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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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ACKNOWLEDGEMENTS

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

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MODERN METHODS IN THE RAPID DIAGNOSIS OF CHOLERA

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ABSTRACT

Cholera is an epidemic life threatening disease caused by *V. cholerae*. The main symptoms of this acute disease are vomiting, profuse watery diarrhea, and severe dehydration. The infection is transmitted by fecal-oral mechanism, food consumption and *V. cholerae* contaminated water (10,15). Often outbreaks are related to wars or natural disasters like hurricanes and floods when water and food sources become contaminated with *V. cholerae* in areas with bad living conditions and poor sanitation. Although cholera is a rare disease eradicated in developed countries it is still common in some parts of the world like Latin America, Africa, India and Asia. Its potential to cause large outbreaks and pandemics with lethal exit creates the need of a better epidemic prevention and an early response (25, 27). In the last decades the cholera is not common disease in our country but the increasing number of refugees from the Middle East and Africa makes it potential threat for Bulgarian population and requests the health authorities to react in case of possible outbreak.

Key words: *Vibrio cholerae,* cholera, molecular methods, diagnosis

V. cholerae is a gram negative aerobic or facultative anaerobic pathogen, with comma shaped mobile monotrichous bacterial cell and more than 200 identified serogroups of which only strains of serogroups O1 and O139 are associated with epidemic cholera. Other serogroups can cause mild diarrhea and cholera-like form of disease which is less severe. Cholera-causing strains produce two main virulence factors: cholera toxin (encoded by bacteriophage CTXf) and so-called "toxin co-regulated pili" – appendages which mediate adhesion to the intestine (and which are encoded by a chromosomal pathogenicity island) (18). Strains of *V. cholerae* which do not produce cholera toxin may produce the Ace and Zot toxins (14). Such strains may give rise to a less severe form of disease.

V. cholerae O1 exists as two biotypes - classical and El Tor. Two of seven investigated cholera pandemics are caused by classical biotype and the last one - by El Tor biotype. Both serogroups O1 and O139 cause clinical disease by producing of enterotoxin (22).

The strains of *V. cholerae* which cause a disease are carrying the gene ctxAB (cholera toxin subunits A and B), the vibrio pathogenicity island (VPI), and other cholera-associated genes (3) (15, 40). Until 1992, only serogroup O1 (out of more than 206 serogroups currently described) was recognized as a cause of cholera. In 1992 a new non-O1 *V.cholerae* strain (subsequently designated *V. cholerae* O139) is found in India and rapidly spread across many countries in Asia(6). Extensive studies demonstrated that the O139 strain was closely related to the O1 El Tor strains of the 7th pandemic, except that the genes responsible for O1-antigen biosynthesis were deleted and replaced with DNA that encodes the O139 antigen. Since the O-antigen is the major protective epitope for the bacterium in the human gut its alteration was sufficient to allow O139 strains to move in epidemic form through populations previously immune to cholera caused by O1. Thus, adults were more commonly affected than children (1,17). Recent studies suggest that the pandemic cluster carries other serogroups as well, including O37, O27, O53, O65, and O75 (28). These findings are corresponding to the hypothesis that pathogenic *V. cholerae* strains are able to acquire easy and/or exchange O-antigen genes, with the new O-antigen allowing strains to avoid preexisting immunity to cholera.

The sampling for the diagnosis of *V. cholerae* is taking place in hospitals and hospices before the start of the treatment by a qualified medical stuff. Feces and vomited materials are used for patients with gastroenteritis. Tubule or rectal tampon is used to take a sample from the rectum of non-gasteroenteritic patient. It is dissolved in alkaline peptone water. Intestinal content and bile are taken from a corpse. Samples from the environment such as water, food, soil etc. can be sampled for *V. cholerae* too (9).

Fast and express methods are developed for the purposes of the diagnosis of the cholera. The express methods don't require culturing and they are completed in 6-8 hours. The fast methods require culturing and all tests are performed on the cultured bacteria, not directly on the clinical sample. The last ones require 16-18 hours.

The methods of diagnosis are subdivided in classical, express and rapid. First preliminary response is given in 6-8 hours and second preliminary response in 16-18 hours. Final response is given in 36-48 hours. Additional classical methods are serology and phage typing (12). Blood is used for serological reactions and stool samples for phage typing.

MICROSCOPE SAMPLE PREPARATION. A smear is prepared from the sample. It is dried and fixed with ethanol or ethanol and ether in ratio 1:1 than stained with fuchsine diluted 1:10. Gram staining is used too. Under a microscope one can see vibrios in parallel rows looking like fish flock. Rods, comma-shaped, S-shaped, helical, filamentary, yeast-like, coccobacteria, etc. may be observed along with the vibrios.

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Immunofluorescence microscopy is technique of observation of bacteria stained with specific immunologically bound fluorescent antibodies. It belongs to the so-called express methods (16, 29).

CULTURING. Direct inoculation primary material is completed independently of the result of the microscope observation during the first days of the investigation only.

Alkaline peptone water is used for inoculation in enrichment medium. Inoculation on solid media is done from non-inhibiting (alkaline agar) and inhibiting (TCBS) medium. *V. cholerae* produces medium colonies, circular, flat and transparent on alkaline agar.

Agglutination of suspected strains is applied with O-cholera 1:100 diluted serum. Positive agglutinated samples have to be further investigated.

Oxidase test. 1-2 drops of 1% water solution of paraphenylene diamine is put on the dish. In 3 minutes oxidase positive colonies are colored brown to dark brown. It is perfect discriminative method for recognition of *V. cholerae* from the members of *Enterobacteriaceae*.

MOBILITY. *V. cholerae* is one of the fastest moving bacteria. For its observation in a hanging drop or a sample with coverslip a dark field or phase contrast microscope is used. Moving vibrios are like “falling stars”.

ANTIGEN STRUCTURE INVESTIGATION. The main compounds of the O-antigens are A, B, C, D and E. Different of these 5 antigens are specific for different strains of *V. cholerae*. For example A-antigen is group-specific. B- and C-antigens are type-specific. A combination of sera against antigens gives important information about the strains and the groups of the cholera vibrios (19).

PHAGE TYPING. The phage typing is a method introduced by S. Basu and S. Mukerjee used for routine identification of *V. cholerae*. They developed a phage typing protocol for *V. cholerae* O1 biotype El Tor for the study of the spread of the E1 Tor biotype of *V. cholerae* O1. This is one of the first methods used in the typing of *V. cholerae* strains. Phage typing is an identification method for testing of specific fagovars. Some of the phages that are infecting bacterial strains are very specific. Others can infect strains of two or more species of the some genus. Difference in lytic susceptibility discriminates different strains from a bacterial species. This phenomenon is on the basis of phage typing - a method for the detection and characterization of bacterial strains using their sensitivity or resistance to different known phages. The phage typing is in use in the epidemiology for the identification and spread determination of the infectious agent (7).

The typing of strains of *V. cholerae* is important for an exact diagnosis and subsequent treatment. **Molecular methods** for gene-typing of organisms including bacteria are widely used in last two decades and they have many advantages (8, 13, 20, 21, 23, 24, 26, 30, 31). Vital microorganisms are not needed and very small amounts of DNA is enough for application of amplification techniques such PCR (Polymerase Chain Reaction). The risk of infection of the laboratory stuff is overcome.

The classical identification methods include culturing, biochemical, morphological and physiological typing including antibiotic susceptibility are time and labor consuming. Serological and ELISA methods so-called express identification techniques are quicker and easier to be performed. Their main disadvantage is the relative low determination capability. The time needed for genetic diagnostics is 2-4 hours. The conventional culturing is taking place in 2-10 days. The rapid diagnosis of *V. cholerae* to strain and serogroup is critical for correct treatment of the patient and the saving of his/her live. Other essential advantage of the molecular methods is that they are easy to be digitized, standardized and software analyzed. Procedures of DNA isolation and further manipulations can be done by much smaller laboratory stuff. Most often used is PCR. Its classical version is amplification of a specific region of the genome up to hundreds or thousands base pairs. In this case oligonucleotides pairs of approximate 20 base pairs so-called primers are used for the application of region related to some gene present missing or different in the strains of the species. The products of amplification – amplicons are loaded on agarose gel stained with ethidium bromide. The most important target sites for PCR amplification are situated in so-called pathogenicity island.

Important variation of PCR is real time PCR. Amplification is software analyzed during the process of the amplification. The primers are labeled with fluorescent dye radiating light with specific wavelength during the process of amplification. In this case different pairs of primers pairs can be labeled with dyes. Counting of number of the bacterial cells is possible using this method. Many commercial kits are designed

The bacterial chromosomal replication origin (ori) sequences are a highly conserved essential genetic element (1). The large chromosomal replication origin sequence of *V. cholerae* (ori CIVC) is targeted for identification of the organism, including the biotypes of serogroup O1. The ori CIVC sequence-based PCR assay specifically amplified an 890 bp fragment from all the *V. cholerae* strains examined. A point mutation in the ori CIVC sequence of the classical biotype of O1 serogroup led to the loss of a Bgl II site which was utilized for differentiation from El Tor vibrions. The PCR assay amplifies a similarly sized ori segment, designated as oriCIVM, from *V. mimicus* strains but fails to produce any amplicon with other strains. Cloning and sequencing of the oriCIVM revealed high sequence similarity (96%) with oriCIVC. The results indicate that *V. mimicus* is indeed very closely related to *V. cholerae*. In addition, the Bgl II restriction fragment length polymorphism (RFLP) between oriCIVM and oriCIVC sequences allows discrimination of these two species. The ori sequence-based PCR-RFLP assay is a useful method for rapid identification and differentiation of *V. cholerae* and *V. mimicus* strains as well as for the delineation of classical and El Tor biotypes of *V. cholerae* O1.

A PCR that amplifies *V. cholerae* RTX (repeat in toxin)

toxin gene is developed (2). Among all clinical and environmental isolates of *V. cholerae* causing epidemics and sporadic cases of cholera in various parts of the world, all are found toxigenicity by both PCR and HEp-2 cell cytotoxicity assay. Standard strains of the classical biotype containing a deletion within the gene cluster exhibited negative results by both assays. This is the first rapid genotyping method for differentiation of *V. cholerae* O1 classical biotype strains from El Tor biotype strains as well as strains of other non-O1 serogroups including serogroup O139. This PCR assay is developed specifically for detection of RTX toxin genes in *V. cholerae*, and clinical isolates of *V. parahaemolyticus*, diarrheagenic *Escherichia coli*, *Aeromonas* species, and *Plesiomonas* species.

Norgen's *Vibrio cholerae* PCR Detection Kit is a ready-to-use system for the isolation and detection of *V. cholerae* without enrichment (4). First, the kit contains components for the rapid isolation of total DNA, including bacterial DNA, from stool samples using spin-column chromatography based on Norgen's proprietary resin (Nucleic Acid Isolation). Second, the kit contains *V. cholerae* Master Mix and controls to allow for PCR amplification, as well as a Control Master Mix to allow for amplification of both an Isolation Control and a PCR Control. The amplified PCR products are then detected using agarose gel electrophoresis. Alternatively, detection can be performed based on real-time PCR using melt curves (5).

V. cholerae Master Mix containing reagents and enzymes for the specific amplification of a 333 bp region of the *V. cholerae* genome is designed. Norgen's *V. cholerae* PCR Detection Kit contains a second Master Mix, the Control 2X PCR Master Mix, which can be used to identify possible PCR inhibition and/or inadequate isolation via a separate PCR with the use of the provided PCR Control (PCRC) or Isolation Control (IsoC). This kit is designed to allow for the testing of 24 samples.

References

1. Saha A, Deb R, Shah S, Ramamurthy T, Shinoda S, Mukhophadyay AK, Bhadra RK. PCR-based identification of Vibrio cholerae and the closely related species Vibrio mimicus using the large chromosomal ori sequence of Vibrio cholerae. FEMS Microbiol Lett. 2006; 257(1): 84-91.
2. Chow K. H., T. K. Ng, K. Y. Yuen, W. C. Yam. Detection of RTX Toxin Gene in Vibrio cholerae by PCR. J Clin Microbiol. 2001; 39(7): 2594–2597.
3. Lipp EK, IN. River, AI. Gil, EM. Espeland, N. Choopun, VR. Louis, E. Russek-Cohen, A. Huq, RR. Colwell. Direct detection of Vibrio cholerae and ctxA in Peruvian coastal water and plankton by PCR. Appl. Environ Microbiol. 2003; 69(6): 3676-80.
4. http://norgenbiotek.com/display-product.php?ID=433
5. Bielawska-Drozd, A., T. Mirski, M. Bartoszsce, P. Cieslik, A. Rozskowiak, A. Michalski. Development of real-time PCR assay for detection of Vibrio cholerae. Pol. J. Environ Stud 2012; 21(2): 279-288.
6. Tamura S, Taniguchi F, Nakamoto C, Nakamoto H, Arakawa E, Fukuchi T, Nakano Y, Fujimoto T. Fatal diarrheal disease caused by Vibrio cholerae O67 in a patient with myelodysplastic syndrome. Intern Med. 2013; 52(14): 1635-9.
7. Katz LS, Petkau A, Beaulaurier J, Tyler S, Antonova ES, Turnsek MA, Guo Y, Wang S, Paxinos EE, Orata F, Gladney LM, Stroika S, Folster JP, Rowe L, Freeman MM, Knox N, Frace M, Boncy J, Graham M, Hammer BK, Boucher Y, Bashir A, Hanage WP, Van Domselaar G, Tarr CL. Evolutionary dynamics of Vibrio cholerae O1 following a single-source introduction to Haiti. MBio. 2013; 4(4): 398-13.
8. Oyedeji KS, Niemogha MT, Nwaokorie FO, Bamidele TA, Ochoga M, Akinsinde KA, Brai BI, Oladele D, Omonigbehin EA, Bamidele M, Fesobi TW, Musa AZ, Adeneye AK, Smith SI, Ujah IA. Molecular characterization of the circulating strains of Vibrio cholerae during 2010 cholera outbreak in Nigeria. J Health Popul Nutr. 2013; 31(2): 178-84.
9. Sawasvirojwong S, Srimanote P, Chatsudthipong V, Muanprasat C. An adult mouse model of Vibrio cholerae-induced diarrhea for studying pathogenesis and potential therapy of cholera. PLoS Negl Trop Dis. 2013; 7(6): 2293.
10. Akoachere JF, Masalla TN, Njom HA. Multi-drug resistant toxigenic Vibrio cholerae O1 is persistent in water sources in New Bell-Douala, Cameroon. BMC Infect Dis. 2013; 13: 366.
11. Octavia S, Salim A, Kurniawan J, Lam C, Leung Q, Ahsan S, Reeves PR, Nair GB, Lan R. Population structure and evolution of non-O1/non-O139 Vibrio cholerae by multilocus sequence typing. PLoS One. 2013; 8(6):e65342.
12. Wang Q, Kan B, Wang R. Isolation and characterization of the new mosaic filamentous phage VFJ Φ of Vibrio cholerae. PLoS One. 2013 1;8(8):e70934.
13. Liang W, Wang L, Liang P, Zheng X, Zhou H, Zhang J, Zhang L, Kan B. Sequence polymorphisms of rfbT among the Vibrio cholerae O1 strains in the Ogawa and Inaba serotype shifts. BMC Microbiol. 2013; 13:173.
14. Anthouard R, DiRita VJ. Small-molecule inhibitors of toxT expression in Vibrio cholerae. MBio. 2013; 4(4). e00403-13.
15. Momtaz H, Dehkordi FS, Rahimi E, Asgarifar A. Detection of Escherichia coli, Salmonella species, and Vibrio cholerae in tap water and bottled drinking water in Isfahan, Iran. BMC Public Health. 2013; 13(1): 556.
16. Nato F, Boutonnier A, Rajerison M, Grosjean P, Dartevelle S, Guénolé A, Bhuiyan NA, Sack DA, Nair GB, Fournier JM, Chanteau S. One-step immunochromatographic dipstick tests for rapid detection of Vibrio cholerae O1 and O139 in stool samples. Clin Diagn Lab Immunol. 2003; 10(3):476-8.
17. Aydanian A, Tang L, Morris JG, Johnson JA, Stine OC. Genetic diversity of O-antigen biosynthesis regions in Vibrio cholerae. Appl Environ Microbiol. 2011; 77(7):2247-53.
18. Rajpara N, Vinothkumar K, Mohanty P, Singh AK, Singh R, Sinha R, Nag D, Koley H, Kushwaha Bhardwaj A. Synergistic Effect of Various Virulence Factors Leading to High Toxicity of Environmental V. cholerae Non-O1/ Non-O139 Isolates Lacking ctx Gene : Comparative Study with Clinical Strains. PLoS One. 2013;8(9): e76200.
19. Instruction for microbiological diagnostics of diseases caused by vibrios. in "Handbook for instructive materials for microbiological diagnostics of bacterial infections." Editors M. Stoyanova and G. Mitov. Medical Academy of Sofia, Center of Scientific Information about Medicine and Health Care. pp.103-136. Sofia 1990 (in Bulgarian).
20. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ. Comparative genomic analysis of Vibrio cholerae: genes that correlate with cholera endemic and pandemic disease. Proc Natl Acad Sci U S A. 2002; 99(3): 1556-61.
21. Stine OC, Sozhamannan S, Gou Q, Zheng S, Morris JG Jr, Johnson JA. Phylogeny of Vibrio cholerae based on recA sequence. Infect Immun. 2000; 68(12): 7180-5.
22. Nair GB, Ramamurthy T, Bhattacharya SK, Mukhopadhyay AK, Garg S, Bhattacharya MK, Takeda T, Shimada T, Takeda Y, Deb BC. Spread of Vibrio cholerae O139 Bengal in India. J Infect Dis. 1994; 169(5): 1029-34.
23. Bik EM, Bunschoten AE, Gouw RD, Mooi FR. Genesis of the novel epidemic Vibrio cholerae O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. EMBO J. 1995; 14(2): 209-16.
24. Comstock LE, Johnson JA, Michalski JM, Morris JG Jr, Kaper JB. Cloning and sequence of a region encoding a surface polysaccharide of Vibrio cholerae O139 and characterization of the insertion site in the chromosome of Vibrio cholerae O1. Mol Microbiol. 1996; 19(4): 815-26.
25. Mooi FR, Bik EM. The evolution of epidemic Vibrio cholerae strains. Trends Microbiol. 1997; 5(4): 161-5.
26. Stroeher UH, Jedani KE, Dredge BK, Morona R, Brown MH, Karageorgos LE, Albert MJ, Manning PA. Genetic rearrangements in the rfb regions of Vibrio cholerae O1 and O139. Proc Natl Acad Sci U S A. 1995; 92(22): 10374-8.
27. Albert MJ, Siddique AK, Islam MS, Faruque AS, Ansaruzzaman M, Faruque SM, Sack RB. Large outbreak of clinical cholera

