral edema. All had temperature in the range of 38.1 to 39.8° C, headache and vomiting, as well as partially manifested neurological signs of meningeal irritation syndrome. Due to a suspicion of neuroinfection, a lumbar puncture was done. The status of the patients was monitored during the hospital stay, as well as their laboratory parameters and this strictly excluded CNS inflammation.

Ten (17.55%) patients from the Group 1 were hospitalized within 3-7 days after the onset of symptoms. Dyspeptic manifestations such as abdominal pain and diarrhea were demonstrated in the three comparable groups, they all had similar frequencies. In viral infections of the CNS, they were observed mainly in EV ones. However, vomiting was not perceived as a manifestation of dyspeptic syndrome, but as a part of the neurological symptoms. In contrast to the classical literature nausea was a common symptom.

Meningeal symptoms were well expressed in most patients from the three groups. Our study showed that the signs of meningeal irritation were more common in EV than in IV neuroinfections. The duration of meningeal syndrome was slightly longer in bacterial neuroinfections ($\bar{x}$ 6.61 ± 2.21) as compared to viral ($\bar{x}$ 4.54 ± 3.82). In the control group, meningeal irritation lasted an average of 3.02 ± 0.11 which was not significantly different. Seizures were initially observed before or shortly after hospitalization in groups 1 and 2. In viral neuroinfections they are most common in IV and HSV, while in bacterial neuroinfections - in S. pneumoniae and N. meningitidis, respectively. Quantitative disorders of consciousness were common. A statistically significant difference was found between the three groups ($p<0.001$ for all comparisons). Cranial nerve palsy was observed with comparable frequence in groups 1 and 2, while peripheral nerve palsy was more common in viral than in bacterial neuroinfections - ($p<0.01$).

Almost all patients included in the study were admitted within 3 days after the onset of the disease. The time of hospitalization was important for the length of the hospital stay, this was done so as to minimize complications, reduce adverse reactions and eventually receive a positive outcome. The average length of hospital stay in bacterial neuroinfections was the longest: from 2 to 31 days ($\bar{x}$ 14.21 ± 9.21), followed by viral infections: from 1 to 22 days ($\bar{x}$ 9.12 ± 3.12), and the control group: from 3 to 5 days (4.11 ± 2.64). No statistically significant differences were detected between the groups for this parameter. Laboratory blood analysis showed leukocytosis with predominance of neutrophils and increased CRP in bacterial neuroinfections. Elevated urea, creatinine, ALAT and ASAT were found in most patients with neuroinfections but they were more common in those of bacterial origin. Statistically significant differences between the studied laboratory parameters were detected only vs. group 3: $p<0.01$ for both comparisons. Laboratory characteristics of cerebrospinal fluid showed inflammatory changes corresponding to a bacterial or aseptic inflammatory process. There were no pathological abnormalities in the control group. (**Table. 1.)**

Table. 1. Indicators of CSF at the initial lumbar puncture of the three patients' groups

Groups of patients Indicators /x̄/	**Group 1 (n=57)**	**Group 2 (n=24)**	**Group 3 (n=10)**	**P**
Leuc.10^6/L	77,60±27,21	1112,87±382,22	2,43±2,11	**1v2=0,003** 1v3=0,43 **2v3=0,000**
Protein g/L	0,75±0,47	1,96±1,33	0,27±0,13	1v2=0,21 1v3=0,34 **2v3=0,04**
Glucosa mmol/L	4,15±2,98	2,04±0,23	3,15±1,19	**1v2=0,003** 1v3=0,43 **2v3=0,000**
Sed.(Ly%)	68,12±28,10	35,14±11,09	/	1v2=0,12

The results of the changes in IFN-γ and IL-6 in CSF and serum in the three groups of patients were presented in **Table 2.**

Table. 2. Mean values of IFN-γ and IL-6 (pg/mL) in cerebrospinal fluid and serum in the groups of viral, bacterial neuroinfections and controls.

Groups of patients / Cytokines(pg/mL)	**Group 1 (n=57)**	**Group 2 (n=24)**	**Group 3 (n=10)**	**P value**
IFN-γ csf	2,15±7,97	96,70±235,29	2,20±1,04	***1vs2=0,000** ***2vs3=0,000** 1vs3=0,161
IFN-γ ser	0,22±0,62	0,45±1,00	0,0006±0,006	***1vs2=0,004** ***1vs3=0,000** ***2vs3=0,000**
IL-6 csf	42,85±113,56	358,30±221,68	2,91±1,25	***1vs2=0,003** ***2vs3=0,000** 1vs3=0,231
IL-6 ser	6,09±12,34	30,61±28,54	4,39±12,02	***1vs2=0,000** ***2vs3=0,000** 1vs3=0,181

Levels of IL-6 and IFN-γ (pg/mL) in both cerebrospinal fluid and serum had been shown to be significantly higher in bacterial neuroinfections than in viral ones. Imaging methods of diagnosis: CT was done for all patients and MRI in 28. The most common finding was cerebral edema. The MRI in five patients with HSV1 neuroinfection demonstrated the classic picture of multistage hemorrhagic-necrotic changes in the brain parenchyma in the frontotemporal regions.

The outcome of neuroinfections in our patients was often favorable. Fatal outcome was observed in 8 patients with viral (14.03%) and 8 (33.33%) with bacterial neuroinfection, no statistically significant difference was found. Residual manifestations - palsy of cranial nerves, most often VI and VII, was observed in 3 patients (5.26%) with viral (HSV1, LCMV) and in 5 (20.83%) with bacterial neuroinfection (S. pneumoniae, N. meningitidis), p = 0.004.

DISCUSSION

Cytokines are known to regulate the intensity and duration of immune response by stimulating or inhibiting the activity, proliferation and / or differentiation of various cells and controlling the secretion of other cytokines and antibodies. Recently, much attention has been paid to the role of cytokines in the regulation of inflammation and host responses to CNS infection [1]. According to literature data, the release of some cytokines, such as interleukin-1 (IL-1), interleukin-8 (IL-8), tumor necrosis factor-α

(TNF-α) and interferon-gamma (IFN-γ), could be responsible for meningeal inflammatory infiltration in purulent and aseptic meningitis and may correlate with the outcome of the disease [2,3,4,5,6].

IL-6 elicits an inflammatory response during the acute phase, which manifests itself with fever and leukocytosis. It also contributes to the transition from acute to chronic inflammation [3]. The etiological diagnosis of bacterial neuroinfections is confirmed by visualization of the etiological agent on a direct Gram-stained preparation and / or by culturing the cerebrospinal fluid on a selective culture medium. The limited number of pathogenic species that could be detected in the laboratory, was most probably associated with the widespread use of pre-hospital antibiotics , which ultimately reduces the effectiveness of etiological diagnosis [7]. In viral infections of the CNS, the detection of genetic material of the causative agent in the cerebrospinal fluid by polymerase chain reaction (PCR) is the gold standard for diagnosis. This otherwise avant-garde method was not always successful, as there might be insufficient viral presence in the biological fluids during the study. While affordable, easy-to-use and inexpensive methods for diagnosis of bacterial neuroinfections have been developed, this is more difficult in viral infections due to their great variety [8]. Bacterial meningitis was associated with the activation of the inflammatory cascade and the production of pro- and anti-inflammatory cytokines [3]. Using the levels of three cytokines, TNFα, IL-6 and IL-8 in the cerebrospinal fluid, some authors distinguish between bacterial and viral meningitis in children with 100% specificity and sensitivity. In our study, a significant difference was found between viral and bacterial neuroinfections with respect to the IL-6 and IFN-γ (pg /mL) in serum and cerebrospinal fluid. Other authors have reported similar observations [3]. IL-6 levels in the cerebrospinal fluid of patients in group 2 were much higher than those cited by Prasad, R et al. [3].

According to Ichiyama T, elevated concentrations of csf pro-inflammatory cytokines such as TNF-α, IL-1 β and IL-6 - showed evidence of acute encephalitis / encephalopathy [9].

In our study, the cytokine profile was examined in bacterial and viral neuroinfections in comparison to healthy controls. In the group of viral neuroinfections, the highest IL-6 values were found in the five patients with herpetic meningoencephalitis. A study of csf cytokines (IL-1 β, IL-2, IL-6, IL-10, TNFα, and IFN-γ) in two cases of nonherpetic limbic encephalitis by Takahashi T et al, found elevated values of IL-6 only [10]. IL-6 elicits acute inflammatory re s ponse, followed by fever and leukocytosis. It also contributes to the transition from acute to chronic inflammation [6]. In our patients we found a directly proportional relationship between the level of IL-6 and the levels of leukocytosis, pleocytosis and CRP.

The sensitivity of csf IL-6 as a diagnostic marker in bacterial meningitis was significantly higher as compared to conventional physicochemical parameters such as leukocytes and protein. When the level of IL-6 in the cerebrospinal fluid exceeded 38.2 pg/mL, its diagnostic sensitivity as a biomarker was 100.0%, as compared to 70.0% for the extent of pleocytosis and 65.1% for proteinorachia [11]. In our study, the level of IL-6 in the cerebrospinal fluid of patients with bacterial CNS infections averaged to 358.30 ± 221.68 pg/ml. In adult patients with bacterial meningitis, IL-6 levels were higher than in children, ($p < 0.001$). This was found by other authors and was explained by the maturity of the immune system of the adult organism [11].

Cytokines at the site of inflammation were a better indicator of the clinical severity of the disease than their serum levels. According to experimental results, the csf / ser IL-6 ratio had a higher diagnostic efficacy than the independent detection of IL-6 in csf [4].

Takahashi W et al. conducted a study in 70 patients, 13 of whom had bacterial meningitis, 21 with aseptic inflammation of the central nervous system and 36 with sepsis. The level of IL-6 in the cerebrospinal fluid was significantly higher in the group with bacterial meningitis as compared to the other 2 groups ($p <.0001$) [11]= This finding was confirmed in our study. We found a strong relationship between serum and cerebrospinal fluid cytokine levels and the etiological agent, which had the value of a diagnostic marker. The higher values of IL-6 and IFN-γ in both biological fluids, the more certain it were that there is a bacterial causative agent.

Mukai AO et al.confirmed this dependence and, like us, did not find a link between cytokine levels and subsequent complications of neuroinfection. It was concluded that cytokines were a good marker for distinguishing bacterial from aseptic meningitis, as well as an index for the intensity of inflammation

[12]. According to Ogha S et al. the levels of IFN-γ concentration was the highest in the cerebrospinal fluid of patients with aseptic meningitis [13]. Our results do not confirm this. In our study IFN-γ showed a positive correlation with disease severity, as found by other authors. [14].

CONCLUSION

Our study of neuroinfections conducted in 91 patients (57 with viral, 24 with bacterial origin and 10 as a control group with meningism), showed that:

1. Viral neuroinfections were more common than bacterial ones and with rare exceptions, had a milder clinical course and a more favorable outcome. Vaccine prophylaxis reduced the chances of viral neuroinfections in mumps, measles, rubella, chickenpox, poliovirus, influenza, rabies.
2. Cytokines in the cerebrospinal fluid were a better indicator of the clinical severity of the inflammatory process of the CNS than their serum levels.
3. In viral neuroinfections, cerebrospinal fluid IL-6 levels increased more than IFN-γ.
4. In aseptic meningitis, we found a proportional relationship between the level of IL-6 and the amount of pleocytosis

Acknowlegement: The authors express special gratitude to the doctors, nurses and laboratory technicians who contributed to the opportunity for this research.