due to Vibrio cholerae non-O1 in Bangladesh. Lancet. 1993; 341(8846): 704.
28. Tobin-D'Angelo M, Smith AR, Bulens SN, Thomas S, Hodel M, Izumiya H, Arakawa E, Morita M, Watanabe H, Marin C, Parsons MB, Greene K, Cooper K, Haydel D, Bopp C, Yu P, Mintz E. Severe diarrhea caused by cholera toxin-producing Vibrio cholerae serogroup O75 infections acquired in the southeastern United States. Clin Infect Dis. 2008; 47(8): 1035-40.
29. Nato, F., A. Boutonnier, M. Rajerison, P. Grosjean, S. Dartevelle, A. Guenole, N. A. Bhuiyan, D. A. Sack, G. B. Nair, J. M. Fournier, S. Chanteau One-Step Immunochromatographic Dipstick Tests for Rapid Detection of Vibrio cholerae O1 and O139 in Stool Samples. Clin Diagn Lab Immunol. 2003; 10(3): 476-8.
30. Velinov, T., Kantardjiev, T., Petrunov, B. PCR for demonstrating the toxicogenicity of *Vibrio cholerae* O 139 strain. Problems of Infectious and Parasitic Diseases. 1997; 24(2): 28-29.
31. Kantardjiev, T., Padeshki, P., Ivanov, I.N. Diagnostic approaches for oculoglandular tularemia: Advantages of PCR. British Journal of Ophthalmology, 2007; 91(9): 1206-1208.

IMMUNE RESPONSE AND CRYPTOCOCCAL INFECTIONS

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ABSTRACT

Cryptococci are universally spread and are commonly isolated from both patients and asymptomatic carriers. The evolution of cryptococcal infection is determined, on one hand, by the immune competence of the individual and the cellular immune response in particular and on the other hand, by the pathogen load and the virulence of the isolate.

Infections with *Cryptococcus* has been on the rise over the last 25 years as a results of the HIV pandemic, the expanding use of immune-suppressive drugs and the increased life expectancy of patients with hematologic malignancies. Cryptococcosis is diagnosed in 5-12% of the HIV patients and in 1-26% of the transplant recipients. Infection is acquired mostly via contaminated skin or by inhalation of conidia from sexually reproducing strains of *C. neoformans.* Cryptococcosis may affect any human organ; however it demonstrates specific lung and CNS tropism. Over the last years, the number of causative agents has expanded, involving new infectious species as *C. albidus, C. laurentii, C. curvatus, C. humicola* and *C. unniguttulatus.*

Key words: Cryptococcus, immune response, diseases, infection

ABBREVIATIONS USED IN THIS PAPER:

CAT - Computed axial tomography, CNS – Central nervous system, CMV – Cytomegalovirus, COPD - Chronic obstructive lung disease, GXM – Glucuronoxylomannan, HIV – Human immunodeficiency virus, INF – Interferon, IL – Interleukin, MCAT – Monoclonal antibody, MRI - Magnetic resonance imaging

Most commonly, cryptococcal infection follows inhalation of yeast cells or basidiospores and their deposition in the alveoli. Basidiospores, formed after the sexual reproduction of *C. neoformans,* are smaller sized compared to yeast cells (in clinical isolates) and are easy to inhale. Following the discovery of haploid budding, it was found out that spores could be shed in the environment without mating. Moreover, in 1977, Neilson (1) described the so called weakly encapsulated yeasts sized similarly to the basidiospores.

In the alveolar spaces, the yeast cells are rehydrated and the specific polysaccharide capsule is formed. The evolution of cryptococcal infection is directly dependent on the state of cellular immunity and on the yeast load and the virulence of the isolate.

The initial immune response involves the alveolar macrophages that ingest the yeast cells but are unable to destroy them completely. However, the macrophage production of pro-inflammatory cytokines as INF-ά, IL-1 and IL-6 results in recruitment of neutrophils, blood macrophages, T-lymphocytes, and NK cells from the circulation to the site of infection – the lungs. Macrophages, with ingested yeasts, act as antigen presenting cells (APC) and thus the specific T and B immune response is activated. The cellular immunity, involving CD4+ and CD8+ T-lymphocytes, is crucial in preventing the dissemination of cryptococci from the lungs to the CNS (3, 4). In immune competent individuals, an adequate immune reaction develops, resulting in granuloma type of inflammation, involving Th1 cytokine response - IL-2, IL-12 and INF-γ. The Th2 immune response accounts for the formation of comparatively weak protective humoral immunity. For instance, the passive immunization of mice with MCAT against the yeast capsule results in protective immunity and modulates the course of infection (5, 6, 7). The humoral immune response results in opsonization and decreased level of GXM in the tissues.

Baker и Haugen (8) described in autopsy material cryptococci in the hilum lymph nodes of patients without lifetime respiratory complaints. These foci serve as a potential reservoir for infection dissemination in case of immune compromise.

In HIV patients, the infection rapidly disseminates due to the decreased numbers of circulating CD4+ T-lymphocytes. In immune compromised patients, cryptococcosis develops as reactivation of previous latent infection or as primary infection due to ineffective primary immune response.

1. INNATE IMMUNE RESPONSE

1.1. Complement. The complement cascade includes serum proteins activated through the classic (antigen - antibody mediated) or alternative pathway (by surface fixed antigens) or by lectins. In all three cases, a C3 convertase is formed, resulting in C3 component cleavage in C3a and C3b fragments. C3b mediates the opsonization of the pathogens thus facilitating their ingestion by phagocytes. It also cleaves C5 into C5a and C5b fragments. C5a and C3a are mediators of the inflammatory response and enhance recruitment of phagocytic cells. C5b plays a major role in the membrane attack complex, formed by complement components C5b, C6, C7, C8 and C9. For this reason, experimental cryptococcosis in guinea pigs and white mice, treated in advance with snake venom to exhaust late complement fractions (C3-C9) results in early lethal outcome and the animal ability to eliminate cryptococcal antigen outside CNS is diminished (9). In Rhodes' opinion (10) white mice deficient in C5 complement

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fragment are far more sensitive to *C. neoformans* and have a lethal outcome due to pneumonia three times quicker compared to mice with normal C5 levels. Patients with cryptococcal fungemia demonstrate decreased levels of C3 and complement factor B (11). Brain sections of patients with cryptococcal meningitis do not show fungus fixed C3 complement fraction (12). Diamond (9) indicates that the alternative complement activation pathway is more effective in cryptococcosis compared to the classic pathway.

The capsule of genus *Cryptococcus* is a virulence factor, acting as phagocytosis inhibitor (13). Cryptococcal non-capsular mutants are not pathogenic for mice (14). Research so far, shows varying degree of complement activation depending on the presence and absence of capsule and the level of encapsulation. The capsule seems to inhibit the mannose bound lectin, thus preventing complement activation via the lectin pathway (15). Moreover, a species specific dependencies have been found regarding the complement activation cascade. For instance, the binding capacity of C3 complement fragment to *C. gatii* serotypes B and C is stronger compared to that to *C. neoformans*, serotypes A and D (16). However, the level of membrane accumulation of C3 in *C. gatii* serotypes B and C is half that in *C. neoformans* serotypes A and D (17, 18).

1.2. Phagocytosis, neutrophils and macrophages. The ingestion of *Cryptococcus* by leucocytes (19), rat and mice peritoneal macrophages (20), lung macrophages in guinea pigs (21), human neutrophils and macrophages (22), and pigs microglial cells (23) have been extensively studied. Phagocytosis is completed by direct recognition of the cryptococci or by receptor mediated recognition through complement or antibodies (24). Antibody – coated cryptococcal particles are recognized by Fcγ receptor molecules on the surface of macrophages, neutrophils or dendritic cells (25, 26). *C. neoformans* expresses a factor called anti-phagocytic protein – 1 (APP-1), that inhibits ingestion through the complement mediated binding to the complement receptors 2 and 3 (27, 28).

Neutrophils also play an important role in the innate immune response to cryptococci. Animal studies have documented significant accumulation of neutrophils at the infection site (29). It is known that neutrophils secrete antimicrobial peptides and proteins (30). Defensins belong to this group and are expressed in humans, rabbits and guinea pigs but not in mice (31). Mice are a frequent animal model of cryptococcosis and the lack of neutrophils explains their high susceptibility to the infection.

A relationship has been found between macrophages and *C. neoformans,* which defines it as an intracellular pathogen (32). Cryptococci are capable of "manipulating' macrophages. Following ingestion by macrophages, *Cryptococcus* survives inside and multiplies there, finally resulting in macrophage destruction (33, 34, 35). Similarly to *Legionella pneumophilla, C. neoformans* does not inhibit the formation of phagolysosome (36). Recently, a novel mechanism of release from the infected host cells has been described. It does not involve host cell lysis or formation of inflammatory response (37, 38). Unlike other intracellular pathogens as *Listera monocytogenes*, *Rickettsia* spp., *Shigella flexneri* and *Burkholderia pseudomallei* (39) *C. neoformans* is released from the cell in less than 60 seconds, without involving the actin cellular skeleton (38). Antibody coated yeasts are released in groups, whereas complement coated cells are released individually (37). Moreover, *C. neoformans* is capable of lateral migration from one macrophage to another (34). Unlike expulsion from the cell, lateral migration is mediated by actin and could be influenced by actin and depolymerization agents as cytohalasin D (35).

The interaction between macrophages and cryptococci illustrates how *Cryptococcus* species cause latent infection and invade new cells, including crossing the blood brain barrier. Their capacity to survive intracellularly enables them to escape the immune response and to multiply freely.

1.3. Dendritic cells. Primary dendritic cells could ingest *C. neoformans* via mannose receptors or the FcγII receptor (T- lymphocyte presenting receptor) (40). Cryptococci are a challenge to dendritic cells due to their large size, rigid cell wall and their ability as mitogens to stimulate T-lymphocytes (41). Dendritic cells are the primary antigen presenting cells that persistently "monitor" the antigen population and are responsible for the adequacy of the immune reaction (42). They also secure the protective cellular immune response (43).

2. ADAPTIVE IMMNUNE RESPONSE

2.1. Humoral immunity in cryptococcosis. Cryptococcosis has been described in patients with primary or acquired B-cellular, humoral or lymphoproliferative immune deficiencies (44). On the other hand, antibodies against cryptococcal proteins and capsular polysaccharides have been detected in individuals without symptomatic infection (latent or non-apparent infection) (45). According to Fleuridor (46) and Mukherjee (6) the passive immunization with anti-capsular antibodies is effective in alleviating the course of infection and in prolonging life in cases of experimental cryptococcosis. Anti-cryptococcal antibodies exert their protective role through opsonization of the pathogen by Fc-receptor mediated phagocytosis or by classic complement activation cascade. Taborda and Casadevall (47) described a direct opsonization of cryptococci, resulting in complement independent macrophage involvement (however, the process is complement receptor dependent through CD18 receptor). So far, it was believed that the two cryptococcal domains function independently: the variable domain is responsible for antigen binding whereas the constant domain accounts for the effector functions. Presently, these considerations undergo revision as it was found out that antibodies with identical variable but different constant domains demonstrate different binding affinity and specificity towards univalent peptide antigens (48, 49). Moreover, antibodies produced by the same B cell could be either protective or non–protective based on their staining properties (producing annular or punctuate patterns). These

antibodies recognize different special regions on the cryptococcal capsule but only those of them (showing punctuate opsonization patterns) are capable of inducing protective immunity in mice. The ratio of protective to non- protective antibodies determines the effectiveness of antibody mediated immunity against *C. neoformans*. Thus, subtle differences in the antibody epitope specificity exert a significant effect on the protective capacity. The protective antibodies demonstrate a punctuate distribution pattern in the capsule, whereas the non – protective demonstrate annular patterns (50). The concentration of antibodies influences their protective function as well. In mice experimental models, the administration of high antibody concentrations is less effective compared to lower antibody concentrations followed by subsequent infection with *C. neoformans.* This prozone (hook) like effect shows that antibodies formed during cryptococcal infection could be protective or non – protective and even could enhance the evolution of infection based on their epitope and concentration (51,52).

Cellular immunity also plays a role in the formation of antibody protective immunity. Mice with defective CD4+, INF-γ, Th1/Th2-associated cytokines develop cryptococcal infection regardless of the passively transferred IgG1 antibodies, on the contrary – mice deficient in the CD8, NK and C3 complement fraction – do not (53, 54, 55).

2.2. Cellular immunity. The number of patients with cryptococcosis after the HIV pandemic increased drastically, especially in the tropical and subtropical areas of Africa (56). Apart from HIV infected individuals, patients with leukemia, lymphoma sarcoidosis, those on prolonged steroid therapy and transplant recipients are also high risk groups (24). All these conditions present with T-cellular immune deficiency. In general, cellular immunity contributes to the elimination of cryptococci by direct cytotoxicity and also indirectly through the regulatory functions of the NK and T-lymphocytes. The NK and CD8+ T-cells have a direct anti-microbial activity against *C. neoformans* (57, 58) as the release of proteins (granulyzin and perforin) result in direct burst and lysis of the cryptococcal cells (59, 60). NK-cells express both proteins, however, perforin seems more effective against *C. neoformans.* It seems to function via PI3K-dependent ERK1/2 signal pathway (61, 62, 63). On the other hand, CD4+ and CD8+ T-cells secrete granulyzin, that is expressed via activation of STAT5 and PI3K in the presence of IL-2/IL-15 and IL-15 respectively (64, 58, 65). Both pathways are malfunctioning in HIV positive patients, resulting in ineffective destruction of *C. neoformans* (65). Th1-related cytokines are important for natural immunity whereas Th2–related immunity has not been shown to be protective in mice experimental models (66, 67). Enhanced expression of Th1 cytokines as TNF-α and INF-γ seems to limit cryptococcal infection (68, 69, 70, 71), on the other hand - INF-γ- deficient mice develop lethal cryptococcosis (72). Müller et al. have provided evidence for the important role of IL-17, which associated with the Th-17 immune response contribute to the survival of mice in experimental models of cryptococcal infection (73, 74). For comparison, Th2 cytokines IL-4 and IL-13 reduce the host capacity to eliminate *C. neoformans in vivo* (75, 76, 77). The frequency of *C. neoformans* infections correlates with the dynamics of HIV infection and increases in parallel to the loss of Th1 immune response in HIV infected patients (73). It also correlates with the Th2 cytokine profile in transplant recipients (78). Thus, the balance between Th1-Th2 and Th17 seems a determinant of survival in patients with cryptococcosis.

According to many authors, besides neutrophils and dendritic cells, lymphocytes, NK and γδT-cells are also incorporated in the regulation of cytokine balance in cases of infection (79, 80, 81, 82). NK-cells produce large quantity of INF-γ and IL-4 that trigger the Th1 but not the Th2 immune response. The activation of the latter seems inhibited by the effect of γδT-lymphocytes (83) – depletion of γδT-лимфоцитите in experimental mice models results in reduced disease severity and decreased INF-γ level. As immunity is pro-inflammatory, intense activation of Th1 in the course of infection has a negative impact on the macro-organism. The γδT-lymphocytes down regulate Th1 thus maintaining the balance between Th1-Th2 (84). Noverr (85) reports that *C. neoformans* is capable of shifting the Th1-Th2 balance towards Th2 through expression of eicosanoids (prostaglandins and leukotrienes) which act as a potential inhibitor of Th1 immune response. Additionally, *C. neoformans* produces urease – a virulence factor that seems to stimulate the Th2 immune response in the lings via a yet unknown mechanism (86).

3. INFECTIONS CAUSED BY GENUS *CRYPTOCOCCUS*

3.1. Pulmonary cryptococcosis

Primary cryptococcosis initially involves the lungs, and is triggered by inhalation of cryptococci. The infection may remain local but also may disseminate via the blood stream reaching most commonly the CNS.

The first description of primary pulmonary cryptococcosis is performed by Campbell (87). In his opinion, in immune competent patients, the disease is asymptomatic in 32 % of the patients (usually discovered accidentally on X-ray films). 54% of the patients present with cough, 46% with chest pain, 32% have sputum production, 26% present with weight loss and temperature, and 18% - with blood sputum (87). Later in the course of infection Menon and Rajamani (88) describe shortness of breath, night sweats and symptoms suggestive of v. cava obstruction with digital clubbing. Most patients are colonized, this stage is common in individuals with underlying pulmonary disease as COPD or cancer (89, 90). According to Kerkering (91) progressive pulmonary form of cryptococcosis is likely to assume a complicated course with extrapulmonary involvement.

Pulmonary cryptococcosis affects commonly immune compromised patients with the following underlying conditions: HIV infection, hepatic cirrhosis, diabetes,

Cushing, syndrome, sarcoidosis, lymphoma, leukemia, in patients on steroid therapy and in transplant recipients (91, 78, 92, 93, 94).

Recently, Baddley (95), Wu (93) and Yu (96) performed a detailed retrospective analysis of pulmonary cryptococcosis in HIV negative patients. In immune compromised patients diffuse pulmonary changes are present, disseminations are not infrequent and the disease adopts a more progressive course compared to immune competent patients (97). Meningeal involvement is more common compared to pulmonary (97). Murray (98) reported an acute respiratory distress syndrome (ARDS) in the course of early cryptococcal pneumonia. Visnegarwala (99) reported a 14% frequency of ARDS in cryptococcal pneumonia with clinical features similar to *P. carinii* pneumonia. In such cases, the lethality is 100% within 2 days following the onset of disease.

Unlike immune competent patients with asymptomatic pneumonitis, according to Kerkering (91) 83% of immuno–compromised patients have persistent symptoms, including : fever - 63%, malaise - 61%, chest pain - 44%, weight loss - 37%, dyspnea - 27%, night sweats - 24%, cough - 17%, blood sputum and headache - 7%. The same author reports that in most patients within 2 to 20 weeks following initial pulmonary infection (confirmed by chest X-ray), dissemination to the meninges takes place. In Chang's opinion (100) the most frequent presentations are: alveolar or interstitial infiltrates, followed by solitary or multiple rounded lesions, tumor-like masses, cavity lesions and pleural effusions. Kerkering (91) and Perfect (101) indicate that this patient contingent requires anti-fungal etiotropic therapy.

In HIV-positive patients with pulmonary cryptococcosis, the meningeal involvement is also prominent (102). However, a number of other authors (103, 104), consider that pulmonary cryptococcosis has a different course in HIV positive immune-compromised compared to other immune-deficient patients. In a case series of 106 HIV patients (105), the following symptoms are prevalent: high fever (81%), cough (63%), dyspnea (50%), weight loss (47%), and headache (41%). Wasser and Talavera (103), as well as Cameron (106) indicate, that in 94% of the patients, dissemination to blood stream and meninges ensues. Cameron (106) mentions concomitant lymphadenopathy, enlarged liver and spleen, increased breathing rate and rales. Moreover, according to Chechani and Kamholz (107) and Clark (108) other opportunistic pathogens could be present as *Pneumocystis jirovecii, Mycobacterium avium-intracellularae*, CMV and *Hystoplasma capsulatum* var. *capsulatum*. Lambertus (109) and Fisk (110) report cases of *P. jiroveci* pneumonia in patients on steroid therapy complicated by superimposing infection with *C. neoformans*. For this reason, in cases of presence of superimposed infections with similar X- ray presentations as well as in cases of unexplained pleural effusions or empyemas Goldman (111) stresses the importance of bronchoscopic confirmation of the diagnosis. Many authors (106, 102, 112) agree that trans-bronchial biopsy and BAL are useful diagnostic tools, which provide diagnostic confirmation in 80 to 100% of the cases. Mulanovich (113) recommends serum and pleural fluid testing for cryptococcal antigen whilst microbiological confirmation is awaited.

3.2. Cryptococcal meningitis and meningoencephalitis

C. neoformans and *C. gattii* have a tropism to the nervous tissues. The usual dissemination route is from the primary asymptomatic or manifested focus in the lungs to the CNS, where the leptomeninges are invaded (114). Infection may also spread to the brain parenchyma, resulting in meningoencephalitis (115), mass lesions or cyst formation (116); if left untreated, mortality is a 100% (117). Lethality rates range from 40 to 70% in case of inadequate management (in terms of available medical equipment and therapy) (118, 119). Mortality drops to 20% in cases of adequate management and strict adherence to the Treatment Guidelines (120).

According to Clark (108) and Ecevit (121) symptoms of cryptococcal infection in HIV-positive and in non-HIV patients are similar and include: headache, fever, meningismus, visual disturbances and mental state changes. However, disease dynamics is different in the two patient contingents due to the severe immune suppression, seen in HIV patients (122). In such cases, symptoms development is more abrupt and meningoencephalitis takes shorter to evolve. *Cryptococcus* multiply in the CNS, leading to fulminant involvement of other organs and tissues. Often, this is accompanied by coma and respiratory arrest (123). Based on Kovacs' (124), Chuck's (125) and Saag's data (126) CNS cryptococcosis in HIV patients is characterized by 3 main symptoms: headache (83%), fever (75%) and malaise (68%); less frequent symptoms are: vomiting (42%), meningismus (32%), clouding of mentation (24%), photophobia (19%), and focal neurological deficits (6%). The above mentioned authors differentiate between the features of cryptococcal meningoencephalitis in HIV-positive and in HIV-negative patients (Table 1.)

In non-HIV patients, cryptococcal meningoencephalitis is present in about 50% of the cases (127). According to Shin (128), the onset is insidious and symptoms are nausea, vomiting, irritability, memory and gait disturbances. More uncommon manifestations are reported by Kwok (129) – a case of 6th nerve palsy and Ahmed (130) – a case with intermittent seizures.

Patterson (131) summarizes the CNS fluid findings in HIV-positive and HIV-negative patients with cryptococcal meningitis, concluding that they are similar except for the presence of larger numbers of cryptococci in the CNS fluid of HIV patients and the lesser magnitude of the immune response (Table 2.)

CAT imaging may be normal or may show thickening of the meninges, solitary or multiple nodes (cryptococcomas), cerebral edema or hydrocephalus (132). MRI proves to be more precise in the differentiation of multiple nodular lesions in the brain parenchyma, meninges, basal ganglia and midbrain. Brain atrophy is common

Table 1.
Comparison between cryptococcal meningoencephalitis in HIV-positive and HIV-negative patients.

Findings	HIV-positive patients	HIV-negative patients
Duration of symptoms	Up to 2weeks	Over 2 weeks
India ink stating of CNS fluid	Approximately 75% (+)	Approximately 50% (+)
Antigen titer in the CNS fluid (1: 1024)	Common	Rare
Positive serum antigen	Common	Rare
Leucocytes in CNS fluid (20/μl)	Very common	Rare
Extra – neurological symptoms	Common	Rare
CNS fluid pressure 200 mm	Common	Very rare

Table 2.
CNS fluid findings in HIV-positive and HIV-negative patients with cryptococcal meningoencephalitis

CNS fluid findings	Percentage in HIV-negative patients	Percentage in HIV-positive patients
Pressure (<180мм H2O)	72%	62%
Low glucose level (<40 mm/dl)	73%	65%
Increased protein level (>45 mg/dl)	89%	64%
White blood cells per /ml (usually lymphocytes)	Over 150/mm^3 in 65-69%	≤ 20/mm^3 in 93%

in HIV patients (132); there are reports of cerebral dementia in patients with cryptococcosis and underlying sarcoidosis (133).

Speed and Dunt (134) in a research conducted in Australia report that *C. neoformans* var. *gattii* more frequently causes neurological complications as hydrocephalus and cerebral nerve damage compared to *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*. This is likely due to the fact that var. *gattii* seems less virulent, thus allowing more aggressive immune response by the host.

According to Sorrell (135), Speed and Dunt (134) and Oliveira (136) brain cryptococcoma are more frequent in *C. neoformans* var. *gatti* infections, compared to the two other serotypes. In such cases, differentiation with space – occupying lesions is necessary; if lesions are sized more than 3 cm – surgical or biopsy testing determines the diagnosis. This invasive approach could be avoided - Himmelreich (137) and Dzendrovskyj (138) suggested a computer based analysis of the MRI spectroscopic signals emitted by the trehalose cytosol of cryptococci in the cryptococcoma.

According to Abadi (139) increased intracranial pressure is typical during the three periods of cryptococcal meningoencephalitis: at the initial stage when diagnosis is made due to the large number of cryptococci and due to the intense immune response against them; during stage two – in the course of anti-fungal therapy (140), when a large number of dead cryptococcal cells and polysaccharide antigens are released thus blocking the normal circulation of CSF; additionally this is typical during the initiation of HAART (Highly Active Antiretroviral Therapy) as well as at its withdrawal when immune response is adequate, resulting in cerebral edema; at stage 3 – during recovery when resolution of infection results in development of ‘ classic” cerebral edema (141).

The role of IRIS (Immune Reconstitution Inflammatory Syndrome) (142) should also be taken into consideration during the evolution of cryptococcal meningoencephalitis. IRIS is characterized by intense surge of immunity, triggered by the institution of successful antiviral therapy. It is seen at variable intervals in approximately 30-35% of HI-infected patients on HAART and results in decreased viral loads and increased CD4+ T-cell counts (143, 144, 145). In non–HIV patients, it is observed in about 5 %, mainly in transplant recipients during 4th to 12th week following the gradual withdrawal of immuno-suppressive therapy (146).

3.3. Other organ locations of cryptococcosis

3.3.1. Eye cryptococcosis

In patients with disseminated cryptococcosis, ocular involvement has been described (147). Based on data from Okun and Butler (148) before the onset of AIDS pandemic, about 45% of the patients with cyrptococcosis had eye manifestations. In part of the cases, these manifestations preceded the development of cryptococcal meningoencephalitis (149, 150, 151). Blackie (147) reports keratitis, edema of the papilla, scotoma, and chorioretinitis; in immuno-compromised patients the

same authors report lesions of the retina and vitreous body. Rex (152) described complete eyesight loss due to cryptococcal meningitis. In his opinion, vision loss is likely to occur through two pathological mechanisms: 1. rapid onset (within 12 hours) vision loss due to infiltration of the eye nerve with cryptococci, resulting in nearly irreversible damage; and 2. slow onset eyesight loss (within weeks or months) due to the increased intra-cranial pressure. In such cases, the process is more likely to be controlled and slowed down (153).

In some cases, the eyes are the portal of entry for cryptococcal infection – for instance during transplant of infected cornea (154, 155). It is usually followed by development of meningoencephalitis. De Castro (156) reports a case of keratitis caused by *C. albidus* in a transplant recipient; Doorenbos-Bot (157) reports post traumatic periorbital cryptococcal necrotizing fasciitis in immune-competent individual.

3.3.2. Cutaneous cryptococcosis

Skin lesions develop in about 10-15% of the patients with cryptococcosis and HIV, or sarcoidosis, as well as in patients on high dose steroid therapy. Patients infected with D and C serotypes of *C. neoformans* seem more prone to skin involvement (158, 159).

In most cases, skin cryptococcosis arises following hematogenous spread (160). Primary skin inoculation or infection during a laboratory accident are rare (161). Cutaneous lesions are variable and could mimic other skin conditions (162). In their classic monograph, Littman and Zimmerman (163) describe: acne like lesions, purpura, vesicles, nodules, abscesses, ulcers, granuloma, pustule and cellulitis. In such cases, the clinical examination should be followed by skin biopsy for cytology and microbiological culturing (163). Some HIV-positive patients may develop Molluscum contagiosum-like skin lesions; even then a differentiation from *Hystoplasma capsulatum, Coccidioides immitis/posadaii* or *Penicillium marneffei* is necessary (164, 165). Other patients present with herpes-like lesions (166). In Singh's opinion (167) interestingly, it seems that *Cryptococcus* has an affinity to the skin of transplant recipient on immune suppression with tacrolimus.

3.3.3. Prostate cryptococcosis

Prostate gland, according to Braman (168), Chang (169) and Shah (170) is rarely involved in cryptococcal infection. Usually it takes the form of asymptomatic carrier state; the yeasts are isolated from the prostate tissue or from the blood after urological procedure (171). Perfect (172) in his review of penile mycotic conditions, indicates the presence of genitourinary cryptococcosis. Data is available in other publications as well (173). In females, ulceration of the vulva has been described (174, 175), however, the authors do not indicate if transmission between the partners is possible.

Prostate gland is an important reservoir for cryptococcal infection as cryptococci accumulate there shielded from the effects of anti-fungal therapy. For this reason, in HIV patients with cryptococcosis, even after prolonged courses of anti-fungal therapy, cultures of semen and urine could be positive for cryptococci (176, 177).