REFERENCES:

1. Zhang JM, An J "Cytokines, inflammation, and pain". International Anesthesiology Clinics. 2007;45 (2): 27–37
2. Titmarsh CJ, Moscovis SM, Hall S et al.Comparison of cytokine gene polymorphisms among Greek patients with invasive meningococcal disease or viral meningitis. J Med Microbiol. 2013;62:694–700
3. Prasad, R., Kapoor, R., Srivastava, R., Mishra, O. P., and Singh, T. B. Cerebrospinal fluid TNF-alpha, IL-6, and IL-8 in children with bacterial meningitis, Pediatr. Neurol., 2014;50: 60–65
4. Qing Ye, Wen-Xia Shao, Shi-Qiang Shang, Hong-Qiang Shen, Xue-Jun Chen, Yong-Min Tang, Yong-Lin Yu, Jian-Hua Mao. Clinical Value of Assessing Cytokine Levels for the Differential Diagnosis of Bacterial Meningitis in a Pediatric Population.Medicine (Baltimore) 2016 Mar; 95(13): e3222
5. Pinto Junior, VL, Rebelo, MC, Gomes, RN, Assis, EF, Castro- Faria-Neto, HC, Boia, MN.IL-6 and IL-8 in cerebrospinal fluid from patients with aseptic meningitis and bacterial meningitis: their potential role as a marker for differential diagnosis, Braz. J. Infect. Dis., 2011;15: 156–158
6. Atreya R, Mudter J, Finotto S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in Crohn disease and experimental colitis in vivo (vol 6, pg 583, 2000). Nature Med 2010; 16:1341–11341.
7. Wang Y, Guo G, Wang H, et al. Comparative study of bacteriological culture and real-time fluorescence quantitative PCR (RT-PCR) and multiplex PCR-based reverse line blot (mPCR/RLB) hybridization assay in the diagnosis of bacterial neonatal meningitis. BMC Pediatr 2014; 14
8. Logan, S. A., and MacMahon, E. Viral meningitis, BMJ. 2008; 336: 36–40
9. Ichiyama T, Nishikawa M, Yoshitomi T, Hayashi T, Furukawa STumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 in cerebrospinal fluid from children with prolonged febrile seizures. Comparison with acute encephalitis/encephalopathy. Neurology. 1998;50(2):407-11.
10. Takahashi T, Kamei S, Miki K, Ogawa K, Mizutani T. The analysis of cytokines in cerebrospinal fluid (CSF) in two cases of non-herpetic acute limbic encephalitis (NHALE) Rinsho Shinkeigaku.2003;43(4):162-9
11. Takahashi W, Nakada TA, Abe R, et al. Usefulness of interleukin 6 levels in the cerebrospinal fluid for the diagnosis of bacterial meningitis. J Crit Care 2014; 29:e691–696.693
12. Mukai AO, Krebs VL, Bertoli CJ, Okay TS. TNF-alpha and IL-6 in the diagnosis of bacterial and aseptic meningitis in children. Pediatr Neurol. 2006 Jan;34(1):25-9
13. Ohga S, Aoki T, Okada K, et alCerebrospinal fluid concentrations of interleukin-1 beta, tumour necrosis factor-alpha, and interferon gamma in bacterial meningitis.Archives of Disease in Childhood 1994;70:123-125.
14. Babu GN, Kumar A, Kalita J, et al. Proinflammatory cytokine levels in the serum and cerebrospinal fluid of tuberculous meningitis patients. Neuroscience Letters ;2008;436(1):48-51

SARCOIDOSIS

Borislava Tsafarova

Department of Microbiology, National
Center of Infectious and Parasitic Diseases (NCIPD),
Sofia, Bulgaria

ABSTRACT

Sarcoidosis is a systemic granulomatous inflammatory disorder that affects multiple organs – lungs, skin, heart, kidneys, liver, eyes, and nervous system, among others. The clinical course of sarcoidosis ranges from spontaneous resolution to chronic progressive disease which can be life-threatening. Most often, patients suffer from cough, shortness of breath, chest pain, and severe fatigue. In more severe cases, there is pulmonary fibrosis and/or irreversible damage to the organs affected by granulomas. Recent studies demonstrate innovative research in the field of sarcoidosis, thus significantly improved our knowledge of epidemiology and causative origins of the disease. Despite numerous studies, the aetiology of sarcoidosis is still not fully understood. It is proposed that the disease is caused by an unknown antigen (antigens) in humans with abnormal immune response, and a genetic predisposition. Here, we overview the current advances in sarcoidosis research.

Keywords: *sarcoidosis, epidemiology, aetiology*

HISTORY AND DEFINITION OF SARCOIDOSIS

The initial description of sarcoidosis is credited to an English physician, Jonathon Hutchinson, who reported the cutaneous form of sarcoidosis in 1875. Caesar Boeck described the skin lesions histologically in 1899. Because they resembled sarcoma but had benign histopathologic and clinical features, Boeck named the lesions 'sarcoid'. Jorgen Schaumann was the first to report systemic sarcoidosis, calling it Lymphogranulomatosis benigna. Sven Löfgren a Swedish physician, was the first to link erythema nodosum with sarcoidosis. The association is now called Löfgren's syndrome. Since then, many scientists have contributed to the current understanding of sarcoidosis as a systemic disease with diverse clinical manifestations (1,2).

An agreed descriptive definition of sarcoidosis was given by the American Thoracic Society, the European Respiratory Society and theWorld Association for Sarcoidosis and Other Granulomatous Disorders in 1999: "Sarcoidosis is a multisystem disorder of unknown cause. It commonly affects young and middle-aged adults and frequently presents with bilateral hilar adenopathy, pulmonary infiltration, ocular, and skin lesions. The liver, spleen, lymph nodes, salivary glands, heart, nervous system, muscles, bones, and other organs may also be involved. The diagnosis is established when clinicoradiographic findings are supported by histologic evidence of noncaseating epithelioid cell granulomas. Granulomas of unknown causes and local sarcoid reactions must be excluded. Frequently observed immunologic features are supression of cutaneous delayed-type hypersensitivity and a heightened Th1 immune response at sites of disease. Circulating immune complexes along with signs of B cell hyperactivity may also be found. The course and prognosis may correlate with the mode of the onset, and the extent of the disease. Acute onset with erythema nodosum or asymptomatic bilateral hilar adenopathy usually heralds a self-limiting course, whereas an insidious onset, especially with multiple extrapulmonary lesions, may be followed by relentless, progressive fibrosis of the lungs and other organs" (3).

EPIDEMIOLOGY

The frequency and prevalence of sarcoidosis, as well as the clinical manifestation of the disease, vary widely according to geographical location, gender, age, and ethnicity (4). The incidence of the disease is highest in the Scandinavian countries (11-24 cases per 100,000 per year) (5-7), and among African Americans (18-71 cases per 100,000 per year), (8-10). It is lowest in Asian countries (1 case per 100,000 per year), (11,12).

Currently, Bulgaria has no reliable epidemiological data available. The incidence calculated around 1980 was 8.7 cases per 100,000 people per year. It was

ADDRESS FOR CORRESPONDENCE:
Borislava Tsafarova
Department of Microbiology
National Centre of Infectous and Parasitic Diseases
44A, Gen. Stoletov Blvd.
1233 Sofia, Bulgaria,
e- mail: b.tsafarova@ncipd.org
Tel. +359 898 718 794

calculated using data from all over the country (13).

The average age of diagnosed patients with sarcoidosis is 40-55 years. In men, the peak occurs slightly earlier (30-50 years) than in women (50-60 years). But women get sick more often (14).

The clinical manifestations of sarcoidosis vary greatly between races. For example, African-Americans have more severe symptoms, as the disease affects larger lung areas and multiple organs than individuals in other ethnicities (4,15).

Löfgren's syndrome is an acute form of sarcoidosis with a characteristic clinical manifestation - fever, bilateral hilar lymphadenopathy, erythema nodosum of the shank (predominant in women), and/or migratory polyarthritis (predominant in men) (16). Löfgren's syndrome is more common in the white population. It is rarely diagnosed in African Americans and Asians. In Sweden, Löfgren syndrome comprises about one-third of all sarcoidosis cases. Patients usually have the HLADRB1*03 (HLA- DR3) allele and have a good prognosis, with remission in 70–80% of these cases (17).

It is assumed that sarcoidosis development is influenced by various environmental factors, as well as some behavioral and physiological characteristics. For example, smoking is associated with a 50% lower risk of developing sarcoidosis, suggesting an immunomodulatory effect of nicotine or another component of cigarette smoke (18). Cigarette smoking stimulates the release of cytokines favoring a Th-2 immune response (interleukin 13) (19), as opposed to the Th-1 immune response typical of sarcoidosis. These findings support the opinion that the host's immune status is a critical determinant of sarcoidosis.

Overweight and obesity increase the risk of sarcoidosis. This has been proven by two large studies involving women from the United States. In the first, monitoring the health of African-American women in America (n = 59,000), obesity was associated with a 40% increased risk of developing sarcoidosis (20). In the other, involving predominantly white women (n = 116439), obesity was associated with a 70% increased risk (21). Both of these studies reported a relationship between a higher body mass index (BMI) at 18 years and increased sarcoidosis incidence in later life.

Markers of higher endogenous estrogen (a consequence of later menopause, later first pregnancy, or recent birth) were associated with a decreased risk of sarcoidosis. The observation that women are more often diagnosed with sarcoidosis later in life (50–60 years of age) than men may be due to hormonal changes around the time of menopause (22).

HISTOLOGY OF SARCOID GRANULOMAS AND IMMUNOPATHOGENESIS OF THE DISEASE

Despite the type of organs affected by sarcoidosis, the histology image is similar and shows the presence of non-caseating, epithelioid granulomas. They are composed of a compact core of macrophages, some of which fuse to form giant multinucleated cells. Another part of the activated macrophages differentiates into epithelioid cells that surround the granuloma core. Scattered CD4+ T-helper cells are present in the granuloma, and significantly fewer CD8+ T-helper cells, fibroblasts, and B-lymphocytes can be found in its periphery. Different structures, such as asteroid bodies, Schaumann's bodies, and Hamazaki-Wesenberg bodies, are often observed in granulomas (23,24). A distinctive feature of sarcoid granulomas is the lack of necrosis, which discriminates them from tuberculous granulomas (25).

It is hypothesized that granulomas form around not completely degraded antigens to restrict and prevent its spread. Candidates for such an antigen are both environmental agents and microbial remnants (23,24).

Immunopathogenesis of sarcoidosis has been studied mainly by respiratory investigations.

Bronchoalveolar lavage studies have shown an increased number of CD4 + T-helper cells in sarcoid patients (24). Granuloma formation is associated with the activation of T-helper cells. There is a predominant proliferation of the Th1 cell populations, which changes the balance of Th1 and Th2 lymphocytes. This results in increased expression of the following cytokines: interleukin 2 (IL-2), interleukin 12 (IL-12), tumor necrosis factor-alpha (TNF-α), and gamma-interferon (IFN-γ), resulting in a slow but persistent inflammatory response in the affected tissues (26). IL-2 is a potent inducer of T cell proliferation and IFN-γ production. IL-2 plays a major role in the immune response in sarcoidosis. IL-12 directs the differentiation of naive T helper cells (Th0) to Th1 cell population and also activates the proliferation of cytotoxic and T cells in general (27). IL-12 plays a major role in the immune response against intracellular

pathogens, including Mycobacterium tuberculosis (28). In people with a genetic defect in the genes for IL-12 and/or for the receptor for this cytokine, the chance for granuloma formation is reduced. These individuals develop an atypical mycobacterial infection (29). Other commonly expressed T-helper populations in sarcoidosis patients are Th17.1 effector cells secreting INFγ and Th17 cells producing IL-17. The exact role of Th17 cells is not entirely clear but there is an increase in their abundance in patients with Löfgren's syndrome, which can be used as a diagnostic marker (30).

AETIOLOGY OF SARCOIDOSIS

The aetiology of sarcoidosis has remained a medical mystery for more than 120 years. It is proposed that the disease is caused by an unknown antigen (antigens) in humans with abnormal immune response, and a genetic predisposition (23,25).

There are two main hypotheses about the nature of this antigen. According to the first, sarcoidosis is caused by the action of an environmental agent/s. The second, indicates the possible involvement of one or more different microorganisms in the pathogenesis of the disease (25). Multiple environmental and occupational exposures have been reported to confer an increased risk of sarcoidosis, including organic dust, solvents, mold/mildew, pesticides, wood stoves, and others (31,32).

More pieces of evidence are accumulating to support the hypothesis of the infectious nature of sarcoidosis, but the specific etiological agent(s) has not been conclusively proven yet. Various microorganisms have been proposed for candidate pathogens in sarcoidosis. Among them are species of the *Mycobacterium spp, Cutibacterium spp, Borrelia spp, Human Herpes Virus 8 (HHV8), Rickettsia helvetica, Chlamydia pneumonia,* and others (33).

Strong evidence for the presence in sarcoid samples and possible involvement in the pathogenesis of sarcoidosis was obtained for *Cutibacterium acnes* (previously known as *Propionibacterium acnes*) and some mycobacteria species (34,35).

C. acnes has been reported as a possible cause of sarcoidosis in many Japanese studies (36-38). It is the only successfully cultured microorganism isolated from sarcoid lesions (39,40). In 2002, a large relevant study was published as a collaboration between Japan, Italy, Germany, and the UK. The results of this international study suggest an association between *C. acnes* and sarcoidosis in not only Japanese patients (positive signal rate of 89.2%) but also Europeans (positive signal rate of 81.4%) (41). However, more international studies with quantitative PCR are needed to clarify the role of *C. acnes* in sarcoidosis.