3.3.4. Bone cryptococcosis

Bone form of cryptococcosis develops in 5-10% of all patients with cryptococcal infection (178), if unrecognized it may affect adversely the disease outcome (179, 180). Bone cryptococcal infection is usually due to hematogeous spread from the lungs or from a lymph node but it could also result from direct skin invasion (181).

Osteomyelitis affects immunocompetent and immunocompromised patients, especially those with sarcoidosis. However, it seems rare in HIV-positive patients, regardless of the fact that they (according to Behram) (182) commonly present with cryptococcal bone marrow involvement. Osteomyelitis most frequently affects the spinal column (183) and the bone epiphyses (184) due to their rich blood supply. There are occasional reports of cryptococcal location in the cranial bones (185).

The presence of asymptomatic patients with X-ray proven bone lesions usually requires differential diagnosis with other conditions, causing similar lesions (181).

3.3.5. Visceral cryptococcosis of other location

In the literature, there are occasional reports of cryptococcosis with unusual visceral locations. Colmers (186) discusses cryptococcal endocarditis, Jones (187) – the problem of cardiac cryptococcosis. Leavitt (188) reports a case of cryptococcal aortitis in intravenous drug user. In his opinion, this is the second case of such involvement described in the literature and the first to be diagnosed in a live patient, Philip (189) describes disseminated lymph node involvement, which clinically mimics tuberculosis lymphadenopathy; Karagüzel (190) – mesenterial cryptococcal lymphadenitis, presenting as acute surgical abdomen; Araújo (191) – intra-abdominal lymph node cryptococcosis in non-HIV patient caused by *C. gattii*, Chong (192) – an unusual cryptococcoma, located in the omentum, Gordon (193) – granulomatous cryptococcosis of the peritoneum, Sing (194) – cryptococcosis masked as tubo-ovarial abscessus, Sundar (195) reports a rare cryptococcosis of the stomach as a presenting symptom of HIV infection, Girardin (196) – a rare case of cryptococcal gastro-duodenitis, Law (197) – cryptococcal colon involvement, Nawabi (198) – intestinal obstruction in HIV positive patients, Chaitowitz (199) reports a perforation of the jejunum in immuno-competent patient with gastro-intestinal cryptococcosis; Matsuda (200) – for liver and cryptococcosis of the adrenal glands with adrenal insufficiency (without CNS involvement) in a patient with type 2 diabetes, Chen (201) and Ranganathan (202) describe a case of cryptococcal vaginitis, Gültaşli (203) - a case of intra-medullar infection in immune competent individual, masked as a solid spinal tumor, Lu (204) in his publication reports early liver cryptococcosis in a transplant recipient. Viriyavejakul (205) in his review of opportunistic infections described visceral cryptococcosis in HIV positive patients in autopsy materials.

CONCLUSION

The development of cryptococcosis is directly dependent on the immune response of the individual as well as on the virulence and the number of inhaled yeasts.

In immunocompromised patients, cryptococcal infection develops as reactivation of dormant latent infection or as primary disease due to ineffective primary immune response.
In general, the defective cell mediated immunity is the main predisposing factor in the evolution of infection.
Clinically, the genus *Cryptococcus* commonly causes pulmonary or meningoencephalitic manifestations. The symptoms vary in HIV positive and HIV negative patients. There are rare forms of infection as: bone, eye, prostate, skin and other visceral cryptococcosis.

References of this paper are available upon request from the editorial board.

GENETIC METHODS IN THE DETECTION OF ANAEROBIC MICROFLORA FROM SUBGINGIVAL DENTAL BIOFILM

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ABSTRACT

***Porphyromonas gingivalis* is one of the relatively specific pathogens in cases with chronic periodontitis. This bacterium possesses the highest number of virulent properties such as the ability to suppress the migration of the polymorphonuclear leucocytes and to counteract their phagocytosis with the help of incapsulation. Its most aggressive property is observed on cell cultures – the ability to invade epithelial cells (1, 2) which results in an ability to be included in the formation of the atheromatous plaque – a highly risk factor for the development of sclerosis (3, 4). Isolation and cultivation of *P. gingivalis* requests special expensive equipment and they are time and labor consuming but very important for proper diagnosis and treatment. In this study we optimized PCR protocol for the detection of *P. ginigivalis* in tooth and atheromatous plaques.**

INTRODUCTION

The infections of the oral cavity are dangerous not only for the health condition of the teeth and gums. They hide a high risk level for complications of the cardio-vascular system and other organs in the human body. Many species of anaerobes inhabit the human oral cavity and some of them are part of the normal flora but in some conditions they can cause inflammation processes such as gingivitis, periodontitis, etc. Others are taking part in the development of myocardial infarctions and other cardiac diseases (5). Oral infectious diseases and dental procedures are causative factors for the transient bacteriaemia (6, 7). For example during mastication endotoxins can be released into the blood stream in patients with periodontitis (8). Infected root canals are a potential source for a blood contamination during the dental procedures (9).

In the last decade scientific investigations showed a relation between dental microflora (bacterial species and the number of their cells). Serological detection for many pathogens like *Chlamydia pneumonie*, *Helicobacter pylori,* and *Cytomegalovirus* is developed (10, 11). There is a suggestion that some members of the microflora are factors in the progression of coronary arterial disease in a combination with other cardiovascular conditions. (12).

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Some authors observe much higher number of species like *Porphyromonas gingivalis, Tannerella forsythensis, Treponema denticola, S. intermedius, S. sanguis* and *Streptococcus anginosus* in patients with coronary syndrome (13).

The aim of this study was to optimize a protocol for the detection of *P. gingivalis* in tooth and atheromatous plaques as it is with the highest percentage of isolation among the others.

MATERIALS AND METHODS:

1. DNA isolation from clinical materials

DNA from 54 samples from subgingival plaque is taken with paper pin from periodontal pockets and from 9 atheromatous plaques cultured on solid medium. Commercial kits of Qiagen® and Invitrogen® for DNA isolation are used. Protocol of the kit manual for human and animal tissues is used with modifications. The incubation step on 56 ◦ C is elongated to 3 h to lyse the bacterial cell wall with vortexing. Final volume in elution buffer is 150 µl with a yield in a range of 100 µg – 1 mg tested on 1% TBE agarose gel stained with ethidium bromide when Qiagen ® kit. Invitrogen ® kit gave no detectable yield.

2. Gene amplification of the gene coding 16S rRNA in *Porphyromonas gingivalis*.

Fragment of the gene coding 16S ribosomal RNA is amplified with the following thermodynamic parameters:

95◦C–15 min
{94◦C–30 sec
50◦C–40 sec
72◦C–1min 10 sec} x 35 times
72 ◦C – 5 min

Perkin Elmer® amplifier with heating led is used. The reaction takes place in a viol with the following reagents:

1 µl DNA matrix (dissolved in AE buffer of Qiagen®)
0.5 µl 5‘ primer 5’-AGGCAGCTTGCCATACTGCG-3’ (1 pmol/µl)
0.5 µl 3’ primer 5′-ACT GTT AGC AAC TAC CGATGT-3′ (1 pmol/µl)
2 µl 10 x PCR buffer Invitrogen®
0.6 µl 50 mM $MgCl_2$ (final concentration 1.5 mM)
0.25 µl dNTP (1mM each of dATP, dTTP, dGTP, dCTP)
0.25 µl Taq polymerase Invitrogen® [5U/ µl]
14.9 µl H_2O
20 µl final volume

The primers used in this study are published in the literature (5 reports).

RESULTS AND DISCUSSION:

Pairs of primers for the region of 16S rRNA coding gene in *Porphyromonas gingivalis* is amplified successfully for the first time in Bulgaria. The method is described by Stelzel M. et al. as well (14) and *Porphyromonas gingivalis* is identified in atheromatous plaque and a tooth plaque.

The product of the PCR reaction is loaded on agarose gel on 100 V for 40 min. 100 b.p.

The visualized on the agarose gel fragment is 404 bp as expected (see fig. 1 and 2). Amplification is successful in 2 of 9 atheromatous plaques and in 11 of 54 tooth samples. These 11 positive samples are from the bacterial plaque of 3 healthy persons, 3 patients with periodontal disease and 5 patients with a coronary syndrome.

AMPLIFIED FRAGMENT:

AGGCAGCTTGCCATACTGCGACTGACACTGAAG-
CACGAAGGCGTGGGTATCAAACAGGATTAGA-
TACCCTGGTAGTCCACGCAGTAAACGATGAT-
TACTAGGAGTTTGCGATATACCGTCAAGCTTCCA-
CAGCGAAAGCGTTAAGTAATCCACCTGGGGAG-
TACGCCGGCAACGGTGAAACTCAAAGGAATT-
GACGGGGGCCCGCACAAGCGGAGGAACATGTG-
GTTTAATTCGATGATACGCGAGGAACCTTACCCG-
GGATTGAAATGTAGATGACTGATGGTGAAAAC-
CGTCTTCCCTTCGGGGCTTCTATGTAGGTGCTG-
CATGGTTGTCGTCAGCTCGTGCCGTGAGGT-
GTCGGCTTAAGTGCCATAACGAGCGCAACCCA-
CATCGGTAGTTGCTAACAG

Fig. 1 Amplification of a fragment of the gene coding 16S rRNA from tooth samples.
On 1-5, 9 and 11 lines are amplified fragments; on 6-8 and 10 - no amplification.

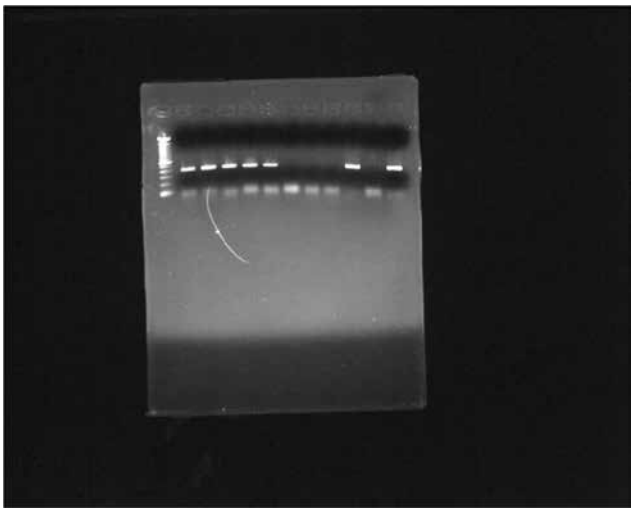
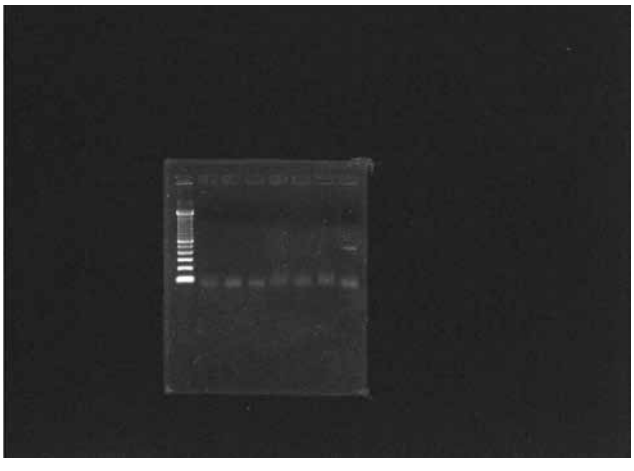


Fig. 2 Amplification of a fragment of the gene coding 16S rRNA from atheromatous plaque.
On line 7 is the amplified fragment.



The contribution of our study is the identification of *Porphyromonas gingivalis* for the first time in Bulgaria with the help of PCR of a bacterial 16S rRNA from a tooth plaque and an atheromatous plaque.
Finding out of *Porphyromonas gingivalis* in atheromatous plaque from a blood vessel of two of our patients supports the hypothesis for direct including of some bacteria participating in the periodontal infection in the pathogenesis of the atherosclerosis as well.

CONCLUSIONS

Based on this study we conclude that *P. gingivalis* is a potentially important predisposition factor for the progression of a coronary syndrome. Observation of *P. gingivalis* in a plaque from a blood vessel supports the hypothesis about its direct participation in the pathogenesis of the atherosclerosis.

REFERENCES:

1. Progulske-Fox, A., et al. *Porphyromonas gingivalis* virulence factors and invasion of cell of the cardiovascular system. J Periodontol Res., 1999, 34, 12, 393-399.
2. Page, R., Beck, J. Risk assessment for periodontal diseases. Int Dent J, 1997, 47, 61-67.
3. Ridker, P., et al. Elevation of tumor necrosis factor- α and increased risk of recurrent coronary events after myocardial infarction. Circulation, 2000, 101, 2149–2153.
4. Geerts, S., et al. Further evidence of the association between periodontal conditions and coronary artery disease. J Periodontol, 2004, 75, 1274-1280.
5. Persona G. R., Ohlssonc O., Petterssond T., Renvertc S. Chronic periodontitis, a significant relationship with acute myocardial infarction. Eur Heart J. 2003; 24(23):2108-15
6. Herzberg MC, Meyer MW. Effects of oral flora on platelets: possible consequences in cardiovascular disease. J Periodontol 1996; 67:1138-42.
7. Daly CG, Mitchell DH, Highfield JE et al. Bacteremia due to periodontal probing: a clinical and microbiological investigation. J Periodontol 2001; 72:210-4.
8. Quitynen M, Mongardini C, de Soete M et al. The role of chlorhexidine in the one-stage full-mouth disinfection treatment of patients with advanced adult periodontitis. Long-term clinical and microbiological observations. J Clin Periodontol 2000; 27:578-89.
9. Debelian GJ, Olsen I, Tronstad L. Bacteremia in conjunction with endodontic therapy. Dent Traumatol 1995; 11: 142-9.
10. Mattila K, Vesanen M, Valtonen V, et al. Effect of treating periodontitis on C-reactive proteins: A pilot study. BMC Infectious Diseases 2002;2:30-32.
11. Ebersole JL, Taubman MA, Smith DJ, Genco RJ, Frey DE. Human immune responses to oral micro-organisms. Association of localized juvenile periodontitis (LJP) with serum antibody responses to Actinobacillus actinomycetemcomitans. Clin Exp Immunol 1982;47:43-52.
12. Pussinen PJ, Jauhiainen M, Vilkuna-Rautiainen T, et al. Periodontitis decreases the antiatherogenic potency of high density lipoprotein. J Lipid Res 2004;45:139-147.
13. Marina, M., Bolyarova, T., Ivanova, K., Dzhemileva, T. Calcium antagonists-induced gingival overgrowth – examination of subgingival microflora. ANAEROBE 2000, International Congress of the Federation of Anaerobe Societies, July 10-12, 2000, Manchester, England, Abstr., p.147, poster VIIb – P8.
14. Page, R. The pathobiology of periodontal diseases may affect systemic diseases: inversion of paradigm. Ann Periodontol, 1998, 3, 1, 108-120

CLINICAL FORMS OF HEPATITIS A IN DIFFERENT AGE GROUPS – ANALYSIS FOR A TWO-YEAR PERIOD, 2011-2012

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ABSTRACT:
Introduction: Hepatitis A is a fecal-oral transmitted disease demonstrating great differences in its epidemiology among countries with varying social and economic development as well as among the social groups within a single country. The disease is a topical health issue for Bulgaria as the morbidity in the country is the highest in the Europe Union. The clinical course of Hepatitis A varies from asymptomatic to fulminant forms and that depends on a number of factors, age being one of them. The objective of the study is to compare the specifics of the clinical course of Hepatitis A in different age groups.
Key words: hepatitis A, clinical form, age groups

Materials and methods:
From 01.01.2011 to 31.12.2012, 325 patients with confirmed acute hepatitis A were treated at the Infectious Diseases Ward of the Multi-profile Hospital of Active Treatment – Pazarzhik. Serologically the disease was confirmed with the presence of anti HAV IgM in the patients' serums (ELISA). The used methods included clinical observation, paraclinical tests, instrumental testing and epidemiologic surveillance.
Results:
From 325 patients treated in the ward, 202 (62.15%) were children up to the age of 18 and the remaining 123 (37.85%) were adults. The largest number of the diseased were children, aged 3-7 years – 104 (32%) and the smallest – patients, elderly than 60 years – 6 (1.8%). A rise in morbidity was observed in the older age groups. Among 202 children treated in the ward, 140 (69.3%) were of Roma origin. In contrast, among the adults, from 123 patients with acute Hepatitis A, only 10 (8.2%) were Romani, while the rest 113 (91.8%) lived in neighbourhoods with good hygienic conditions.