European and American research teams propose *M. tuberculosis* or another member of the genus as the most likely etiological agent (42-44). *M. tuberculosis* has been the longest hypothesized and the most investigated microorganism, due to the histological similarity between tuberculosis and sarcoidosis. Cultures and acid-fast stains of sarcoid specimens do not demonstrate the presence of mycobacterial organisms. PCR methods have detected mycobacterial DNA or RNA in different percentages in sarcoid patients (from 0 to 80%) (45). Recent studies have shown the presence of a mycobacterial DNA - the katG marker gene, coding for the KatG *Mycobacterium tuberculosis* catalase-peroxidase protein in 38% of the biopsy materials and evidence for circulating IgG to mycobacterial KatG in 50% of blood samples from sarcoid patients (46). Lack of mycobacterial DNA or its remnants in many sarcoid probes excludes *Mycobacterium spp.* as the sole etiologic agent in sarcoidosis.

A meta-analysis from 2016 shows the relationship between the most commonly associated with sarcoidosis microorganisms, and the disease itself. It includes 58 scientific case-control studies, published from 1980 to 2015, in which the presence of microorganisms in sarcoid samples was detected by cultural or molecular methods. The results of over 6000 patients were summarized and presented by odds ratio (OR) and 95% confidence intervals (95% CI). The possible etiological link between sarcoidosis and *Cutibacterium acnes* was with an OR of 18.80 (95% CI 12.62, 28.01), between sarcoidosis and mycobacteria, with an OR of 6.8 (95% CI 3.73, 12.39), between sarcoidosis and *Borrelia* - an OR of 4.82 (95% CI 0.98, 23.81), and between sarcoidosis and *HHV-8* with an OR of 1.47 (95% CI 0.02, 110.06). The authors suggest that more than one microbial species could be involved in the pathogenesis of sarcoidosis. The most probable etiological agents are *C. acnes* and members of the *Mycobacterium* and *Borrelia* genus. They also note the possible link between the geographical location and the predominance of

certain microorganisms in samples from patients with sarcoidosis (33).

CONCLUSIONS

Sarcoidosis develops in individuals with an immunogenetic predisposition to the disease. Many occupational and environmental exposures may increase the risk of developing sarcoidosis. The underlying inflammatory process is an antigen-driven, strongly polarized Th1 immune response. Although there has been some progress in understanding sarcoidosis over the past years, much is still unknown. Several important questions remain to be answered: whether one or more than one aetiological agents are leading to the disease; what are the exact geographical or racial impacts on the disease manifestation; what is the role of the genetic factors that increase susceptibility to sarcoidosis?

ACKNOWLEDGEMENTS: The study was funded by the National Science Program "VIHREN" and the Bulgarian National Science Fund contract N: КП-06-ДВ/10 - 21.12.2019, 'SARCOIDOSIS'.

REFERENCES:

1. James, D. G. *Descriptive definition and historic aspects of sarcoidosis.* Clin. Chest Med. 1997; 18:663–679.
2. Hosoda Y, Odaka M. *History of sarcoidosis.* Semin. Respir. Med. 1992; 13:359–367.
3. Hunninghake GW, Costabel U, Ando M, et al. *ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/ World Association of Sarcoidosis and other Granulomatous Disorders.* Sarcoidosis Vasc Diffuse Lung Dis. 1999 Sep; 16(2):149-73.
4. Judson MA, Boan AD, Lackland DT. *The clinical course of sarcoidosis: presentation, diagnosis, and treatment in a large white and black cohort in the United States*. Sarcoidosis Vasc. Diffuse Lung Dis. 2012; 29:119–127.
5. Arkema EV, Grunewald J, Kullberg S, et al. *Sarcoidosis incidence and prevalence: a nationwide register- based assessment in Sweden.* Eur. Respir. J. 2016; 48:1690–1699.
6. Byg KE, Milman N, Hansen S. *Sarcoidosis in Denmark 1980–1994. A registry- based incidence study comprising 5536 patients.* Sarcoidosis Vasc.Diffuse Lung Dis. 2003; 20:46–52.
7. Milman N, Selroos O. *Pulmonary sarcoidosis in the Nordic countries 1950–1982. Epidemiology and clinical picture.* Sarcoidosis. 1990; 7:50–57.
8. Baughman RP, et al. *Sarcoidosis in America. Analysis based on health care use.* Ann. Am. Thorac. Soc. 2016; 13:1244–1252.
9. Cozier YC, et al. *Sarcoidosis in black women in the United States: data from the Black Women's Health Study.* Chest. 2011; 139:144–150.
10. Dumas O, Abramovitz L, Wiley AS, et al. *Epidemiology of sarcoidosis in a prospective cohort study of U.S. women.* Ann. Am.Thorac. Soc. 2016; 13: 67–71.
11. Morimoto T, et al. *Epidemiology of sarcoidosis in Japan.* Eur. Respir. J. 2008; 31:372–379.
12. Yoon HY, Kim HM, Kim YJ, et al. *Prevalence and incidence of sarcoidosis in Korea: a nationwide population- based study.* Respir. Res. 2018; 19:158.
13. Costa M, Dobrev P, Ivanov S. *Epidemiology of sarcoidosis. Outlook. Sarcoidosis another granulomatous disorders.* ExceptaMedica. 1988; 329-337.
14. Arkema EV, Cozier YC. *Epidemiology of sarcoidosis: current findings and future directions.* Ther. Adv. Chronic Dis. 2018; 9: 227–240.
15. Rabin DL, Richardson MS, Stein SR, et al. *Sarcoidosis severity and socioeconomic status.* Eur. Respir. J. 2001; 18: 499–506.
16. Grunewald J, Eklund A. *Sex- specific manifestations of Lofgren's syndrome.* Am. J. Respir. Crit. Care Med. 2007; 175: 40–44.
17. Grunewald J, Eklund A. *Lofgren's syndrome: human leukocyte antigen strongly influences the disease course.* Am. J. Respir. Crit. Care Med. 2009; 179: 307–312.
18. Carlens C, et al. *Smoking, use of moist snuff, and risk of chronic inflammatory diseases.* Am. J. Respir. Crit.Care Med. 2010; 181: 1217–1222.
19. Cozen W, Diaz-Sanchez D, Gauderman WJ, et al. *Th1 and Th2 cytokines and IgE levels in identical twins with varying levels of cigarette consumption.* J Clin Immunol. 2004; 24:617-22.
20. Cozier YC, et al. *Obesity and weight gain in relation to incidence of sarcoidosis in US black women: data from the Black Women's Health Study.* Chest. 2015; 147:1086–1093.
21. Dumas O, Boggs K M, Cozier YC, et al. *Prospective study of body mass index and risk of sarcoidosis in US women.* Eur. Respir. J. 2017; 50:1701397.
22. Cozier YC, et al. *Reproductive and hormonal factors in relation to incidence of sarcoidosis in US Black women: the Black Women's Health Study.* Am. J.Epidemiol. 2012; 176:635–641.
23. Saidha S, Sotirchos ES, Eckstein C. *Etiology of Sarcoidosis: does infection Play a role?* Yale J. Biol. Med. 2012; 85:133-141.
24. Grunewald J, Eklund A. *Role of CD4+ T cells in sarcoidosis.* Proc Am Thorac Soc. 2007; 4(5):461-464.
25. Grunewald J, Grutters JC, Arkema EV, et al. *Sarcoidosis.* Nat Rev Dis Primers. 2019; 5, 45
26. Darlington P, Haugom-Olsen H, Von Sivers K, et al. *T-cell phenotypes in bronchoalveolar lavage fluid, blood and lymph nodes in pulmonary sarcoidosis-indication for an airborne antigen as the triggering factor in sarcoidosis.* J Intern Med. 2012; 272(5):465–471.
27. Ziegenhagen MW, Müller-Quernheim J. *The cytokine network in sarcoidosis and its clinical relevance.* J Intern Med. 2003; 253(1):18–30.
28. Locksley RM. *Interleukin 12 in host defense against microbial pathogens.* Proc Natl Acad Sci USA. 1993; 90(13):5879-80.
29. Lammas DA, De Heer E, Edgar JD, et al. *Heterogeneity in the granulomatous response to mycobacterial infection in patients with defined genetic mutations in the interleukin 12-dependent interferon-gamma production pathway.* Int J Exp Pathol. 2002; 83(1):1-20.
30. Ostadkarampour M, et al. *Higher levels of interleukin IL-17 and antigen- specific IL-17 responses in pulmonary sarcoidosis patients with Lofgren's syndrome*. Clin. Exp. Immunol. 2014; 178:342–352.
31. Newman LS, Rose CS, Bresnitz EA, ACCESS Research Group, et al. *A case control etiologic study of sarcoidosis: environmental and occupational risk factors.* Am J Respir Crit Care Med. 2004; 170(12):1324-30.
32. Barnard J, Rose C, Newman L, ACCESS Research Group, et al. *Job and industry classifications associated with sarcoidosis in A Case-Control Etiologic Study of Sarcoidosis (ACCESS).* J Occup Environ Med. 2005; 47(3):226-34.
33. Esteves T, Aparicio G, Garcia-Patos V. *Is there any association between Sarcoidosis and infectious agents?: a systematic review and meta-analysis.* BMC Pulm Med 2016; 16: 165.
34. Celada LJ, Hawkins Ch, Drake WP. *The Etiologic Role of Infectious Antigens in Sarcoidosis Pathogenesis.* Clin Chest Med. 2015; 36:561–568.
35. Ezzie M, Crouser E. *Considering an infectious etiology of sarcoidosis.* Clinics in Dermatology. 2007; 25: 259–266.
36. Zhao MM, Du SS, Li QH, et al. *High throughput 16SrRNA gene sequencing reveals the correlation between Propionibacterium acnes and sarcoidosis.* Respir Res. 2017; 18:28.
37. Negi M, Takemura T, Guzman J, et al. *Localization of Propionibacterium acnes in granulomas supports a possible etiologic link between sarcoidosis and the bacterium.* Modern Pathology. 2012; 25(9):1284–1297.
38. Nagata K, Eishi Y, Uchida K, et al. *Immunohistochemical Detection of Propionibacterium acnes in the Retinal Granulomas in Patients with Ocular Sarcoidosis.* Sci Rep. 2017; 7,15226.
39. Abe C, Iwai K, Mikami R, et al. *Frequent isolation of Propionibacterium acnes from sarcoidosis lymph nodes.* Zentralblatt fur Bakteriologie Mikrobiologie und Hygiene. 1984 Series A, 256(4):541–547.
40. Homma JY, Abe C, Chosa H, et al. *Bacteriological investigation on biopsy specimens from patients with sarcoidosis.* Japanese Journal of Experimental Medicine. 1978; 48(3):251–255.

41. Eishi Y, Suga M, Ishige I, et al. *Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis.* J Clin Microbiol. 2002; 40(1):198–204.

42. Saboor SA, Johnson NM, Mcfadden J. *Detection of mycobacterial DNA in sarcoidosis and tuberculosis with polymerase chain reaction.* Lancet. 1992; 339(8800):1012–5.

43. Gazouli M, Ikonomopoulos J, Trigidou R, et al. *Assessment of mycobacterial, propionibacterial, and human herpesvirus 8 DNA in tissues of Greek patients with sarcoidosis.* J Clin Microbiol. 2002; 40:3060–3063.

44. Li N, Bajoghli A, Kubba A, et al. *Identification of mycobacterial DNA in cutaneous lesions of sarcoidosis.* J Cutan Pathol. 1999; 26:271–278.

45. Gupta D, Agarwal R, Aggarwal AN, et al. *Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis.* Eur Resp J. 2007; 30: 508-516.

46. Song Z, Marzilli L, Greenlee BM, et al. *Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis.*J Exp Med. 2005; 201(5):755-67.

CLINICAL CASE OF CRYPTOCOCCAL MENINGITIS IN A LIVER TRANSPLANT PATIENT

L. Boyanova, Z. Ivanova

National Reference Laboratory of Mycoses and STIs
National Center of Infectious and Parasitic Diseases

ABCTRACT

The incidence of infections caused by Cryptococcus neoformans has increased significantly in recent years, especially in the settings of immune deficiency (HIV infection transplantation, etc.). Most often after inhalation of spores dissemination of yeast to the brain parenchyma occurs, leading to meningitis (meningo-encephalitis). Our clinical case, is a patient with cryptococcal meningitis after liver transplantation, who died despite the onset of antifungal therapy. This is further evidence of the severe prognosis of CNS cryptococcosis, especially in immunocompromised patients

Keywords: *meningitis, Cryptococcus neoformans, immunosuppression*

INTRODUCTION

The incidence of infections caused by Cryptococcus neoformans has increased significantly in recent years, especially in immunocompromised patients (14). Among these patients, the most common are HIV-positive and organ transplant recipients. The appearance of infections at a late stage after organ transplantation indicates a probable exogenous origin (inhalation of basidiospores or from untreated locus in sites with special blood circulation (1).

Scientific research has shown that in a large percentage of cases cryptococcal meningitis occurs more than 6 months post-transplantation as a result of immunosuppressive treatment against an acute graft rejection.

ADDRESS FOR CORRESPONDENCE:
Boyanova L.
National Center of Infectious and Parasitic Diseases
Department of Microbiology
26 Yanko Sakazov Blvd.
1504 Sofia, Bulgaria
Email: miraanbo@abv.bg
Phone: +359 2 944 6999/ 318

Depending on the immunological status of the host acute dissemination with brain organotropism - infection of subarachnoidal space may occur prior to an invasion of brain parenchyma (meningo-encephalitis), (1). Clinically, it is most often described with headache, increased intracranial pressure, basal discharge symptoms, nausea, vomiting, a positive symptom of Kernig and Brudzinski.

Inadequate diagnosis, lack of microbiological examination, and consequently, r untimely antifungal therapy can lead to increased intracranial pressure and fatal result.

MATERIALS AND METHODS

Our clinical case is a 71 years old man of, diagnosed with hepatitis B in 2004, subsequently complicated by liver cirrhosis. On this occasion, in 2016 the patient was subjected to liver transplantation.

A broncho-alveolar lavage (BAL) sample was received at the National Reference Laboratory of Mycoses (National Center of Infectious and Parasitic Diseases) for detection of medically important fungi, with the observation of pulmonary mycosis. The microbiological culture from BAL was negative, but microscopy detected a lot of leukocytes.

A serum sample was tested alongside for the presence of antibodies to yeasts of the genus Candida and molds of the genus Aspergillus by indirect immunofluorescence (IIF), and for Cryptococcus antigen by latex – agglutination test (3).

The patient was transferred to the Clinic of Nervous Diseases with suspected meningitis, and on this occasion another clinical sample was received at the National Reference Laboratory - cerebrospinal fluid, again for fungal testing. A late - agglutination test was performed, which is a rapid test for detection of Candida and Cryptococcus antigens (2; 6) .

The cerebrospinal fluid was also cultured for bacteria and fungi detection. The strains were identified by biochemical tests and microscopy and the antifungal susceptibility was determined.

Serological testing with indirect immunofluorescence (IIF) did not show antibodies specific for fungi of the genus Candida and Aspergillus (Ig G 1:40 at a rate of up to 1: 160, IgA-negative, IgM-negative and Aspergillus-negative).

However, the latex–agglutination test for Cryptococcus antigens turned out positive (Fig.1). This is a qualitative

test for the detection of polysaccharide antigens (glucurono-xylomanan is the main component of Cryptococcus capsule) using latex particles loaded with monoclonall antibodies (7).

Fig.1 *Positive latex agglutination test*

The cultures were negative for bacrerial pathogen. On the universal culture medium for fungi Sabouraud dextrose agar a pure culture of white to cream-colored yeast and mucoid colonies were isolated in a significant amount, (Fig.2).

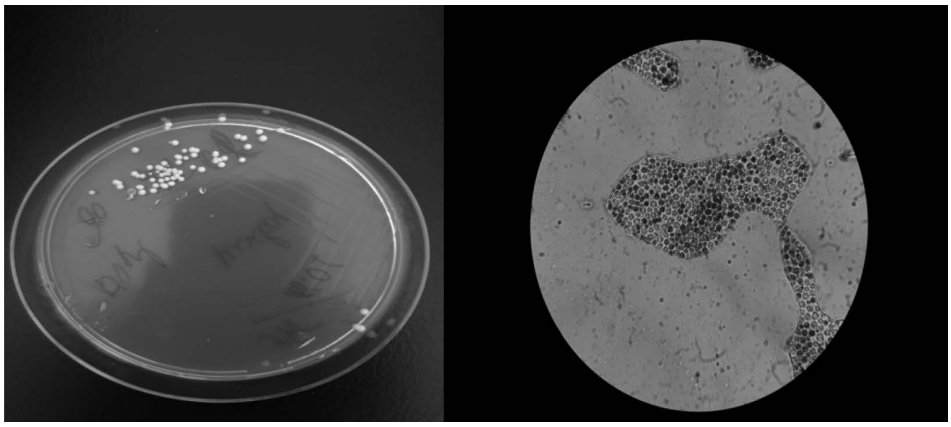


Fig.2 - *Macroscopic and microscopic view of cryptococci*

Single oval and budding yeast cells were visualized on a microscope slide (Fig. 2) .
The fungi from the pure culture were identified as Cryptococcus neoformans by Auxacolor biochemical identification test (Fig .3).

Fig.3 - *Biochemical identification of yeast*

Cryptococci were also confirmed as urease-positive by an urease activity test (Fig .3).

The strain was further tested for sensitivity to several antifungals using the so-called. E - test and disco-diffusion test. The results are as follows: Fluconazole-S, Itraconazole-S, Voriconazole-S, Miconazole-S, Nystatin-S, Anidulafungin-R, Caspofungin R. Cryptococci are less sensitive to echinocandins due to the lower amount of target (β D-glucan) in their cell wall, but have been shown to be sensitive to azoles and polyenes.

On our recommendation, a serum sample was also tested for antibodies to Cryptococcus neoformans, and proved negative (3).

The recommended treatment scheme (SANFORD guide) for Cryptococcus neoformans includes three types of antifungals - Fluconazole, Amphotericin B and Flucytosine , followed by single use of Fluconazole (Amphotericin B and Flucytosine are not available on our market), (12; 13), (Table 1) .

Table 1. *Recommendations for therapy of SANFORD guide, USA*

Cryptococcosis (meningitis)	- **Liposomal Amphotericin B**(L-AmpB) 3-4 mg/kg iv q24h or - **Amphotericin B lipid complex**(ABLC) 5 mg/kg iv q 24h+Fluconazole 25 mg/kg po q6h -also in combination with :**Fluconazole** 400-800 mg po/ day/8 weeks	- **Liposomal Amphotericin B**(L-AmpB) 3-4 mg/kg iv q24h or **Amphotericin B lipid complex** 5 mg/kg iv q 24h or **Amphotericin B** 0.7-1 mg/kg iv q24h+**Fluconazole** 800-1200mg/day iv/po/2 weeks **- Liposomal Amphotericin B**3-4 mg/kg iv q24h or **Amphotericin B lipid complex** 5 mg/kg iv q 24h or **Amphotericin B** 0.7-1 mg/kg iv q24h/4-6 weeks -**Fluconazole** 800-1200/day iv/ po+**Flucytosine** 25 mg/kg po q6h/4-6 weeks -**Fluconazole** 1200-2000 mg po/ day/10-12 weeks

The patient was treated with Fluconazole i.v.-a loading dose of 800 mg, with supporting dose of 400 mg. According to the attending physician and relatives, his condition was improving as he became contact and conscious. However, a control puncture and cerebrospinal fluid examination were not performed, because a few days later the exitus letalis was reached.

DISSCUSSION

Infections caused by Cryptococcus spp are reported worldwide, including the United States and Europe.A patient with HIV-positive status and cryptococcal meningitis was also reported in Egypt (18). An association with eucalyptus trees has been demonstrated, but they can be isolated from various environmental locations, including birds.

In the recent years in Europe, infections caused by Cryptococcus neoformans Cryptococcus gatii and Cryptococcus deuterogatii have been on the rise (especially in HIV-positive patients),(17). In Spain, a case of cerebral cryptococcus has been described in an immunocompromised patient (19). Cryptococcal meningitis has also been reported in HIV-negative patients, but with other risk factors such as organ transplantation and chemotherapy. The patient in our clinical case is after an organ liver transplantion.

The culture study with isolation of yeast in pure culture once again proves that the previous latex-agglutination test is the best laboratory method, with great reliability in the diagnosis of cryptococcosis of the CNS. The test is highly sensitive and specific, and gives positive reaction even at very low microbial counts of cryptococci in the cerebrospinal fluid (1; 8).

According to EORTS (European Organization for Research and Treatment of Cancer) / MSGERC (Mycoses Study Group Education and Research Consortium), this serological test is accepted as criterion for proven invasive fungal disease (IFD), ie.

cryptococcal meningitis can be reliably diagnosed by antigen testing (Table 2), (16). Microscopic detection of encapsulated yeast should not be neglected, either.

Table 2.- *Criteria for "proven" IFD*

Type of fungus	Microscopic analysis	Cultural examination of sterile clinical material	Blood culture	Serological method	DNA
Yeast Molds	Histopathological, cyto pathological examination of biopsy material or direct microscopic examination in which yeast, pseudohyphae, true hyphae of biopsy material are visualized	Isolation of fungal strain from clinical material from the site of infection, with the exception of BAL,urine, paranasal sinus or mastoid sinus secretion	When positive for Candida, Cryptococcus Trichosporon, mold and others	Not applicable, except for detection of Cryptococcus antigen in cerebrospinal fluid, which confirms the diagnosis	Amplification of fungal DNA in combination with DNA sequencing

The symptoms of cryptococcal meningitis (headache, fever and fatigue) are not typical enough and are often confused with tuberculosis (20). Therefore, a fungal cause should be always considered. Cryptococcosis is one of the leading causes of illness and death in severely immunocompromised individuals. Timely application of antifungal therapy is vital in order to increase the chances for favorable outcome.

REFERENCES:

1. Kantardjiev T. *Etiologic diagnosis and etiotropic therapy of mycoses*. 2012, Sofia, National Centre of Infectious and Parasitic Diseases.
2. Kantardjiev, T., Kouzmanov, A., Velinov, Tz., Christozova, E.Latex agglutination test for diagnosis of the cryptococcal meningitis(2000) *Problems of Infectious and Parasitic Diseases*, 28 (2), pp. 25-26.
3. Kantardjiev T, .Detection of antibodies to germ tube of Candida albicans by microscopic indirect immunofluorescence method for diagnosis of systemic candidosis. Problems of Inf.and Paras.diseases, Vol.39 (2):20; 2011
4. Kantardjiev T. Immunological diagnosis of systemic candidosis. PhD th.Nat Cent Inf.and Parasitic Diseases – Sofia; 1990;
5. Kantardjiev**,** T., E. Popova. Anti-Candida antibodies in serum and saliva of patients with denture stomatitis. *Folia Medica,* 2002, 44 (4), 39-44.
6. Kantardjiev, T., Kouzmanov, A., Velinov, Tz., Christozova.Antigen detection in body fluids for diagnostic of systemic mycosis by latex-agglutination. Infectology; 2000, 4:23-26
7. Kantardjiev, T., Kouzmanov, A., Baikushev R, Velinov, Tz.Comparative studies on latex-agglutination test on Sanofi Diagnostics Pasteur in patients with systemic and mucocutaneus candidosis. Infectology; 1999, 1:28-31
8. Frederic Dalle, Pierre E. Charles. Cryptococcus neoformans galactoxylomannan contains an epitope(s) that is cross-reactive with Aspergillus galactomanna. JCM 2005; 43 (6):2929-31
9. Kantardjiev, T., V. Levterova, S. Panaiotov, I. Ivanov, E. Hristozova. Molecular taxonomy of Cryptococcus neoformans varieties displaying phenotypic similarities. Biotechnology and Biotechnological Equipment, 2006, 20 (2), 101-103.
10. Kantardjiev**,** T., E. Popova. Anti-Candida antibodies in serum and saliva of patients with denture stomatitis. *Folia Medica,* 2002, 44 (4),39-44.
11. CDC. Preventing Deaths from Cryptococcal meningitis, X, 2018;
12. Ruschel M, Thapa B.-Cryptococcal meningitis, NCBI, 10 august 2020
13. Ming DK, Harrison TS. Cryptococcal meningitis ,BrJ Hosp Med, 2017, 78 (8): 125-127
14. Poley M, Koubek R, Walsh L. Cryptococcal meningitis in an apparent immunocompetenet patient, J Investig Mrd High Impact case, rep.2019
15. Gassiep I, Aye Armstrong. Correlation between serum cryptococcal antigen titre and meningitis in immunocompetent patient-J Med Microbiol.2018, oct; 67 (10): 1515-1518;
16. Clin Inf Diseases-Revision and Update of the Consensus Definitions of Invasive Fungal Disease from the European Organization for Research and Treatment of Cancer and the Mycoses study group education and research consortium, 2019;
17. M.Bauer, C.Wickenhauser, A.Haauet et all- Case report: A fatal case of cryptococcosis in an immunocompetent patient due to Cryptococcus deuterogatii – JMM Case Rep.2018, oct, 5 (10);
18. A. Mansour, I.Nakhla, M.El Sherif et. all- Case report: Cr. Neoformans var.gatii meningitides in Egypt: a case report;
19. M. Franciska Colon, Susana Frases, Consuelo Ferrer et all- First Case of Human Cryptococcosis due to Cryptococcus neoformans var.gattii in Spain- J.Clin. Microb. July, 2005, p.3548-3550, vol.43;
20. H.Khutan, Pr. Grover – Cryptococcal meningitis in an immunocompetent young male-Asian journal of sc.techn.,vol.8, p.4869-4871, 2017