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Clinical manifestations (symptoms and signs): Astheno-adynamia among the children was observed more rarely – in 84 (41.6%) cases, while among the adults the number was 104 (84.5%) cases. The average increase of the total bilirubin among the children was much lower than that among the adults. The results obtained were identical in relation to the cytological enzymes (ALT and AST). Among all patients ultrasound examination of the abdominal organs was performed which demonstrated more pathological findings among the adults compared to the children. The average hospital stay among children was 5.2 days and for the adults it was 7.9 days.
CONCLUSIONS:
Among the children the lighter forms prevailed and the stay in hospital was shorter. The adults have expressed clinical symptoms; the disease most often progressed as an icteric form and compared to the children the subjective complaints, paraclinical abnormalities, the average hospital stay and the convalescence period were more prolonged. Acute viral hepatitis A remains a health problem in Bulgaria. The Roma population gets the disease primarily during their childhood years while the other ethnic groups get sick in their adult years.
Introduction
Viral Hepatitis A is a fecal-oral transmitted disease, demonstrating great epidemiological differences between developed and developing countries as well as among the different social and economic strata within the individual countries (1, 2, 3, 4). Every year about 1.5 million people in the world have hepatitis A. In some European countries still epidemic outbreaks of Hepatitis A occur (5,6,7). The morbidity in Bulgaria is the highest in the Europe Union. In some regions of the country there are periodic epidemic outbreaks (8). The greatest among those was registered in 2006, when in one of the neighbourhoods of Plovdiv 1004 patients were affected .
The clinical course of the disease varies from asymptomatic infection to fulminant hepatitis (9,10,11). In about 70-80% of the cases among children, the disease course is asymptomatic while among the adults most cases are clinically manifested. In most of the patients the disease has the course of a self-limiting infection but in certain cases unusual clinical manifestations of Hepatitis A are observed including cholestatic, relapsing and fulminant hepatitis. Hepatitis A accounts 93% of the cases of acute hepatitis in Argentina including 7% of atypical clinical cases. Hepatitis A is the major cause of fulminant hepatitis and has been reported to account for 10% of liver transplants in children in France and 20% in Argentina. One-year survival after liver transplantation is 64%. Prevention must be considered as the main means of averting this severe illness (12). The clinical manifestation depends on a number of factors, one of which is the age (13, 14, 15).
Hepatitis A is a serious health problem for Bulgaria. The proof of that statement includes the periodically occurring epidemic outbreaks in various regions of

the country and its highest morbidity in Europe. The objective of the present study is to compare the clinical course of Hepatitis A among adults and children.

MATERIALS AND METHODS

The study covered two-year period, 2011 – 2012, during which 325 patients with serologically confirmed acute Hepatitis A were treated in the Infectious Diseases Ward at the Multi-profile Hospital of Active Treatment – Pazardzhik. The following methods have been used: clinical observation; paraclinical tests: hematological tests (blood count, biochemical characteristics) and ultrasound examination of the abdominal organs. The cases were serologically confirmed by proving the presence of anti HAV IgM (ELISA). An epidemiologic analysis of the following parameters was conducted: demographic and age distribution, hygiene habits and living conditions, mechanism and factors of disease's transmission.

RESULTS AND DISCUSSION

Epidemiological analysis:

The age distribution of the patients with Hepatitis A is presented in Table 1.

Table 1: Age distribution of the patients with HAV

Age (years)	number	percent
0-3	25	7,69
3-7	104	32.00
8-18	73	22,46
19-40	77	23,69
41-60	40	12,31
> 60	6	1,85

The epidemic process had a different intensity in the different age groups: in the group up to 18 years of age 202 patients were treated while those above 18 years of age were 123. Our observations indicated that children aged 3-7 years got ill most often and elderly people above 60 years of age got ill most rarely. Although, there was a relative increase of the cases with Hepatitis A among the higher age groups. This concerned primarily the Bulgarian population. We consider the factor age as related to the ethnical belonging of the patients – the morbidity in childhood years is much higher among Roma population (compared to Bulgarian population) because the Roma population is living in considerably worse hygiene conditions. In contrast, the cases among the Bulgarian adult population increased during the last years.

Demographic structure: The results obtained demonstrated that from 202 children treated in the ward, 140 (69.3%) were of Roma origin. They contracted the disease as early as in their childhood, being ill in the asymptomatic, subclinical or clinically manifested form and then became immune as adults. The reasons for contracting the disease in their childhood are related to their social standing and living conditions: not established personal hygiene routines, low health care culture, gathering of many people in one place and the consequences of it. The mechanism of transmission was realized predominantly by person-to-person contact. In contrast with the Roma the people from the other ethnic groups got ill of Hepatitis A in their more adult years of age – from 123 adult patient only 10 (8.2%) were Roma people, and the remaining 113 (91.8%) were from other ethnic groups.

The following clinical symptoms have been observed (Table 2).

1.Fever: We observed it in 72 (35.64%) of the children and in 58 (47.2%) of the adults. The fever period was longer and the temperature's values were higher among the adult patients. In contrast to the the children who were bearing it more easily the adult patients were most often in bed and took higher dosages of anti-pyretic, analgesic and non-steroid anti-inflammatory medications.

2.Nausea and vomiting: they were among the basic symptoms for the patients with Hepatitis A. Their frequency among adults and children was almost identical (table 2). We observed, however, that among the adult patients dyspeptic manifestations continued longer in comparison with the children which confirmed the data provided by other authors (11, 12, 15).

3.Fatigue (asthenia and adynamia): Asthenia and adynamia were more often manifested symptoms among the adult patients – 84.5% versus 41.6% among the children. The duration among the adults was greater - throughout the entire hospital stay, even in the early convalescence period.

4.Lack of appetite: a basic subjective symptom expressed differently among adults and children (table 2). Unlike the adults, among the children the lack of appetite was a more frequently announced symptom than astheno-adynamia regardless of the fact that the evaluation may be subjective (the requirements of relatives concerning the nutrition regimen of children are unreasonably high). These symptoms among the children disappeared for shorter periods of time and this was related to the duration of the intravenous therapy and the stay in hospital. According to data provided by a number of authors, among children the most frequent forms of the disease are the asymptomatic and the oligosymptomatic forms (11,13,15). We studied in outpatient conditions several children who were in close contact with patients with hepatitis A (living in the same homes). One of them – a 10-month-old infant – had several unstable stools and the other 5 were clinically healthy. The biochemical tests conducted

Table 2: Symptoms, observed among children and adults:

Symptom	Children		Adults	
	Number	Percentage	Number	Percentage
1. Fever	72	35.64	58	47.20
2. Nausea (vomiting)	113	55.90	67	54.50
3. Astheno-adynamia	84	41.60	104	84.50
4. Lack of appetite (anorexia)	106	52.40	104	84.50
5. Abdominal pains	99	49.01	43	34.96
6. Diarrhea	9	4.45	2	1.63
7. Arthralgia	3	1.48	32	26.02
8. Cough	28	13.86	6	4.88
9. Hyperpyrexia	1	0.49	14	11.38
10. Rash	1	0.49	1	0.81
11. Sleepiness	28	13.86	6	4.88
12. Itching	7	3.46	11	8.94
13. Headache	3	1.48	3	2.44
14. Enlarged lymph nodes	3	1.48	0	0
15. Heaviness in the right under rib area	0	0	12	9.76

did not establish any deviations but the serological testing results were all with positive values for anti HAV IgM.

Other symptoms, manifested in various degrees within the two age groups: abdominal pain, diarrhea, arthralgia, cough, chills, rash, sleepiness, itching, headache, enlarged lymph nodes and heaviness in the right under rib area (table 2).

Abdominal pain was a common symptom among the children – 99 (49%) of the children announced of a paraumbilical pain,which passed away for several days. This pain was not dependent on the nutrition regimen and was more expressed at home and during the first 1-2 days after admission into the hospital. Among the adult patients the stomachaches were rare – in 43 (34.9%) of the patients; they were localized epigastrally, often accompanied by heartburn and a feeling of heaviness in the right under rib area. The aches persisted in the pre-icteric and more rarely in the icteric period.

Cathars of the upper respiratory tract were observed more often among the children as compared to the adult patients. Similar results have been announced by other authors (9, 10).

Accompanying disorders among patients with Hepatitis A:

We found pneumonia in 21 (10.39%) of the children and in 1 (0.81%) of the adults; calculose cholecystitis – in none of the children and in 4 (3.25%) of the adults; anemia – in 22 (10.89%) of the children and 2 (1.63%) of the adults; diabetes mellitus – in 1 (0.49%) of the children and 3 (2.44%) of the adults.

The accompanying disorders rarely found among the children included: epilepsy, retardatio mentalis, tracheobronchitis, otitis, tonsillitis, and among the adults they included: arterial hypertension, pyelonephritis, psychic deviations, ulcerative colitis, ethylism.

Paraclinical tests:

The following tests were conducted of the patients with hepatitis A: total blood count, differential blood count, erythrocyte sedimentation rate (ESR), fibrinogen, prothrombine time, INR, blood sugar, total bilirubin with fractions, AST, ALT, GTP, AP, urine testing and serological test for acute Hepatitis A, and by estimation – hepatitis B, C, herpes viruses. The results obtained are presented in Table 3.

Table 3: Biochemical characteristics among adults and children

Characteristics	Children – average values	Adults – average values
AST – admission	Max. value – 4088 U/l, average value 778.2 U/l	Max. value – 4040 U/l, average value 1024.1 U/l
AST – discharge	Average value – 139.8 U/l	Average value – 133.2 U/l
ALT - admission	Max. value – 4806 U/l, average value 1557.9 U/l	Max. value – 5864 U/l, average value 1999.6 U/l
ALT – discharge	Average value – 327.7 U/l	Average value – 367.9 U/l
GTP	Average value – 175.1 U/l	Average value – 278.8 U/l
AP	Average value – 698.8 U/l	Average value – 392.6 U/l
Total bilirubin	Max. value – 285 mmol/l, average value – 70.4 mmol/l	Max. value – 287 mmol/l, average value – 108.1 mmol/l

The results indicated that cytolysis was expressed in both groups – adults and children. The patients were admitted with high values of ALT, AST and they often correlated to higher values of total bilirubin. In spite of that there was a certain difference among the children and adults – the values of the adult patients were higher both upon admission and upon discharge. From the children patients, 21 (10.4 %) had normal bilirubin values – non-icteric. In contrast with them, we observed normal values of bilirubin in only 2 (1.63%) of the adult patients. This data confirmed the observations of other authors that among the adults Hepatitis A most often progressed as an icteric form (9, 10, 15).

In 69.8% of the children the bilirubin values were not significantly increased – they were less than 100 mmol/l, while only 40.6 % of the adult patients had such bilirubin values. With the advance in age the number of patients with higher bilirubin values also increased.

OTHER DEVIATIONS IN THE PARACLINICAL TESTS:

Anemic syndrome: we observed it more often in children but it was not connected to the main disease – hepatitis A. Most parents were aware of the existing anemia but the treatment prescribed had not been conducted.

Deviations in the number of leukocytes: we found them in 44 (21.8%) children: in 18 (8.9%) of them there was leukocytosis while in 26 (12.8%) there was leukopenia with maximum low leukocyte values 2.8 G/l. The leukocytosis was not strongly expressed and was related to the accompanying diseases – pneumonia, tonsillitis, etc. Among the adults leukopenia was found in 29 patients (23.6%) as the leukocyte levels were lowered to 1.3 G/l. With the end of the intoxication and the improvement of the general conditions the leukocyte values normalized.

Thrombocytopenia was found in 5 children and 11 adults but not in any critical values and it was not accompanied by hemorrhagic manifestations.

Hepatomegalia was found in 199 (98.5%) of the children as the size of the liver varied within broad limits – from 1 to 5-6 cm under the rib arc.

Splenomegalia was found in 57 (28.2%) of the children as the size of the spleen was up to 3 cm under the rib arc. During the hospital stay organomegalia underwent considerable reverse development among the children and upon their discharge their organs were normal in size or reached normal size within a month after discharge.

Among the adults hepatomegalia was found in 123 patients – 100% and splenomegalia was found in 16 of them (13%). Like to the children, the organ sizes varied within broad limits but their enlargement lasted longer - for 2-3 months after the discharge and most often correlated to increased values of the cytological enzymes.

Among 65 children (32.2%) we found tachycardia and in one child – bradicardia (sinus). The frequency of the cardiac activity was about 120-140 strokes per minute and normal frequency was restored with the end of the intoxication. Compared to them, tachycardia was found among the adults in 29 (23.6%) cases. The frequency reached 120 strokes per minute and with the passing of the intoxication it became normal.

Hypotonia was a frequent symptom among the adult patients – we observed it in 35 (28.5%) patients upon admission and analogously to the tachycardia it passed along with the gaining control over the dyspeptic and intoxication manifestations. Bradicardia was found upon admission and in the icteric period in only 4 adult patients – sinus bradicardia with a frequency of 52-56 strokes per minute.

Ultrasound examination of the parenchyma organs was performed of all 123 adults patients and of 118 children. Among the children hepatomegalia or hepatosplenomegalia was found. Only in one child a thickened wall of the gallbladder was described. Among the adult patients

the findings were more diverse. In 4 of them calculous cholecystitis was found, in 1 - choledocholythiasis; in 18 - acalculous cholecystitis. Acalculous cholecystitis in the course of acute viral hepatitis has been described by other authors too (A. Galev).

Clinical forms:

According to the degree of intoxication (16) the clinical forms of Hepatitis A are:

A) light form – moderately reduced appetite, non-permanent nausea, not strongly expressed adynamia, quickly disappearing with the icter occurrence.

B) moderate form – bad appetite, persistent nausea, general fatigue getting stronger during the second half of the day, headache – up to 3-4 days after the icter occurrence.

C) grave form – anorexia, aversion to food, nausea and vomiting, adynamia, inversion of sleep, vertigo, headache – persisting for more than 10 days after the icter occurrence.

Upon the evaluation of the graveness we took into consideration the point system – score, proposed by Petrov (17) whereby subjective characteristics, clinical signs and paraclinical parameters are graded with a specific number of points.

Applying these criteria we observed the following clinical forms:

a) Among the children: light form – 91.09%; moderate form – 8.42%; grave form – 0.5% (diagram 1).

b) Among the adults: light form – 51.22%; moderate form – 46.34%; grave form – 2.44% (diagram 2).

The average hospital stay for the children was 5.2 days and for the adults – 9.7 days.