OCULAR TOXOPLASMOSIS: BRIEF LITERATURE REVIEW

Iskren Kaftandjiev[1], Rumen Harizanov[1]

[1]National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria, Department of Parasitology and Tropical Medicine

ABSTRACT

Toxoplasmosis in humans is a zoonotic parasitic disease caused by a ubiquitous protozoan, *Toxoplasma gondii. Toxoplasmosis* is an opportunistic infection that can cause serious damage in immunocompromised patients.While in the non-immunocompromised individuals it is most often latent and asymptomatic, about one-third of the world's population is estimated to be infected. Toxoplasmosis is the most common cause of posterior uveitis in non-immunocompromised individuals and the second most common cause of chorioretinitis after cytomegalovirus infection in people with HIV / AIDS. The infection can be acquired congenitally or postnatally and ocular lesions may present during or years after the occurance of the acute infection. Molecular biology techniques to diagnose ocular toxoplasmosis have been available for many years and are now accessible as standard laboratory tests in many countries. Aqueous humor or vitreous evaluation to detect parasite DNA by polymerase chain reaction or specific antibodies may provide evidence for diagnosis. Oral pyrimethamine and sulfadiazine plus corticosteroids are an effective therapy for ocular toxoplasmosis. Recent data supports the use of other treatment options, including intravitreal antibiotics. The aim of the present review is to discuss briefly the new diagnostic and treatment approaches for ocular toxoplasmosis.

Keywords: *ocular toxoplasmosis; diagnosis; treatment*

ADDRESS FOR CORRESPONDENCE:
Rumen Harizanov
Bulgaria, 1504 Sofia, 26, Yanko Sakazov Blvd.,
National Centre of Infectious and Parasitic Diseases,
Department of Parasitology
and Tropical Medicine;
email: harizanov@ncipd.org;
Phone: +35929446999; ext. 360 /Fax: +35928438002

INTRODUCTION

Toxoplasma gondii is an ubiquitous obligate intracellular parasite, which infects both humans and warm-blooded animals as a zoonotic pathogen widespread in nature (1,2). Approximately one-third of humans worldwide are estimated to be chronically infected with *T. gondii* (2,3). Ocular toxoplasmosis is the most frequent cause of posterior uveitis, presenting with a unilateral chorioretinal lesion associated with vitritis (4, 5). Although ocular toxoplasmosis in adult life was presumed to be the recurrence of the congenitally acquired infection, more recent reports indicate that acquired infections may account for a larger portion of ocular involvement than congenital toxoplasmosis (6, 7). Visual symptoms during acute toxoplasma retinochoroiditis are typically secondary to vitritis or less frequently from the involvement of the macula or optic nerve. Vision loss may become permanent due to formation of a macular scar or optic atrophy, and up to 24% of patients may have 20/200 vision or less in at least one eye (8, 9). A toxoplasmosis scar can be associated with severe visual field loss when it occurs close to the optic disk (10).Felidae are the definitive hosts for *T. gondii*, and humans and other mammals act as intermediate hosts. The transmission occurs by many routes, including ingestion of raw or undercooked meat infected with tissue cysts, ingestion of food and water contaminated with oocysts, ingestion of eggs and milk contaminated with tachyzoites, blood transfusion, organ transplantation or transplacental transmission (11).

Accurate diagnosis depends heavily on the characteristic clinical features of this disease, but atypical presentations, especially in immunocompromised patients, may create diagnostic challenges and lead to misdiagnosis and inappropriate treatment (12). The aim of the present review is to discuss briefly the new diagnostic and treatment approaches for ocular toxoplasmosis.

EPIDEMIOLOGY

T. gondii is a common parasite that infects almost all mammalian species including humans. Approximately 25–30% of the human population is infected with *T. gondii* (13). However, seroprevalence varies widely, from 10 to 80% between different geographic areas and countries and even within countries. Reports of low seroprevalence in the range of 10–30% come from Southeastern Asia, North America and Northern Europe with (14). Prevalence between 30 and 50% has been reported for Central and Southern Europe, whereas high seroprevalences are observed in Latin America and in tropical African countries (15).

The population structure of *T. gondii* is highly clonal. There are three predominant clonal lineages in North America and Europe, namely I, II and III. The lineages are based on murine model virulence studies (16). It has been suggested that the type II clonal lineage of *T. gondii* may be responsible for the majority of acquired ocular lesions, while type I may be more frequently seen in congenital toxoplasmosis. Recently, it has been shown that type I as well as atypical strains may play an important role in acquired infection (16-17). Type II strains appear to be responsible for the majority of symptomatic human cases in France and the United States (18), while types I and III are found in only 10% and 9% of *Toxoplasma* isolates from patients, respectively (19).

Most patients present with uveitis secondary to ocular toxoplasmosis in their second to fourth decade of life. Disease severity is typically higher in older patients (20, 21). In a study by Nguyen et al. (22) toxoplasmosis was the most common etiology of uveitis in patients referred to a tertiary center and had a prevalence of 14% among all other etiologies. A survey of 1,916 patients from Europe found ocular toxoplasmosis to be the most frequent diagnosis in patients with posterior uveitis and the cause of 4.2% of uveitis cases (23). Multiple studies from different regions of the globe have identified ocular toxoplasmosis as the most common form of posterior uveitis (24).

The incidence of congenital infection ranges from 1/770 to 1/10,000 and largely depends on the geographical region (25–27). Most cases of congenital toxoplasmosis are asymptomatic, and initially remain unrecognized. Severe cases resemble other acute intrauterine infections such as rubella or cytomegalovirus. Low birth-weight, hydrocephalus, prematurity, seizures, enlargement of liver or spleen, and jaundice may occur. Evidence of retinal infection may be found in 75-80% of the infected babies. The disease affects both eyes in 85% of cases (27). In pediatric cases, ocular disease is the most common manifestation of congenital toxoplasmosis, with 95% of patients showing signs of chorioretinitis in the presence of systemic findings, and occurs in the absence of systemic involvement in 26% of children (28, 29). The majority of ocular toxoplasmosis infections are acquired orally, either by consuming or handling raw meat containing tissue cysts, or by drinking water contaminated with oocysts (11).

CLINICAL FEATURES

Most of the acute systemic toxoplasmosis cases in healthy hosts tend to be subclinical, but some may present with mild flu-like symptoms. If parasites reach an eye and they yield a focus of inflammation, the lesion progresses to retinitis and involves the choroid secondarily. Is The release of rhoptry protein kinase from the parasite is a key-point in the pathogenesis of *T. gondii* infection of. The former interacts with JANUS kinase - an enzyme that is part of the signal transducer and transcriptional activator - STAT (JAK-STAT) pathway, which reduces cytokine production and leads to an ineffective local immune response l (30). Host immune responses appear to induce conversion of the parasitic forms from tachyzoites to bradyzoites and their encystment (31). The cyst may remain inactive in the scar or nearby for a long time. However, when the cyst ruptures with release of organisms into the surrounding retina, retinitis may be reactivated (32). The reactivation of retinitis is known to develop at the border of old scars and is attributed to the rupture of tissue cysts which are located within old lesions **(Fig. 1)**. Sometimes however, new lesions are found at locations distant from old scars. In general, the hallmark of the ocular lesion is retinitis, adjacent to an inactive retinochoroidal scar. Necrosis of the retina and choroid with destruction of the surrounding tissues is found within the active lesion. The inflammatory response is mononuclear cell reaction in nature, and

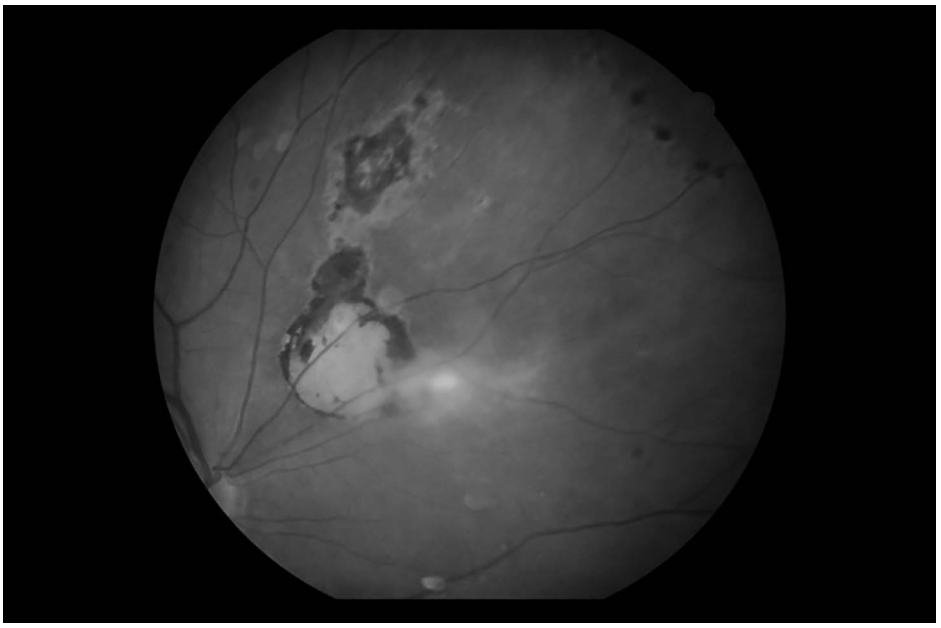


Fig.1. *Reactivation of toxoplasmic retinitis at the border of an old scars.*

consists of lymphocytes and macrophages at the edge of the lesion. Viable and intact cysts may be present, either adjacent to the scars or within the area of retinal necrosis, and rarely tachyzoites may be identified in the extracelluar space (33). Ocular toxoplasmosis often presents with classic ophthalmic findings, and the diagnosis is reached by clinical examination without any laboratory confirmation of *T. gondii* infection (34). Seropositivity for T. gondii infection indicates previous systemic exposure to the parasite, though this finding is not sufficient to confirm the diagnosis of ocular toxoplasmosis. Visual impairment may be secondary to a macular lesion, while lesions located at the peripheral retina often lead to vision loss secondary to severe vitreous inflammation (35, 36). Although the optic nerve is not commonly affected its damage may cause visual field loss and color vision deficiency. The involvement of the vitreous body in the inflammation leads to blurry vision, an important symptom of ocular toxoplasmosis. When the parasite becomes inactive, retinochoroidal scars are formed and from their size and location depends the severity of visual field deficits. Classical ocular manifestation of toxoplasmosis is a nidus of fluffy white, focal necrotizing retinitis or retinochoroiditis adjacent to a variably pigmented chorioretinal scar. Often the active lesion is obscured by severe vitritis producing the classic 'headlight in the fog' sign (37) **(Fig. 2)**. The severity of anterior uveitis may range from minimal reaction to an intense inflammation, masking the posterior

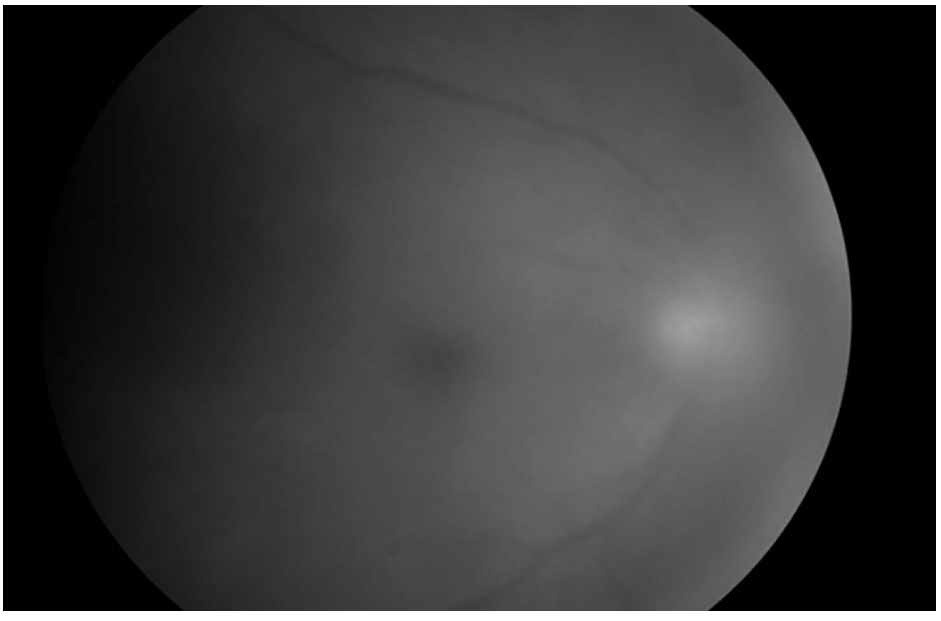


Fig.2. *An active lesion is obscured by severe vitritis producing the classic 'headlight in the fog' sign.*

segment involvement. Anterior uveitis may be either granulomatous or nongranulomatous inflammation. In children with congenital toxoplasmosis, cataract may be associated with retinochoroiditis and may follow severe iridocyclitis (36). Other common clinical signs of ocular toxoplasmosis include satellite lesion adjacent to an inactive retinochoroidal scar, retinochoroidal scar, focal or widespread vasculitis, and inflammatory ocular hypertension syndrome (38). Atypical findings include multifocal retinochoroiditis, low-grade or absent vitreal infiltration, an active lesion more than 2 disk diameters without an associated retinochoroidal scar, absence of a retinochoroidal scar, bilaterality, optic disk involvement, choroiditis without retinitis, hemorrhagic vasculitis, serous retinal detachment, andretinal neovascularization (38). Spectral-domain optical coherence tomography (SDOCT) imaging is an important diagnostic tool to identify the morphological features of the vitreoretinal changes in ocular toxoplasmosis (40-41). The stage of the disease is determinant for the SD-OCT findings of chorioretinal lesions. In children with congenital toxoplasmosis, cataracts may occur as a complication of retinochoroiditis, and may follow severe iridocyclitis. Cataract may cause severe amblyopia in children and may need to be removed surgically (42). Inflamation of the vitreous is usually more intense near the lesion of active retinochoroiditis. In cases of intense vitritis, epiretinal membranes may develop and vitreoretinal traction adjacent with consequent retinal detachment to the area may occur (**Fig. 3 and 4)**. A bright white reflex seen when one shines the light of the indirect ophthalmoscope into the back of the eye - headlight in the fog sign which results from severe vitritis.

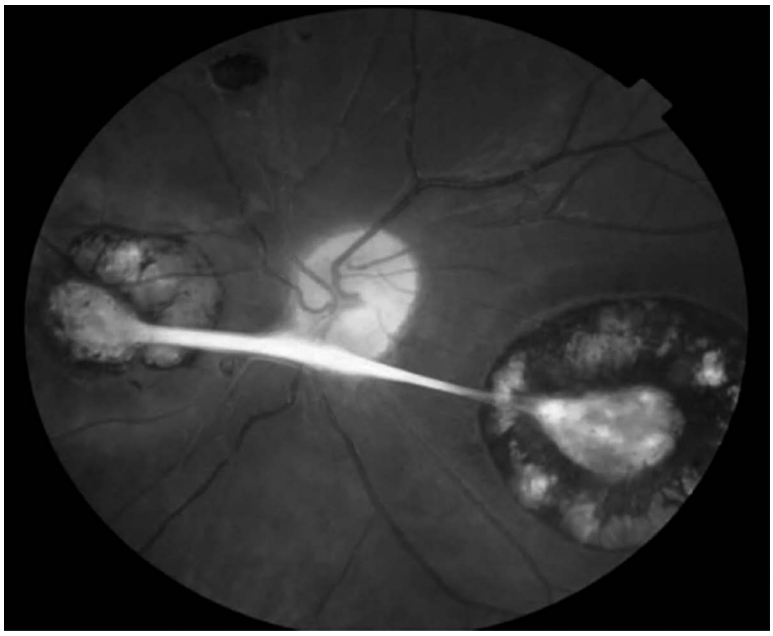


Fig. 3. *Severe vitritis with formation of epiretinal membranes that may develop vitreoretinal traction with consequent retinal detachment to the area.*

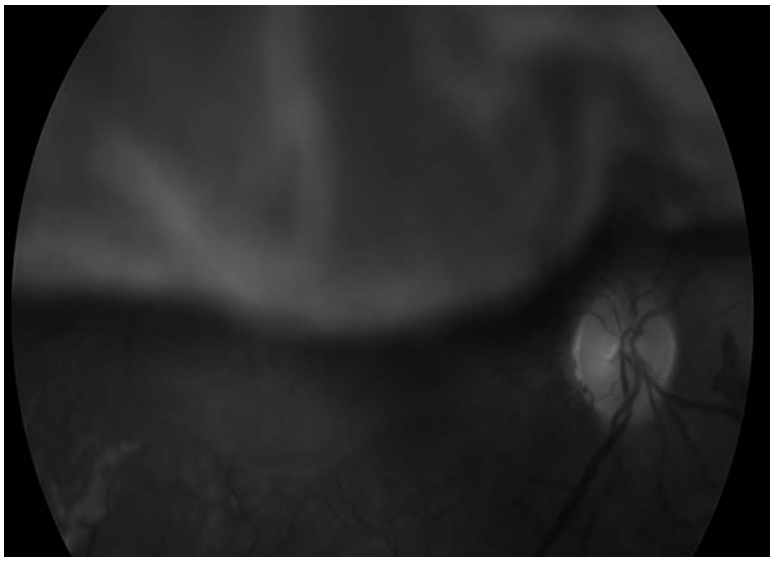


Fig. 4. *Retinal detachment as a result of severe toxoplasmic vtritis.*

DIAGNOSIS

The diagnosis of ocular toxoplasmosis is typically clinical. There is no reliable diagnostic test to identify toxoplasmic uveitis. The presence of anti *T. gondii* IgG antibodies does not confirm the toxoplasmic aetiology, but a negative IgG generally discards the possibility. Such antibodies can often persist at high titers for years after the acute infection and there is a high prevalence of such antibodies in the general population (43). *T. gondii* antibody titers in ocular fluids or polymerase chain reaction (PCR) of aqueous and vitreous samples are other newer tools with high sensitivity and specificity to confirm the diagnosis (44, 45). Many diagnostic laboratories are capable of measuring IgG and IgM antibody levels using enzyme-linked immunosorbent assay (ELISA) or immunofluorescent antibody commercial kits. ELISA has an advantage over the immunofluorescent antibody testing because it permits automation for simultaneous testing of large numbers of samples and the results are objective. The Sabin-Feldman dye test, the classic gold standard serology test, uses live *T. gondii* tachyzoites to detect IgG antibodies (46). It as high sensitivity and specificity, but this test is not frequently performed, owing to the risk for laboratory-acquired infections. Serum IgM and IgG antibodies to *T. gondii* develop within 1–2 weeks after infection (47). Patients suspected of acute toxoplasmosis may initially be analyzed for IgG serology, and if the result is positive for IgG, IgM antibody levels may be measured. IgM levels rise within the first week and become undetectable after 6–9 months. Elevated levels of antibodies alone should not be considered as an evidence of recent infection, nor should low serum IgG levels be considered as inactive disease. If the laboratory testing is unequivocal, serological tests should be repeated in 15–21 days (46).

Asymptomatic patients with IgG reactivity alone may have latent infection with a history of primary exposure. This serological pattern is most important for immunosuppressed patients, including HIV infection and transplant recipients, and defines the risk for reactivation of the disease (12). In patients with reactivation disease, IgM and IgG responses may not be seen. In immunocompromised patients with seronegativity but strong clinical evidence, further tests to exclude *Toxoplasma* infection should be performed. These include IgG antibody testing or *T. gondii* PCR of thevitreous and aqueous humor. Serology is also used to assess the risk of transplacental transmission. IgG serology is performed in women considering pregnancy routinely in countries with endemic toxoplasmosis (48) . Elevated levels of IgG before pregnancy in immunocompetent women indicate a low risk for transplacental transmission. Although it is classically known that only during acute infection the mother could transmit the infection to the foetus, there are a few reports supporting the possibility that chronically infected women may be transmitting the disease congenitally (43). Those with undetectable IgG levels are advised to avoid undercooked meat consumption or cat feces. Negative IgM serology excludes infection in the last 6 months; if positive, it may persist up to 2 years after exposure to T. gondii . The IgG avidity test provides information about the time of exposure if IgG and IgM serologic tests are reactive. An IgG avidity test resulting in high-avidity IgG antibodies in the sera of first trimester embryos indicates that the infection was acquired before conception, because high-avidity IgG antibodies take 3–4 months to appear (49). Low-avidity IgG antibodies could not confirm the diagnosis of recent infection, due to their persistence for many months afte rthe acute infection (12,48). Detection of Toxoplasma -specific antibodies or DNA of the parasite in ocular specimens is the main basis for diagnosis (13). Intraocular antibody production is established by the Goldmann-Witmer coefficient (GWC), which compares the Toxoplasma -specific antibodies in ocular fluids and in serum (50). Although a ratio >1 should indicate intraocular antibody production, this may also occur in healthy controls, and therefore a ratio of at least 3 is rather used to confirm diagnosis (51).The contribution of PCR to the diagnosis is more controversial. In immunocompetent patients with clinical diagnosis of ocular toxoplasmosis, DNA of T. gondii could be amplified by PCR techniques only in 30– 40% cases (52, 53). However, in immunocompromised individuals, T. gondii DNA was amplified in 75% of the clinically diagnosed patients (53). Montoya et al. (54) reported that the diagnostic value of PCR in

intraocular specimens for T. gondii chorioretinitis was 67%. The sensitivity of PCR in patients meeting clinical diagnostic criteria for toxoplasmic chorioretinitis was lower in other studies, ranging from 27 to 36% (51, 54, 55) . Despite low sensitivity, the specificity of PCR is 100% (56) . The sensitivity of PCR also depends on the immune status of the patient. When the clinical symptoms first manifest in immunocompetent patients, the intraocular inflammatory response reduces the parasitic burden in the aqueous humor and vitreous, thus decreasing the amount of target DNA for PCR amplification. To improve the sensitivity of PCR, Sugita et al. (57) established a 2-step PCR protocol as a novel PCR technique for the diagnosis of ocular toxoplasmosis. In the first step, this technique uses a qualitative multiplex PCR approach to detect the Toxoplasma genome in the ocular sample. In the second step, quantitative real-time PCR is used to measure the genomic DNA of T. gondii. By using this 2-step PCR method, it was possible to detect an exceedingly small amount of nucleic acid in small amounts of an ocular sample with a sensitivity of 85%.

TREATMENT AND MANAGEMENT

There is no treatment for inactive toxoplasmosis. In immunocompetent patients, Toxoplasma - related chorioretinitis is usually a self-limited infection and generally resolves spontaneously in a period of 4–8 weeks (58). However, the highly variable severity of ocular toxoplasmosis raises significant issues regarding appropriate therapeutic strategies, mand even the need for any treatment at all, in this self-limiting disease (59). Traditionally, antibiotics and corticosteroids have been the mainstay of pharmacologic therapy against T. gondii. Treatment is given to reduce the risk of permanent visual impairment (aiming to reduce the size of the retinochoroidal scar), the risk of recurrence, and the severity and duration of acute symptoms. Antibiotics are usually given for 6 to 8 weeks. Steroids are also sometimes used to decrease the severity of intraocular inflammation symptoms (60). The aim of the treatment of gestational toxoplasmosis is to prevent fetal infection (61). Antibiotics used for the treatment have included trimethoprim-sulfamethoxazole, pyrimethamine, sulfadoxine, sulfadiazine, clindamycin, tetracyclines, clarithromycin, azithromycin, atovaquone, minocycline, spiramycin, rifabutin, trimetrexate, lincomycin, dapsone, sulfafurazole, ciprofloxacin, doxycycline, miokamycin, erythromycin, macrolide, sulfonamide, sulfamerazine, nifurtimox, methotrexate, alone or in combination (60, 62–65). The most frequent chemotherapeutic regimen for ocular toxoplasmosis consists of pyrimethamine and sulfadiazine, plus corticosteroids. Trimethoprim/sulfamethoxazole plus oral prednisolone is an alternative treatment option. This treatment was shown recently to have similar efficacy to classical therapy in a randomized clinical trial (66, 67). Other treatment option is intravitreal clindamycin injection and dexamethasone which is a promising approach (67-69). Intravitreal drug administration bypasses ocular barriers, and thereby delivering a high drug concentration directly to the intraocular tissues, avoiding systemic exposure and its risk of complications. Clindamycin 1.5 mg, given intravitreally, was non-toxic to the retina and had a half-life of 5.6 days. Following 1 mg intravitreal clindamycin injection, its concentration remained≥1.6 μg/ml during about 40 hr, which was higher than the 50% inhibitory concentration for T. gondii (67, 70).