Diagram 1: Clinical forms among children with Hepatitis A

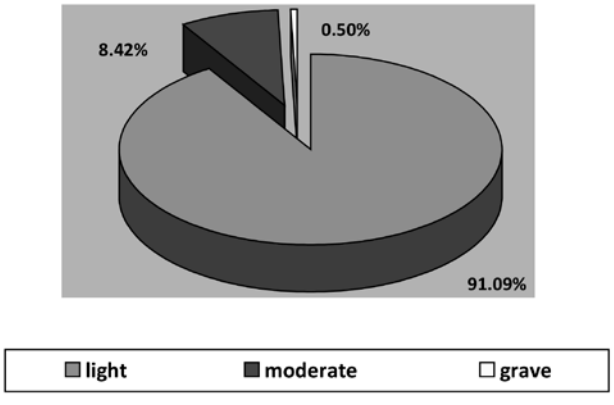
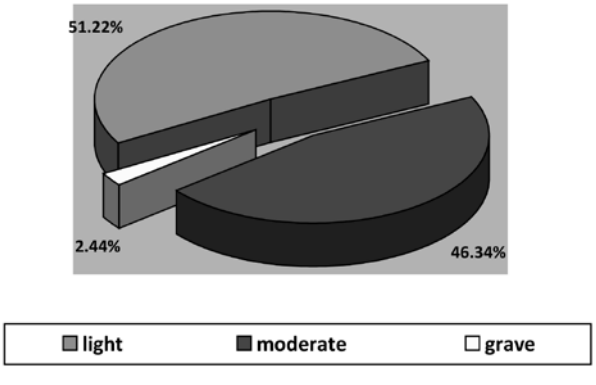


Diagram 2: Clinical forms among adults with Hepatitis A



Conclusion

We found significant differences in the clinical course of Hepatitis A among children and adults. They referred primarily to the following symptoms: fever, astheno-adynamia, lack of appetite, abdominal pain, arthralgia, hyperpyrexia, sleepiness and itching. Light forms prevailed among the children. The relative share of the moderate and grave forms among the adults was considerably higher than that among the children. This led to the need of a longer stay in hospital.

The disease prevailed among the Roma population that lived in poor hygiene conditions. Among the Bulgarian population an increase of cases was reported among the older age groups.

In our opinion, Hepatitis A remains an important health and social problem for Bulgaria, as the morbidity in the country is the highest in Europe. We believe it is appropriate to include the vaccination against Hepatitis A in the routine vaccines. This would lead to a positive health and economic effect.

REFERENCES:

1. ECDC. Annual Epidemiological Report on Infectious Diseases; 2012.
2. Franco E, Meleleo C, Serino L, Sorbara D, Zaratti L: Hepatitis A: Epidemiology and prevention in developing countries. World J Hepatol. 2012; 4(3):68-73.
3. B. Pham, B. Duval, G. De Serres, V. Gilca, A. C. Tricco, J. Ochnio and D. W. Scheifele, "Seroprevalence of hepatitis A infection in a low endemicity country: a systematic review," BMC Infect Dis. 2005; 5: 56 - 66.
4. Stoycheva M, Vatev N, Petrov A, Venchev C, Atanasova M. Epidemiological study of hepatitis A in Plovdiv region – Bulgaria, 2005-2008. World Journal of Vaccines. 2011; 1(4):162-8.
5. Cástková, J., Beneš, C. (2009). Increase in hepatitis A cases in the Czech Republic in 2008 – update. Euro Surveill., 14(3), pii=19091.
6. Hrivniaková, L., Sláčiková, M., Kolcunová, S. (2009). Hepatitis A outbreak in a Roma village in eastern Slovakia, August-November 2008. Euro Surveill., 14(3), pii=19093.
7. Perevoscikovs, J., Lucenko, I., Magone, S., Brila, A., Curikova, J., Vennema, H. (2009). Community-wide outbreak of hepatitis A in Latvia in 2008 – an update. Euro Surveill., 14(3), pii=19092.
8. Ministry of Health, Republic Bulgaria. Annual epidemiological report on Infectious Diseases, 2012 (in Bulgarian).
9. Hepatitis A. Matheny SC, Kingery JE. Am Fam Physician; 2012 Dec 1; 86(11):1027-34; quiz 1010-2.
10. Tong MJ, el-Farra NS, Grew MI. Clinical manifestations of hepatitis A: recent experience in a community teaching hospital. J Infect Dis. 1995;171 Suppl 1:S15-8.
11. Polson J, Lee WM; American Association for the Study of Liver Disease. AASLD position paper: the management of acute liver failure. Hepatology. 2005;41:1179-97.
12. Ciocca M.Clinical course and consequences of hepatitis A infection. Vaccine. 2000 Feb 18;18 Suppl 1:S71-4.
13. Debray D, Cullufi P, Devictor D, Fabre M, Bernard O. Liver failure in children with hepatitis A. Hepatology. 1997;26:1018-22
14. Schiodt FV, Atillasoy E, Shakil AO, et al. Etiology and outcome for 295 patients with acute liver failure in the United States. Liver Transpl Surg. 1999;5:29-34.

15. Zahid Hussain (2011). Hepatitis A: Clinical, Epidemiological and Molecular Characteristics, Viral Hepatitis - Selected Issues of Pathogenesis and Diagnostics, Dr. Sergey Mukomolov (Ed.), ISBN: 978-953-307-760-4, InTech, Available from: http://www.intechopen.com/books/viral-hepatitis-selected-issues-of-pathogenesis-and-diagnostics/hepatitis-a-clinical-epidemiological-and-molecular-characteristics
16. Bluger A, Novitskyi I. Viral hepatitis. 1988 (In Russian).
17. Petrov A. Upon some contemporary aspects in the clinical course and diagnosis of viral hepatitis A. Thesis. 2007 (In Bulgarian). Table 3: Biochemical characteristics among adults and children

RELATION BETWEEN HEPATITIS B SURFACE ANTIGEN KINETIC AND VIRAL REPLICATION IN PATIENTS WITH RESOLVED HEPATITIS B VIRUS INFECTION

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ABSTRACT:

Serum hepatitis B surface antigen (HBsAg) is a reliable marker in the diagnosis of hepatitis B virus infection. HBsAg levels reflect the transcriptional activity of the virus at the same time and are considered a surrogate marker of infected cells. The relationship between the presence of HBV DNA and HBsAg kinetics in sera of inactive carrier was analyzed in the present study; patient with treated HBV mono-infection; and patient with self-limited HBV mono-infection. The sera levels of HBsAg were measured by ELISA and HBV DNA concentration – by real time PCR. The HBsAg kinetics was modeled and correlation between these two parameters was evaluated in order to determine the importance of HBsAg kinetic as a marker for HBV infection clearance.

Key words: HBsAg, HBV DNA, HBsAg kinetic

ABBREVIATIONS USED IN THIS PAPER:

HBsAg - hepatitis B surface antigen, HBV – hepatitis B virus, PCR – polymerase chain reaction, ELISA – enzyme linked immunosorbent assay, anti-HBe - hepatitis B e antigen, ant-HBc IgM – antibodies class IgM against hepatitis B core antigen, CO – cut off, IU/ml – International Units per milliliter.

INTRODUCTION

The detection of hepatitis B surface antigen (HBsAg) in serum is the main serological marker for diagnosis of hepatitis B virus (HBV) infection. The elimination of HBV is a final goal of successful outcome of chronic and acute hepatitis B infection in treated and untreated patients (1). Serum HBsAg levels and HBV DNA concentration differentiate inactive carriers from patients with active disease (2). It was established that serum HBsAg correlate with transcriptionally active covalently closed circular DNA and is considered as a surrogate marker of infected cells (3). The incidence of spontaneous HBsAg seroclearance which is rare annual event in the natural history of HBV infection varied from 0.12% to 2.38% in cohorts from Asian countries and from 0.54% to 1.98% in cohorts from western countries (4). HBsAg levels differ significantly during the natural history of HBV infection – progressively decline from immune tolerance to inactive phase. At the same time they decline more rapidly during antiviral treatment (5). According Wiegand at.al (6) a decline in HBsAg predicts clearance but does not correlate with quantitative HBV DNA levels in a cohort of organ transplant patients. The relationship between the concentration of HBV DNA and HBsAg kinetic was analyzed in the sera samples of patients with three different cases of HBV infection in the present study : 1) inactive carrier; 2) patient with treated HBV mono-infection; and 3) patient with self-limited HBV mono-infection. We evaluated the correlation between these two parameters in order to determine their importance as a marker for resolving HBV infection.

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PATIENTS AND METHODS

The patient cohort consists of three separate cases:

Patient 1: At intervals of three time points for one year (2012) the blood samples of a 48-year-old asymptomatic HBV carrier were tested for the presence of HBsAg and the HBV DNA. The patient was anti-HBe antigen positive during the followed period. Correlation between HBsAg levels and concentration of HVB DNA was monitored in order to follow spontaneous HBsAg seroclearance.

Patient 2: A 32 -year-old women diagnosed at November 2011 with acute viral hepatitis type B. She was HBsAg and anti-HBc IgM positive. The patient was hospitalized with elevated levels of hepatic enzymes – ASAT, ALAT and GGT and treated with Zeffix 100 mg per day in combination with hepatic protectors – transmetil, karzil and cynarix. The period of hospitalization was 18 days. During the whole period the patient was anti-HBe positive.

Patient 3: A 59 -year-old man was tested for the presence of HBsAg and HBV DNA to confirm the suspicion for HBV infection. At the beginning of

Table 1. Correlation between HBV DNA concentration and HBsAg levels.

Sera samples (total = 31)	HBV DNA		HBV serology	
	[IU/ml]	[copies/ml]	HBsAg (S/CO)	Others
		Patient 1		
1.1	2.41E+01	140	+ (12.01)	Anti-HBe (+)
1.2	< 2.00E+01	<116	+ (4.13)	Anti-HBe (+)
1.3	< 2.00E+01	<116	negative (0.91)	Anti-HBe (+)
		Patient 2		
2.1	5.00E+05	2 910 000	+ (61.14)	Anti-HBc IgM (+) Anti-HBe (+)
2.2	8.41E+01	472	negative (0.35)	Anti-HBe (+)
		Patient 3		
3.1	2.02E+06	11 756 400	+ (48.11)	Anti-HBc IgM (+) HBeAg (-) Anti-HBe (+)
3.2	< 2.00E+01	<116	negative (0.31)	HBeAg (-) Anti-HBe (+)

infection the patient was anti-HBc IgM positive, HBeAg negative and anti-HBe positive.

Serological assays (ELISA)

Detection of HBsAg was done by SURASE B-96 ELISA kit (GmbH, Germany) for qualitative detection elevated levels of HBsAg in serum or plasma according manufacture instructions. Results were reported using S/CO ratios (signal-to-cut off ratio) and HBsAg was considered positive when the S/CO ratio was greater then 1.

Detection of anti-HBe was done by HBe Ag&Ab ELISA kit (DIA.PRO, Italy); and of anti-HBc IgM – by HBc IgM ELISA kit (DIA.PRO, Italy) according manufacture instructions.

Determination of HBV DNA concentration

Serum HBV viral load was determinate with COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Diagnostics GmbH) with the analytical measurement range that can be directly measured on a specimen without any dilution from < 2.00E + 01 IU/ml to > 1.70E + 08 IU/ml. The conversion factor between HBV copies/ml and HBV IU/ml is 5.82 copies/IU using the WHO International Standard for HBV DNA for nucleic acid technology assays testing – NIBSC 97/746.

RESULTS

A correlation between HBsAg levels and concentration of HVB DNA was monitored in the case of patient 1 in order to follow spontaneous HBsAg seroclearance. HBV DNA was measured at three time points and the concentration decreased from 140 copies/ml for the first samples to under limit of detection for the second and third samples <116 copie/ml but still detectable. A correlation between DNA concentration and serum levels of HBsAg (S/CO) was 12.01 for the first sera sample (1.1), 4.13 for the second sample (1.2) and negative value for the

third (1.3) as can be seen from table 1.

The serum levels of HBsAg and HBV DNA were measured at a two time points in the cases of treated and untreated acute HBV infection – patient 2 and patient 3. The patient 2 was HBsAg positive with S/CO equal of 61.14 and DNA concentration - of 2 910 000 copies/ml (2.1) before treatment (November 2011). The patient showed improvement and the followed parameters were measured again after finishing the treatment, December 2011. The sera sample was negative for the presence of HBsAg and the HBV concentration rapidly decreased to 472 copies/ml (2.2).

The serum level of HBsAg (S/CO) was 48.11 and concentration of HBV DNA was 11 756 400 copies/ml in the first sera samples of patient 3 (3.1) that was collect at the beginning of infection. We established that the patients were negative for the presence of HBsAg and concentration of HBV DNA rapidly decreased to <116 copie/ml but was still detectable for the second sera sample (3.2).

The kinetics of serum HBsAg in patient 1 was differing from those in patients with acute HBV infection (Figure 1). The seroclearance in a setting of inactive carrier lead to a more slowly reduce of the mean HBsAg levels from 12.01 through 4.13 to negative value – S/CO < 1. The patterns of HBsAg kinetics in settings of treated (patient 2) and untreated (patient 3) with acute HBV infection were similar with rapid decline from 61.14, respectively 48.11 to negative values.

Figure 1. HBsAg kinetics in sera of inactive HBV carrier, in treated and in untreated patients with acute HBV infection. HBsAg was considered positive when the S/CO ratio was greater then 1.

DISCUSSION

Results of the present study demonstrated clearly that it is important to perform correct interpretation of correlation between the main serological markers and quantity of HBV DNA in the settings of HBV infection. Serum HBV DNA levels have become very important as a marker for clinical stage and prognosis of HBV infection. It was established that in 20% of patient in low-replicative phase of HBV infection the HBsAg levels were high even the concentration of HBV DNA was below the detection limit of the TaqMan PCR (7) in a study of European HBsAg-positive patients. In the case 1, where the process of seroclearance was followed, the strong correlation between HBsAg and HBV DNA serum levels was measured (Table1). A progressive reduction of HBV DNA was established - from 140 copies/ml in the first sera sample to less than 116 copies/ml in the second and the third samples, with simultaneously slow decrease of HBsAg serum levels respectively from 12.01 in the first sample, 4.13 – in the second and to negative – in the third(Figure 1). The presence of HBV DNA in concentration less then 20 IU/ml, (116 copies/ml), in case of HBsAg clearance from chronic HBV carriers was established and by other authors (8). Recently it was established that during seroclearance serum HBsAg levels correlate with intrahepatic DNA levels (9) and decline with immune clearance (10). High serum levels of HBsAg, respectively 61.14 and 48.11, were matched with HBV DNA concentration > 2 000 000 copies/ml in cases 2 and 3 with active HBV mono-infection. The strong positive correlation between serum HBsAg and HBV-DNA was observed during prospective study of acute HBV and by Jaroszewicz J., et.al. (7). HBV DNA concentration simultaneously declined from 2 910 000 copies/ml to 472 copies/ml in the case of the treated patients (2.1 and 2.2) with the rapid decreased of HBsAg (figure 1) – from 61.14 to negative value. The rapid decline of HBsAg levels during antiviral treatment was established and by other authors (4). The same correlation was measured and in the case of self-limited HBV infection (3.1 and 3.2), with rapid decrease in HBsAg levels (figure 1) from 48.11 to negative value and simultaneous rapid decrease in HBV DNA concentration from 11 756 400 copies/ml to less then 116 copies/ml. In untreated patients HBsAg levels differ significantly during the four phases of HBV infection with progressive declines from immune tolerance to inactive phase (7) that is seen in the present study.

Hepatitis B surface antigen is the first serological marker used to confirm presence of HBV infection and its disappearance marks outcome of infection. Determination of HBsAg levels into blood sera not only helps to follow the course of HBV infection but also predicts the clinical outcome of infection and the identification of the inactive carriers. The detection of HBsAg by ELISA assay is done routinely in many diagnostic laboratories. It is possible to predict changes in HBV DNA concentration based on the value of HBsAg and the kinetic of its reduction. This will help to facilitate the identification of patients with good survival prognosis and will reduce the costs for determination of HBV DNA.

Conflict of interest: The authors have no conflict of interest to declare.