However, the potential toxicity of, or intolerance to, many drug combinations has prompted research for alternative treatment regimens with better adverse events profiles. Azithromycin is an acid-stable, orally administered macrolide antibiotic, structurally related to erythromycin, with a similar antimicrobial spectrum (71). In vitro and in vivo efficacy of azithromycin against T. gondii has been demonstrated in several animal models, as well as for the treatment of T. gondii encephalitis in patients with AIDS (72, 73). The efficacy of azithromycin alone (74) or in combination with pyrimethamine (75) and trimethoprim/ sulfamethoxazole (76) in the management of active toxoplasmic retinochoroiditis has been demonstrated in previous studies. Aprospective randomized clinical trial compared the effects of two treatment regimens, pyrimethamine and azithromycin versus pyrimethamine and sulfadiazine, for the treatment of sight-threatening (near optic disk or fovea) ocular toxoplasmosis. The efficacy of the multidrug regimen

with pyrimethamine and azithromycin was similar to the standard treatment with pyrimethamine and sulfadiazine. The frequency and severity of adverse effects were significantly lower in patients receiving pyrimethamine and azithromycin. This data supports multidrug therapy with the combination of pyrimethamine and azithromycin as an acceptable alternative for the treatment of sight-threatening ocular toxoplasmosis (77). As in other ocular infections, the host immune response promotes intraocular inflammation against tachyzoites within the retina. The role of corticosteroids is to suppress the accompanying inflammation and minimize chorioretinal damage. The timing of initiation and the appropriate dose of corticosteroids are important to balance the suppression of the immune response to the parasite while minimizing the disease severity (78). Corticosteroid therapy without antiparasitics may lead to large retinal lesions even in immunocompetent patients (79, 80). The baseline indications for the use of corticosteroids include severe vitreous inflammation, decreased vision, proximity of lesions to the fovea or optic disk and the large size of the active lesion (65). The preferred oral corticosteroid drug is prednisone. In a survey by Holland and Lewis (65), 17% of physicians reported using oral corticosteroids for all immunocompetent patients with ocular toxoplasmosis regardless of clinical findings. Corticosteroids were started simultaneously with antiparasitic drugs by 36% of the respondents in this survey, while 64% deferred the start of corticosteroid therapy 1–7 days after starting antiparasitic therapy, with the majority waiting steroid initiation. Corticosteroid therapy is contraindicated in immunocompromised patients lacking the normal inflammatory response to the parasite (80). Most ophthalmologists prefer topical steroids for ocular toxoplasmosis patients. The main indications for use of topical corticosteroids are ocular pain, redness, photophobia, moderate to severe anterior chamber inflammation, and elevated intraocular pressure (21, 65). The treatment of ocular toxoplasmosis during pregnancy requires special consideration due to the potential adverse effects of antiparasitic agents on the fetus (48). If the mother acquires Toxoplasma infection during or immediately prior to pregnancy, there is a significant risk of placental transmission, and the risk increases with gestational age. Dunn et al. (81) reported an overall vertical transmission rate of 29%. The risk of transmission in early pregnancy was low, 6% at 13 weeks of gestation, and increased significantly in the second and third trimesters of pregnancy reaching 72% at 36 weeks. Although the transmission rate is low, toxoplasmosis severity and morbidity are much higher in infants acquiring the infection in the early gestational period (81). If a pregnant woman becomes infected up to 18 weeks into the pregnancy or within the 6 months prior to conception, treatment with the macrolide antibiotic spiramycin is recommended. Spiramycin does not readily cross the placenta, and there is no evidence for spiramycin teratogenicity. Alternatively, local intervention with intravitreal clindamycin and dexamethasone could be considered to prevent the possible teratogenic effects of systemic pyrimethamine and sulfadiazine. If the maternal infection is acquired 18 weeks or later after conception, treatment with pyrimethamine, sulfadiazine and folinic acid is advised (48).

CONCLUSIONS

Ocular toxoplasmosis is mainly acquired postnatally. Although food is considered as the main source of infection, contaminated water should also be considered as a mechanism of acquisition of the disease. Diagnosis is based on the clinical picture and, in particular, on the presence of retinochorioiditis accompanied by inflammation of the vitreous body . Old retinochoroidal scars may be also observed. Laboratory confirmation relies on the analysis of serum or intraocular samples for antibody detection, but PCR is becoming more widely available for direct identification of the parasite DNA in the eye, and sensitivity of PCR is improving with new methods of detection. The combination of pyrimethamine, sulfadiazine, and corticosteroid provides an effective therapeutic approach. Alternative regimens may include treatment with trimethoprim- sulfamethoxazole alone, intravitreal injection of clindamycin with dexamethasone, or combination of azithromycin with pyrimethamine. All have shown efficacy in

the therapy of ocular toxoplasmosis. The use of the trimethoprim-sulfamethoxazole combination is now preferred by many due to better patient compliance, faster resolution of chorioretinitis, and improved visual acuity. Trimethoprim-sulfamethoxazole is recomended for long-term prophylaxis in patients with frequent relapses. The intravitreal injections of clindamycin and dexamethasone as local agents in the vitreous cavity and retina permit avoiding most systemic side effects.The combination of pyrimethamine and azithromycin has been reported to have very good therapeutic efficiency and fewer side effects in comparison with the combination of pyrimethamine and sulfadiazine. Continuous progress in improving diagnosis and treatment is very important to minimize the vision loss from ocular toxoplasmosis. An in-depth clinical and pathophysiological knowledge of the disease can lead to more effective approaches for its prevention and treatment. In case of surgical treatment, the application of antibiotics for prevention or treatment of the complications should be considered..

In summary, the time of toxoplasma infection leading to ocular disease is rarely known. However, current evidence suggests that many more people are affected by postnatal than by prenatal toxoplasmosis. This has major public health implications. Considerable expertise and expenses are concentrated on screening and health information to reduce the risks of toxoplasmosis due to prenatally acquired infection, principally to reduce the risks of ocular morbidity in the long term. Primary preventive strategies should include children and adults at risk of ocular disease as a result of postnatal infection and should not be confined only to the pregnant women.

REFERENCES

1. Holland GN. Ocular toxoplasmosis: a global reassessment. Part I: Epidemiology and course of disease. Am J Ophthalmol 2003; 136: 973-988.
2. Subauste CS, Ajzenberg D, Kijlstra A. Review of the series 'Disease of the year 2011: toxoplasmosis' pathophysiology of toxoplasmosis. Ocul Immunol Inflamm 2011; 19: 297-306.
3. Tenter AM, Heckeroth AR, Weiss LM. Toxoplasma gondii: from animals to humans. Int J Parasitol 2000; 30: 1217-1258.
4. McCannel CA, Holland GN, Helm CJ, et al: Causes of uveitis in the general practice of ophthalmology. UCLA Community-Based Uveitis Study Group. Am J Ophthalmol 1996; 121: 35–46.
5. Balasundaram MB, Andavar R, Palaniswamy M, Venkatapathy N: Outbreak of acquired ocular toxoplasmosis involving 248 patients. Arch Ophthalmol 2010; 128: 28–32.
6. Perkins ES: Ocular toxoplasmosis. Br J Ophthalmol 1973; 57: 1–17.
7. Atmaca LS, Simsek T, Batioglu F: Clinical features and prognosis in ocular toxoplasmosis. Jpn J Ophthalmol 2004; 48: 386–391.
8. Bosch-Driessen LE, Berendschot TT, Ongkosuwito JV, Rothova A: Ocular toxoplasmosis: clinical features and prognosis of 154 patients. Ophthalmology 2002; 109: 869–878.
9. Kim SJ, Scott IU, Brown GC, et al: Interventions or toxoplasma retinochoroiditis: a report by the American Academy of Ophthalmology. Ophthalmology 2013; 120: 371–378.
10. Harrell M, Carvounis PE: Current treatment of toxoplasma retinochoroiditis: an evidencebased review. J Ophthalmol 2014; 2014: 273506.
11. Montoya JG, Liesenfeld O: Toxoplasmosis. Lancet 2004; 363: 1965–1976.
12. Cem Ozgonul, Cagri Giray Besirli: Recent Developments in the Diagnosis and Treatment of Ocular Toxoplasmosis. Review. Ophthalmic Res 2017;57:1–12, DOI: 10.1159/000449169.
13. Maenz M, Schluter D, Liesenfeld O, et al: Ocular toxoplasmosis past, present and new aspects of an old disease. Prog Retin Eye Res 2014; 39: 77–106.
14. Dubey JP, Jones JL: Toxoplasma gondii infection in humans and animals in the United States. Int J Parasitol 2008; 38: 1257–1278.
15. Robert-Gangneux F, Dardé ML: Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev 2012; 25: 264–296.
16. Howe DK, Sibley DL 1995. Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis 172: 1561-1566.
17. Belfort-Neto R, Nussenblatt V, Rizzo L, Muccioli C, Silveira C, Nussenblatt R, Khan A, Sibley LD, Belfort R Jr 2007. High prevalence of unusual genotypes of Toxoplasma gondii infection in pork meat samples from Erechim, Southern Brazil. An Acad Bras Cienc 79: 111-114.
18. Nowakowska D, Colón I, Remington JS, Grigg M, Golab E, Wilczynski J, Sibley LD 2006. Genotyping of Toxoplasma gondii by multiplex PCR and peptide-based serological testing of samples from infants in Poland diagnosed with congenital toxoplasmosis. J Clin Microbiol 44: 1382-1389.
19. Howe DK, Honoré S, Derouin F, Sibley LD 1997. Determination of genotypes of Toxoplasma gondii strains isolated from patients with toxoplasmosis. J Clin Microbiol 35: 1411-1414.
20. Holland GN: Ocular toxoplasmosis: the influence of patient age. Mem Inst Oswaldo Cruz 2009; 104: 351–357.
21. Dodds EM, Holland GN, Stanford MR, et al. Intraocular inflammation associated with ocular toxoplasmosis: relationships at initial examination. Am J Ophthalmol 2008; 146: 856 865. e2.
22. Nguyen AM, Seve P, Le Scanff J, et al: Clinical and etiological aspects of uveitis: a retrospective study of 121 patients referred to a tertiary centre of ophthalmology (in French). Rev Méd Interne 2011; 32: 9–16.
23. Jakob E, Reuland MS, Mackensen F, et al: Uveitis subtypes in a German interdisciplinary uveitis center – analysis of 1,916 patients. J Rheumatol 2009; 36: 127–136.
24. Furtado JM, Winthrop KL, Butler NJ, Smith JR: Ocular toxoplasmosis. I. Parasitology, epidemiology and public health. Clin Exp Ophthalmol 2013; 41: 82–94.
25. Guerina NG, Hsu HW, Meissner HC, et al: Neonatal serologic screening and early treatment for congenital Toxoplasma gondii infection. The New England Regional Toxoplasma Working Group. N Engl J Med 1994; 330: 1858–1863.
26. Webster JP, Lamberton PH, Donnelly CA, Torrey EF: Parasites as causative agents of human affective disorders? The impact of antipsychotic, mood-stabilizer and anti-parasite medication on Toxoplasma gondii 's ability to alter host behaviour. Proc Biol Sci 2006; 273: 1023–1030.
27. Vasconcelos-Santos DV, Machado Azevedo DO, Campos WR, et

al: Congenital toxoplasmosis in southeastern Brazil: results of early ophthalmologic examination of a large cohort of neonates. Ophthalmology 2009; 116: 2199–2205. e1.
28. Safadi MA, Berezin EN, Farhat CK, Carvalho ES: Clinical presentation and follow-up ofchildren with congenital toxoplasmosis in Brazil. Braz J Infect Dis 2003; 7: 325–331.
29. Berrebi A, Assouline C, Bessieres MH, et al: Long-term outcome of children with congenital toxoplasmosis. Am J Obstet Gynecol 2010; 203: 552. e1–6.
30. Barbara A. Butcher, Barbara A. Fox, Leah M. Rommereim, Sung Guk Kim, Kirk J. Maurer, Felix Yarovinsky, De'Broski R. Herbert, David J. Bzik, Eric Y. Denkers. Toxoplasma gondii Rhoptry Kinase ROP16 Activates STAT3 and STAT6 Resulting in Cytokine Inhibition and Arginase-1-Dependent Growth Control. PLoS Pathog, 2011 Sep;7(9):e1002236. doi: 10.1371/journal.ppat.1002236.
31. Shimada K, O'Connor GR, Yoneda C. Cyst formation by Toxoplasma gondii (RH strain) in vitro. The role of immunologic mechanisms. Arch Ophthalmol 1974; 92: 496-500.
32. Brézin AP, Kasner L, Thulliez P, Li Q, Daffos F, Nussenblatt RB, Chan CC. Ocular toxoplasmosis in the fetus. Immunohistochemistry analysis and DNA amplification. Retina 1993; 14: 19-26.
33. Ryan SJ. Retina. 3rd ed. St. Louis, USA, Mosby. 2001, p 1531-1544.
34. Dodds EM: Toxoplasmosis. Curr Opin Ophthalmol 2006; 17: 557–561.
35. Mets MB, Holfels E, Boyer KM, et al: Eye manifestations of congenital toxoplasmosis. Am J Ophthalmol 1996; 122: 309–324.
36. Delair E, Latkany P, Noble AG, et al: Clinical manifestations of ocular toxoplasmosis. Ocul Immunol Inflamm 2011; 19: 91–102.
37. Park YH, Nam HW: Clinical features and treatment of ocular toxoplasmosis. Korean J Parasitol 2013; 51: 393–399.
38. Wakefield D, Cunningham ET Jr, Pavesio C, et al: Controversies in ocular toxoplasmosis. Ocul Immunol Inflamm 2011; 19: 2–9.
39. Alwassia AA, Cho H, Adhi M, et al: Sequential optical coherence tomography images of retinal necrosis in acute ocular toxoplasmosis. Retin Cases Brief Rep 2013; 7: 98–101.
40. Goldenberg D, Goldstein M, Loewenstein A, Habot-Wilner Z: Vitreal, retinal, and choroidal findings in active and scarred toxoplasmosis lesions: a prospective study by spectral-domain optical coherence tomography. Graefes Arch Clin Exp Ophthalmol 2013; 251: 2037–2045.
41. Oréfice JL, Costa RA, Scott IU, et al: Spectral optical coherence tomography findings in patients with ocular toxoplasmosis and active satellite lesions (MINAS Report 1). Acta Ophthalmol 2013; 91:e41–e47.
42. Noble A. Cataracts in congenital toxoplasmosis. J AAPOS 2007; 11:551-554.
43. Alessandra G Commodaro, Rubens N Belfort, Luiz Vicente Rizzo, Cristina Muccioli, Claudio Silveira, Miguel N Burnier Jr, Rubens Belfort Jr. Ocular toxoplasmosis - an update and review of the literature. Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 104(2): 345-350, March 2009.
44. Harper TW, Miller D, Schiffman JC, Davis JL: Polymerase chain reaction analysis of aqueous and vitreous specimens in the diagnosis of posterior segment infectious uveitis. Am J Ophthalmol 2009; 147: 140–147.
45. Rothova A, de Boer JH, Ten Dam-van Loon NH, et al: Usefulness of aqueous humor analysis for the diagnosis of posterior uveitis. Ophthalmology 2008; 115: 306–31.
46. Suzuki LA, Rocha RJ, Rossi CL: Evaluation of serological markers for the immunodiagnosis of acute acquired toxoplasmosis. J Med Microbiol 2001; 50: 62–70.
47. Marcolino PT, Silva DA, Leser PG, et al: Molecular markers in acute and chronic phases of human toxoplasmosis: determination of immunoglobulin G avidity by Western blotting. Clin Diagn Lab Immunol 2000; 7: 384–389.
48. Montoya JG, Remington JS: Management of Toxoplasma gondii infection during pregnancy. Clin Infect Dis 2008; 47: 554–566.
49. Montoya JG, Huffman HB, Remington JS: Evaluation of the immunoglobulin G avidity test for diagnosis of toxoplasmic lymphadenopathy. J Clin Microbiol 2004; 42: 4627– 463.
50. Goldmann H, Witmer R: Antibodies in the aqueous humor (in German). Ophthalmologica 1954; 127: 323–330.
51. De Groot-Mijnes JD, Rothova A, Van Loon AM, et al: Polymerase chain reaction and Goldmann-Witmer coefficient analysis are complimentary for the diagnosis of infectious uveitis. Am J Ophthalmol 2006; 141: 313–318.
52. Fekkar A, Bodaghi B, Touafek F, et al: Comparison of immunoblotting, calculation of the Goldmann-Witmer coefficient, and real-time PCR using aqueous humor samples for diagnosis of ocular toxoplasmosis. J Clin Microbiol 2008; 46: 1965–1967.
53. Garweg JG, de Groot-Mijnes JD, Montoya JG: Diagnostic approach to ocular toxoplasmosis. Ocul Immunol Inflamm 2011; 19: 255–261.
54. Montoya JG, Parmley S, Liesenfeld O, et al: Use of the polymerase chain reaction for diagnosis of ocular toxoplasmosis. Ophthalmology 1999; 106: 1554–1563.
55. Fardeau C, Romand S, Rao NA, et al: Diagnosis of toxoplasmic retinochoroiditis with atypical clinical features. Am J Ophthalmol 2002; 134: 196–203.
56. Villard O, Filisetti D, Roch-Deries F, et al: Comparison of enzyme-linked immunosorbent assay, immunoblotting, and PCR for diagnosis of toxoplasmic chorioretinitis. J Clin Microbiol 2003; 41: 3537–3541.
57. Sugita S, Ogawa M, Inoue S, et al: Diagnosis of ocular toxoplasmosis by two polymerase chain reaction (PCR) examinations: qualitative multiplex and quantitative real-time. Jpn J Ophthalmol 2011; 55: 495–501.
58. Butler NJ, Furtado JM, Winthrop KL, Smith JR: Ocular toxoplasmosis. II. Clinical features, pathology and management. Clin Exp Ophthalmol 2013; 41: 95–108.
59. de-la-Torre A, Stanford M, Curi A et al: Therapy for ocular toxoplasmosis. Ocul Immunol Inflamm. 2011, 19(5): 314–20.
60. Pradhan E, Bhandari S, Gilbert RE, Stanford M. Antibiotics versus no treatment for toxoplasma retinochoroiditis. Cochrane Database Syst Rev. 2016;5:CD002218.
61. Rajapakse S, Chrishan Shivanthan M, Samaranayake N, Rodrigo C, Deepika Fernando S. Antibiotics for human toxoplasmosis: a systematic review of randomized trials. Pathog Glob Health. 2013;107(4):162–9.
62. Wakefield D, Cunningham ET, Pavesio C, Garweg JG, Zierhut M. Controversies in ocular toxoplasmosis. Ocul Immunol Inflamm. 2011;19(1):2– 9.
63. Engstrom RE, Holland GN, Nussenblatt RB, Jabs DA. Current practices in the management of ocular toxoplasmosis. Am J Ophthalmol. 1991;111:601–10.
64. Gilbert RE, Harden M, Stanford M. Antibiotics versus control for toxoplasma retinochoroiditis. Cochrane Database Syst Rev. 2002(1). Art. No.: CD002218.
65. Holland GN, Lewis K. An update on current practices in the management of ocular toxoplasmosis. Am J Ophthalmol. 2002;134(1):102–14.
66. Holland GN. Prospective, randomized trial of trimethoprim/sulfamethoxazole vs. pyrimethamine and sulfadiazine in the treatment of ocular toxoplasmosis: discussion. Ophthalmology 2005; 112: 1882-1884.
67. Soheilian M, Sadoughi MM, Ghajarnia M, Dehghan MH, Yazdani S, Behboudi H, Anisian A, Peyman G A. Prospective randomized trial of trimethoprim/sulfamethoxazole versus pyrimethamine and sulfadiazine in the treatment of ocular toxoplasmosis. Ophthalmology 2005; 112: 1876-1882.
68. Soheilian M, Ramezani A, Azimzadeh A, Sadoughi MM, Dehghan MH, Shahghadami R, Yaseri M, Peyman GA. Randomized trial of intravitreal clindamycin and dexamethasone versus pyrimethamine, sulfadiazine, and prednisolone in treatment of ocular toxoplasmosis. Ophthalmology 2011; 118: 134-141.

69. Kishore K, Conway MD, Peyman G A. Intravitreal clindamycin and dexamethasone for toxoplasmic retinochoroiditis. Ophthalmic Surg Lasers 2001; 32: 183-192.
70. Fichera ME, Bhopale MK, Roos DS. In vitro assays elucidate peculiar kinetics of clindamycin action against Toxoplasma gondii. Antimicrob Agents Chemother 1995; 39: 1530-1537.
71. Peters DH, Friedel HA, McTavish D: Azithromycin. A review of its antimicrobial activity, pharmacokinetic properties and clinical efficacy. Drugs, 1992; 44: 750–99.
72. Konstantinos Balaskas, Jean Vaudaux, Noémie Boillat-Blanco, Yan Guex-Crosier. Azithromycin versus Sulfadiazine and Pyrimethamine for non-vision-threatening toxoplasmic retinochoroiditis: A pilot study. Med Sci Monit, 2012; 18(5): CR296-302.
73. Alireza Lashay, Ahmad Mirshahi, Najaf Parandin, Hamid Riazi Esfahani, Mehdi Mazloumi, Mohammad Reza Lashay, Mohammad Karim Johari, Elham Ashrafi. A prospective randomized trial of azithromycin versus trimethoprim/ sulfamethoxazole in treatment of toxoplasmic retinochoroiditis. Journal of Current Ophthalmology 29 (2017) 120-125.
74. Rothova A, Bosch-Driessen LE, van Loon NH, Treffers WF: Azithromycin for ocular toxoplasmosis. Br J Ophthalmol, 1998; 82: 1306–8
75. Bosch-Driessen LH, Verbraak FD, Suttorp-Schulten MS et al: A prospective, randomized trial of pyrimethamine and azithromycin vs pyrimethamine and sulfadiazine for the treatment of ocular toxoplasmosis. Am J Ophthalmol, 2002; 134: 34–40
76. Yazici A, Ozdal PC, Taskintuna I et al: Trimethoprim/Sulfamethoxazole and azithromycin combination therapy for ocular toxoplasmosis. Ocul Immunol Inflamm, 2009; 17: 289–91.
77. Bosch-Driessen LH, Verbraak FD, Suttorp- Schulten MS, et al: A prospective, randomized trial of pyrimethamine and azithromycin vs pyrimethamine and sulfadiazine for the treatment of ocular toxoplasmosis. Am J Ophthalmol 2002; 134: 34–40.
78. Oray M, Ozdal PC, Cebeci Z, et al: Fulminant ocular toxoplasmosis: the hazards of corticosteroid monotherapy. Ocul Immunol Inflamm 2015, Epub ahead of print.
79. Garweg JG, Stanford MR: Therapy for ocular toxoplasmosis – the future. Ocul Immunol Inflamm 2013; 21: 300–305.
80. Holland GN, Engstrom RE Jr, Glasgow BJ, et al: Ocular toxoplasmosis in patients with the acquired immunodeficiency syndrome. Am J Ophthalmol 1988; 106: 653–667.
81. Dunn D, Wallon M, Peyron F, et al: Motherto- child transmission of toxoplasmosis: risk estimates for clinical counselling. Lancet 1999; 353: 1829–1833.

CONFLICT OF INTEREST STATEMENT (AUTHORS)

I certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Author name Date Signature

When there is conflict of interest, specify the company title and the relationship with the Author.

CONFLICT OF INTEREST STATEMENT (REVIEWERS)

I certify that have no personal or financial conflict of interest with authors of the manuscript provided me for review.

Reviewer name Date Signature

When there is conflict of interest, please specify the relationship with the Author.

STATEMENT ABOUT PROTECTION OF HUMAN SUBJECTS
AND ANIMALS IN RESEARCH

I certify that this study involving human subjects is in accordance with the Helsinky declaration of 1975 as revised in 2000 and that it has been approved by the relevant institutional Ethical Committee.

Author name Date Signature

I certify that this study involving animals followed the institutional and national guide for the care and use of laboratory animals.

Author name Date Signature