REFERENCES:

1. Liaw Y-F. *Clinical utility of hepatitis B surface antigen quantitation in patients with chronic hepatitis B: a review*. Hepatology. 2011; 53(6): 2121-2129.
2. Sonneveld MJ, Rijckborst V, Boucher CA, et al. *A comparison of two assays for quantification of Hepatitis B surface Antigen in patients with chronic hepatitis B*. J Clin Virol. 2011; 51(3): 175-178.
3. Brunetto MR. *A new role for an old marker, HBsAg*. J Hepatol. 2010;52(4):475-477
4. Chu C-M, Liaw Y-F. *Hepatitis B surface antigen seroclearance during chronic HBV infection*. Antivir Ther. 2010; 15(2):133-143.
5. Vigano M, Lampertico P. *Clinical implications of HBsAg quantification in patients with chronic hepatitis B*. Saudi J Gastroenterol. 2012; 18(2): 81-86.
6. Wiegand J, Wedemeyer H, Finger A, et.al. *A decline in hepatitis B virus surface antigen (HBsAg) predicts clearance, but does not correlate with quantitative HBeAg or HBV DNA levels*. Antivir Ther. 2008, 13(4): 547-554.
7. Jaroszewicz J, Serrano BC, Wursthorn K, et.al. *Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: A European perspective*. J Hepatol. 2010; 52(4): 514-522.
8. Cheng HR, Liu CJ, Tseng TC, et.al. *Host genetic factors affecting spontaneous HBsAg seroclearance in chronic hepatitis B patients.* PLoS ONE. 2013; 8(1): e53008.
9. Chan HL, Wong VW, Tse AM, et al . *Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response*. Clin Gastroenterol Hepatol. 2007; 5(12): 1462-1468.
10. Chan HL, Wong VW, Wong GL, et al. *A longitudinal study on the natural history of serum HBsAg changes in chronic hepatitis B*. Hepatology. 2010; 52(4): 1232-1241.

GASTROENTERAL OUTBREAK OF MIXED VIRUSES IN SAMOKOV, BULGARIA AUGUST-OCTOBER 2013

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ABSTRACT

Background

Acute gastroenteritis (AGE) is a notifiable disease in Bulgaria in accordance with the national legislation. Annual CDs analyses of Sofia region show that for the last 7 years the incidence of acute gastroenteritis rises from 105.02 per 100 000 in 2006 to 193.94 per 100 000 in 2012 but the proportion of affected children < 5 years decreases from 68.5% in 2006 to 52.92% in 2012. Most of the cases are from Roma minorities living in the region, which comprise 7.4% of all local population [1]. The seasonality of the disease is from May to October and only people with severe illness are reported as cases of AGE based on their hospital admission to infectious wards.

Key words: outbreak, noroviruses, rotaviruses, enteroviruses, Roma population

MATERIALS AND METHODS

The outbreak analysis was done by using data of conducted epidemiological investigations provided by Regional Health Inspectorate – Sofia region together with results of laboratory testing performed by National Reference Laboratory of Enteroviruses.

Conclusion

Between 1 August and 16 October 2013, an outbreak of mixed viral gastroenteritis caused by norovirus genogroup II, enterovirus and rotavirus group A occurred in Samokov, Bulgaria affecting local Roma community. Sixty-six per cent of the cases (56) were children < 5 years. The conducted outbreak investigation revealed that the most probable route of infection is person-to-person transmission in the living area with poor hygiene conditions and existence of illegal landfill sites.

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INTRODUCTION

Acute gastroenteritis (AGE) is a notifiable disease in Bulgaria in accordance with the national legislation. All medical doctors, mostly GPs, infectious diseases specialists and hospital doctors are oblige to report to the regional public health authorities any person who meets the criteria for AGE (Box 1).

Box 1. Definition of case of AGE
Clinical criteria: Any person with malaise, nausea, vomiting and/or diarrhoea syndrome, with or without fever
Laboratory criteria:
- Isolation of bacterial causative agents for gastroenteritis or enterocolitis other than Campylobacter, Salmonella, Shigella, pathogenic E. Coli, Vibrio cholerae, Listeria, Yersinia enterocolitica;
- Biochemical bacterial identification;
- Isolation of bacterial nucleic acid;
- Antigen detection of some viral causative agents (excl. *Rotavirus*) for gastroenteritis or enterocolitis in clinical specimen *(Adenovirus*, *Norovirus, Astrovirus);*
- Detection of viral nucleic acid in clinical specimen (*Adenovirus*, *Norovirus, Astrovirus)*
and
Epidemiological criteria:
- Person-to-person transmission
- Common source of infection
- Consumption of contaminated food/drinkable water
- Animal-to-human transmission
- Environmental exposure
Case classification:
Possible case: a case that meets clinical criteria.
Probable case: a case that meets clinical and epidemiological criteria.
Confirmed case: a case that meets clinical and laboratory criteria.

MATERIAL AND METHODS, RESULTS

In the beginning of August 2013, the number of AGE cases in Sofia region started to increase reflecting the increasing of the reported cases from Samokov, a town located in southwestern part of the region with approx. 27,000 population.

For the period, 01 August - 16 October 2013, 85 cases were registered and investigated as 81% of them developed the disease between 1 August and 10 September (Figure 1). The detected cases were only those people who sought medical care and afterward were admitted to hospital for adequate treatment.

The Regional Healthcare Inspection (RHI) initiated an outbreak investigation aiming to identify the causative agent, to describe the extent and severity of the outbreak, and to identify potential risk factors.

The disease spread throughout the town but 75 % of cases were localized in Roma minority neighborhood. Seven family clusters with two affected persons were identified. Sixty-six per cent of all cases were children < 5 years. The sex distribution was equal - 44 females: 41 males. The clinical course of the disease was with duration of 1-4 days and included fever ≤ 38°C, malaise, nausea, repeated vomiting, abdominal pain and 1-2 diarrheal stools.

All GPs and Emergency services in the region were asked to report on a daily basis the number of ill people who visit or consult health services with symptoms of nausea, malaise, vomiting and anorexia regardless if they have or have not diarrhea. From 21 August to 23 September 2013, a total of 360 visits were reported to RHI compared with 42 officially registered cases of AGE for the same period.

In order to determine the source or route of ongoing infection RHI formal informed the Regional Food Safety Agency for the situation but no specific food neither foods item were detected as a potential risk factor.

Following complaint of Samokov residents about regular problems with municipal water supplying the monitoring of drinking water quality from the waterworks and affected households was intensified. The local water authorities denied any water supply stoppage or pipes reconstructions and the analyses showed that water was safe for drinking. Therefore no environmental sampling and testing was performed. Nevertheless, the water chlorine concentration was raised to 0.6 mg/l for 10 days as a precautious measure.

Intensified direct personal contacts were considered as the most probable route of infection transmission in the Roma neighborhood, an area with poor hygiene and living conditions and existence of illegal landfill sites. The city government was timely alerted to take immediately sanitation actions in order to confine spread of the disease.

General information about the nature of the disease, ways of transmission and prevention was populated among public through leaflets and TV announcements.

LABORATORY ANALYSIS

Laboratory analysis for bacterial agents (Salmonella, Shigella and E.coli) was performed for all hospitalized patients but only in one person (1.18%), the test was positive for Sh.flexneri. Virological testing was done for children < 5 years as a part of National health insurance fund' hospital treatment requirements. Faecal specimens from 25 children were sent to the National Reference Laboratory of Enteroviruses and were tested for the presence of rotaviruses group A using enzyme linked immunosorbent assay (ELISA) and for noroviruses genogroup I and II and enteroviruses by 2-step reaction of reverse transcription followed by real-time polymerase chain reaction (RT-qPCR). Eighty per cent of all examined patients were positive for one or more viral pathogens revealing that the outbreak was with viral origin (Table 1).

Table 1. Results of virological laboratory testing

No of children examined	Positive for Norovirus genogroup II	Positive for Enterovirus	Positive for Rotavirus group A
25	11/44.0%	8/32.0%	7/28.0%

One child was with mixed infection caused by Sh.flexneri and Enterovirus and six children - with dual viral infection caused by Norovirus genogroup II and Enterovirus.

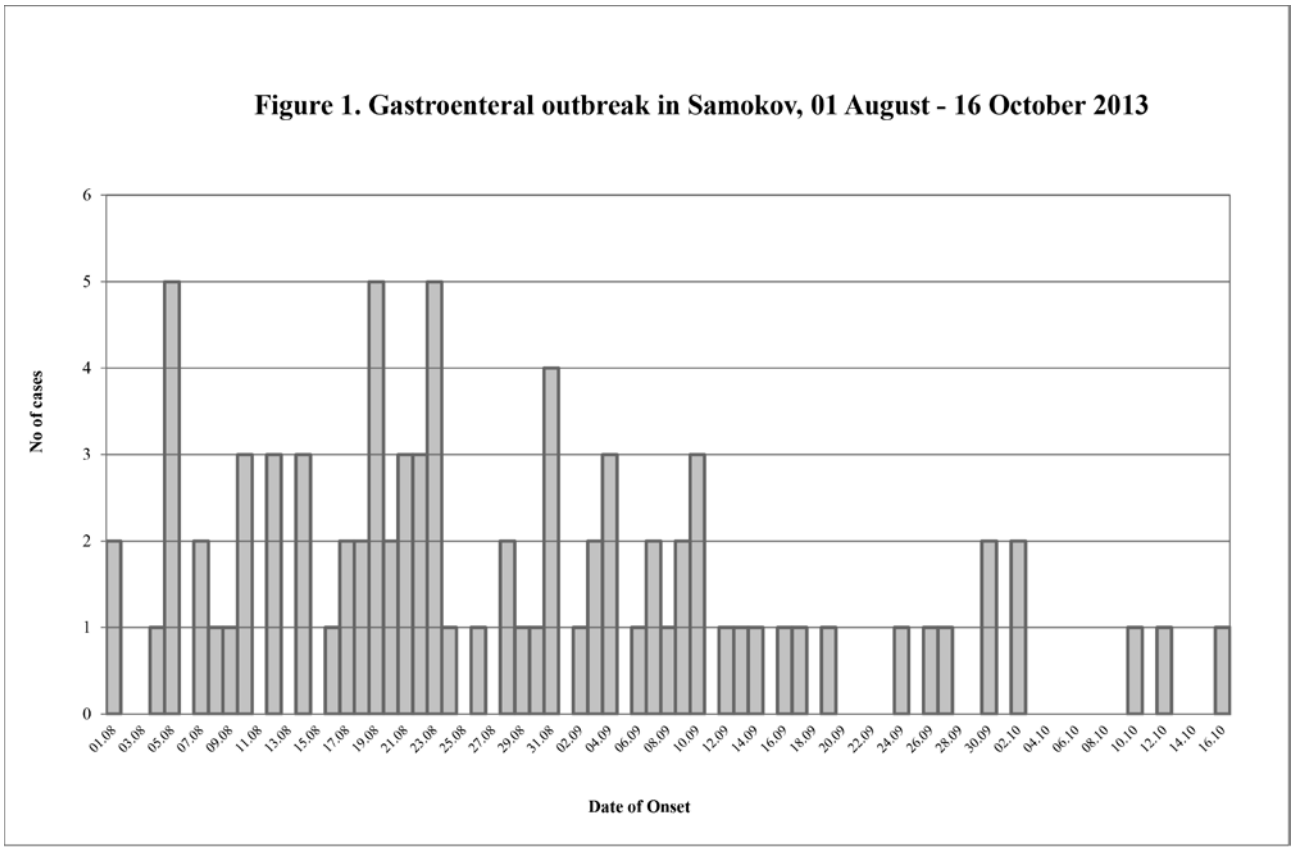
DISCUSSION

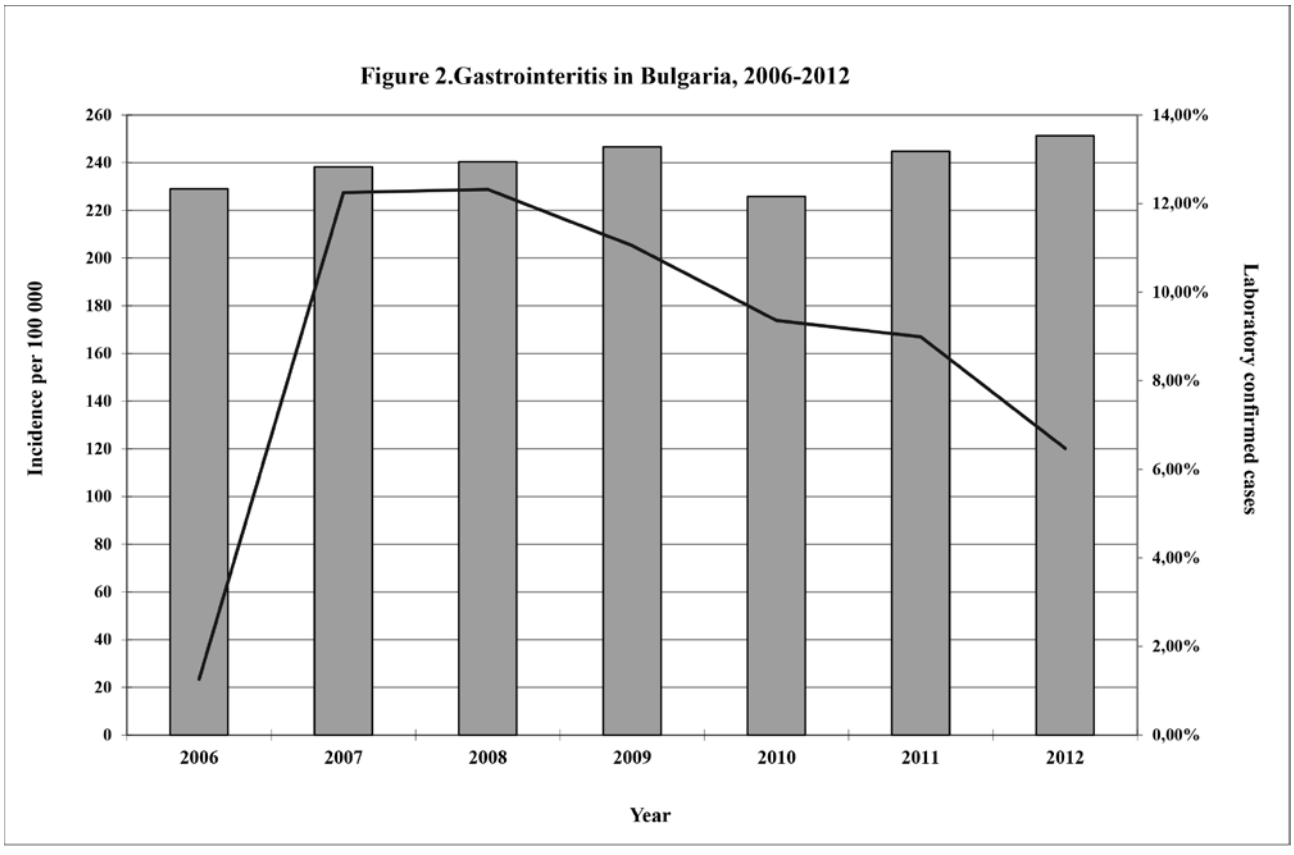
The incidence of acute gastroenteritis in Bulgaria is usually high but less than 10 % of all reported cases are laboratory confirmed mainly as caused by bacteria (Figure 2). Up to 86 gastroenteral outbreaks were registered in the country for the last 7 years (2006-2012) with 1,530 affected people. 55.81% of them were as a result of food poisoning or other bacterial origin; 29.07% were unspecified and only 15.12% were confirmed as viral outbreaks due to rotaviruses (11 outbreaks / 77 cases), enteroviruses (1 outbreak / 15 cases) or noroviruses (1 outbreak / 89 cases) [2].

Outbreaks of viral gastroenteritis are widespread among infants and children and different viruses are responsible for their suffering mainly noroviruses and rotaviruses [3-6]. 83.5% of all affected people in Samokov outbreak were children, 56 of them less than 5 years.

The gastroenteritis outbreak presented in this report was caused by several viral pathogens each of them have been often identified in Bulgaria during the end of the summer and early in the autumn (August to October) [7,8]. Enterovirus infections are typical for the summer months but the rotavirus and norovirus outbreaks have been also reported for this season.

The cases of viral gastroenteritis are underreported in Bulgaria. On one side, the GPs report only cases of diarrheal infections with severe clinical manifestation. Most of the other cases are misclassified as acute viral infection, which is not notifiable infectious category in Bulgaria. During the intensified monitoring of AGE prevalence in Sofia region from late August to late September was highlighted that only 11.67% of all ill people was registered as cases. This leads to underestimating of the real presence of viral gastroenteritis as well as confounding the existence of viral outbreaks. On the other side, the laboratory testing for noroviruses and adenoviruses in the country is provided only at National Reference Laboratory of Enteroviruses, located at Sofia city. The remoteness of laboratory settings for diagnose' verification and confirmation is a crucial obstacle for promptly viral outbreak detection and management. The lack of regional laboratory network for viral agents and absence of routine virological testing make difficult surveillance of cases of viral gastroenteritis in terms of their real magnitude defining and social burden [9]. The viral outbreaks emerge in consequence





of food or water contamination but person-to-person transmission is probably the often mode of infection [10]. The Roma minority in Samokov town comprises approx. 18.5% of all local residents mainly concentrated in one neighborhood [1]. Existence of ethnical communities living in overcrowded conditions with inadequate water and sanitation systems, more often expose to sick family members or other contacts who have delayed infectious disease treatment because of poor or different understanding of infectious risks is an additional contributable factor for widespread viral infection transmission.

References:

1. http://www.nsi.bg/census2011/pagebg2.hp?p2=175&sp2=190.
2. Communicable diseases in Bulgaria. Basic epidemiological indicators. 2006-2012.
3. Li CSY, Paul KS, Tang CJW. Prevalence of diarrhea viruses in hospitalized children in Hong Kong in 2008. J Med Virol 2009 81(11):1903-1911.
4. Oh DY, Gaedicke G, Schreier E. Viral agents of acute gastroenteritis in German children: prevalence and molecular diversity. J Med Virol 2003 71:82–93.
5. Mellou et al.: Epidemiological investigation of two parallel gastroenteritis outbreaks in school settings. BMC Public Health 2013 13:241.
6. Nelson EAS, Bresee JS, Parashar UD, Widdowson M-A, Glass RI (2008) Rotavirus epidemiology: the Asian Rotavirus surveillance network. Vaccine 26:3192–3196.
7. Mladenova Z, Korsun N, Geonova T, Iturriza-Gómara M; Rotavirus Study Group. Molecular epidemiology of rotaviruses in Bulgaria: annual shift of the predominant genotype. Eur J Clin Microbiol Infect Dis. 2010; 29(5):555-562. doi: 10.1007/s10096-010-0895-1.
8. Mladenova Z, Buttinelli G, Dikova A, Stoyanova A, Troyancheva M, Komitova R, Stoycheva M, Pekova L, Parmakova K, Fiore L. Aseptic meningitis outbreak caused by Echovirus 30 in two regions in Bulgaria, May-August 2012. Epid & Infec, in press.
9. Hedberg CW, Osterholm MT. Outbreaks of food-borne and waterborne viral gastroenteritis. Clin Microbiol 1993 Rev 6(3):199–210.
10. Lopman BA, Reacher MH, van Duijnhoven Y, Hanon FX, Brown D, Koopmans M (2003) Viral gastroenteritis outbreaks in Europe, 1995–2000. Emerg Infect Dis 9(1):90–96.

SOIL – TRANSMITTED HELMINTH INFECTIONS IN BULGARIA: A RETROSPECTIVE STUDY OF SOME EPIDEMIOLOGICAL FEATURES

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ABSTRACT

Background. Soil transmitted helminth infections are widespread throughout the world and represent significant problem for public health care in many countries. In Bulgaria with local distribution are two parasitic diseases - ascariasis and trichuriasis (trichocephaliasis) but exist favorable climatic conditions for local distribution of ancylostomiasis and necatoriasis. We did retrospective study of prevalence of ascariasis and trichuriasis in Bulgaria for 23 year period and compared our data to these collected in 1952-1954 when the first systematic studies on the subject were held.

Key words: Soil - transmitted helminthiases, ascariasis, trichiuriasis, prevalence, insidence

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Introduction. Soil - transmitted helminthiases (STH) are among the most frequent and widespread helminth diseases affecting people living in poor areas of developing countries. Four most common parasitic worm infections in this group are: ascariasis – caused by the roundworm *Ascaris lumbricoides*, trichuriasis (trichocephaliasis) – caused by the whipworm *Trichocephalus trichiurus (Trichiuris trichiura)*, ancylostomiasis – caused by the hookworm *Ancylostoma duodenale* and necatoriasis – a parasitic infection caused by the hookworm *Necator americanus.* The clinical course and severity of STH depends on the extent of invasion. Approximately 300 million people with a high degree of parasitic invasion develop severe clinical symptoms each year and more than 150,000 die (1, 2). According to WHO data from 2002 about 29% of the world population is infected with STH. Over 270 million preschool children and over 600 million school-age children are living in areas of intense transmission, and in need of anthelminthic treatment and other measures for prevention and control of STH (3).

In the Republic of Bulgaria ascariasis and trichuriasis are with local transmission as actual and potentially endemic for the two geohelminthiases are the semi mountainous and high valley areas in the country. First detailed studies on the subject were held in 1953-1954, from Genov et al. The research covered over 650 000 people and has been found that the mean prevalence of ascariasis in the country is 5.75% and of trichuriasis is 1.48% (4, 5). The high proportion of affected population at that time necessitated to impose measures reducing the widespread of ascariasis and trichuriasis. Such measures included careful study of the local epidemiological patterns of distribution of these two geohelmintic infections, sanitation of the affected settlements, extensive health education among the population and mass anthelmintic treatment. As a result of the above actions was achieved a long term steady decrease in the incidence and prevalence of ascariasis and trichuriasis in the country.

MATERIALS AND METHODS. In Bulgaria all laboratory confirmed cases of human infections caused by STH are subject to a compulsory notification. This is a retrospective study of data collected from the annual analyses of parasitic diseases in the country (6 - 12) and particularly ascariasis and trichuriasis for the period 1990 – 2012. Data were compared with those from the first large-scale study of the two parasitic infections committed by Genov et al. 1952 – 1954 (4). The annual analyses are based on annual reports from the Regional Health Inspectorates (RHI) for surveillance and control of the parasitic diseases.

Calculated were the prevalence proportions for ascariasis and trichuriasis by year, by 5 - year periods, and also the average prevalence for the whole period 1990 – 2011. Mapped were the country areas with highest level of invasion.

Results. In 14 out of 28 districts in Bulgaria the geographic and climatic conditions are favorable for distribution and transmission of STH (Fig.1).

Fig. 1 Geographical distribution of STH in Bulgaria. The districts in Bulgaria with favorable geographic and climatic conditions for distribution and transmission of STH are in yellow.

Table 1. Number of recorded cases, prevalence and incidence of ascariasis and trichiuriasis in Bulgaria by years

	Ascariasis			Trchiuriasis		
Year	Number of cases per year	Annual prevalence (%)	Annual incidence per 100 000	Number of cases per year	Annual prevalence (%)	Annual incidence per 100 000
1990	651	0.24%	7,24	282	0,10%	3,14
1991	395	0.20%	4,40	87	0,04%	0,97
1992	537	0.19%	6,33	77	0,03%	0,91
1993	774	0.31%	9,15	148	0,07%	1,75
1994	638	0.26%	7,57	187	0,08%	2,22
1995	640	0.23%	7,63	145	0,05%	1,73
1996	638	0.26%	7,65	187	0,08%	2,24
1997	520	0.24%	6,28	180	0,08%	2,17
1998	783	0.61%	9,51	100	0,17%	1,22
1999	862	0.74%	10,52	122	0,11%	1,49
2000	109	0.71%	13,42	218	0,14%	2,68
2001	996	0.71%	12,62	258	0,18%	3,27
2002	932	0.56%	11,88	252	0,15%	3,21
2003	957	0.50%	12,27	262	0,17%	3,36
2004	736	0.43%	9,48	126	0,10%	1,62
2005	714	0.38%	9,25	192	0,10%	2,49
2006	829	0.24%	10,80	211	0,06%	2,75
2007	942	0.20%	12,33	142	0,04%	1,86
2008	779	0.16%	10,24	129	0,03%	1,70
2009	775	0.15%	10,25	109	0,02%	1,44
2010	700	0.13%	9,33	109	0,02%	1,45
2011	535	0.10%	7,26	118	0,02%	1,60
2012	593	0,10%	8,09	54	0,01%	0,74

Cases of ascariasis and trichuriasis are registered by turnover, clinical and prophylactic indications in both endemic and non-endemic regions in the country.

Our study found that for a period of 23 years (1990-2012) were recorded a total of 17 020 persons (children and adults) with ascariasis and 3695 with trichuriasis. The average annual number of cases for the period of ascariasis was 740, and for trichuriasis - 161. Data on the number of recorded cases, prevalence and incidence are shown in Table 1.

Ascariasis. Comparison between the prevalence of ascariasis established for the country from the first study in 1952-1954 with the prevalence found for the period from1990 to 2011 shows a decrease over 50 times – from 5.75% in 1954 to 0.10% in 2012. On Fig.2 are presented the average values of prevalence for every 5 years. Unlike the prevalence, the incidence per 100 000 exhibits greater stability over time, as the average of this index for the period was 28.9 per 100 000 (Fig. 3).

Trichuriasis. Data for trichuriasis showed that its degree of prevalence was reduced about 37 times - from

Fig. 2 Average values of ascariasis prevalence for every 5 years

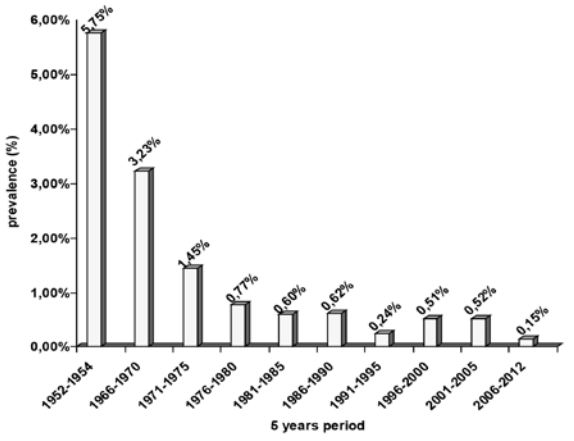


Fig. 3 Annual incidence of ascariasis and trichiuriasis per 100 000 by years (1990-2012)

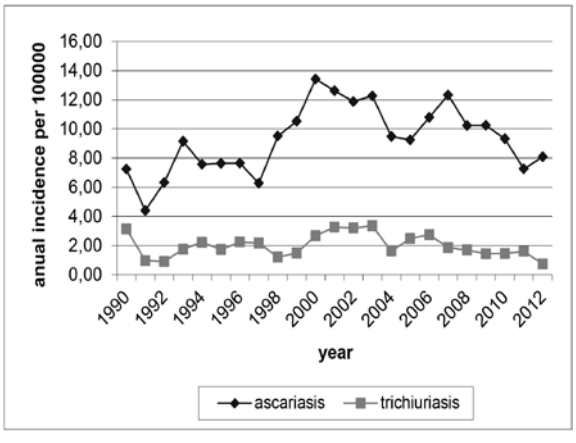


Fig. 4 Average values of trichiuriasis prevalence for every 5 years

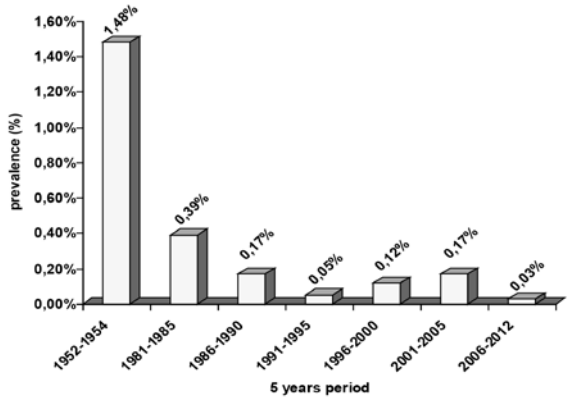


Fig. 5 Distribution of ascariasis cases in the country and social facilities

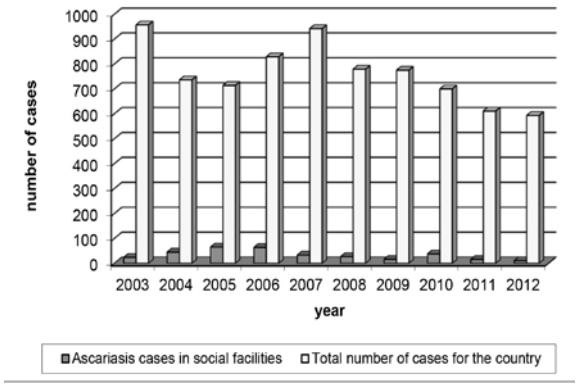


Fig. 6 Distribution of trichiuriasis cases in the country and social facilities

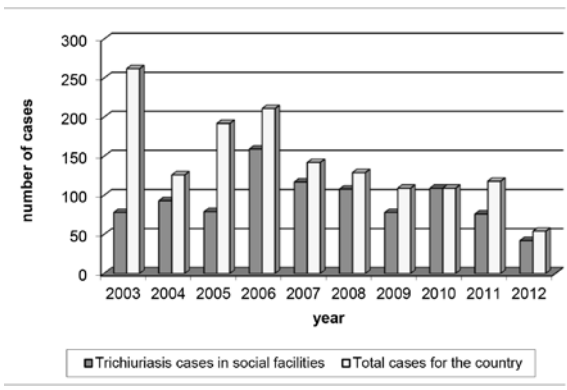
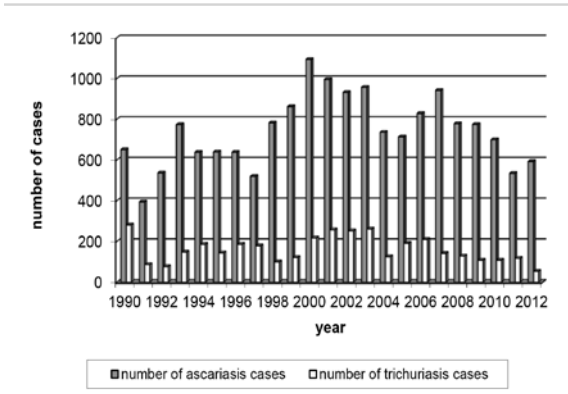


Fig. 7 Number of ascariasis and trichiuriasis cases by years (1990-2012)



1.48% (1952-1954.) to 0.03% (2006-2011) (Fig.4). It should be noted that this geohelminthosis is found predominantly among residents of care facilities such as orphanages and nursing homes but also in care facilities for children or adults with mental disabilities. As with ascariasis, the incidence was more stable over time and average incidence per 100 000 population for the period was 1.2 (Fig.3).

Ascariasis and trihuriasis in organized groups. Study on the STH in Bulgaria covering a period of 5 years – from 2003 to 2007 (13), provides interesting information on the prevalence of these diseases among different social groups of the population. Collected were data from annual reports for prevention control measures carried out in institutions for children deprived of parental care, as well as for children and adults with physical and / or mental disabilities. For the studied period in Bulgaria were functioning 222 social institutions, and average number of inhabitants for the period was 11,347.

The annual prevalence of ascariasis among people in care facilities is as follows: in 2003 from total of 957 persons with ascariasis for the whole country 24 (2.53%) were residents of care facilities; in 2004 – 45 from 736 (6.11%); in 2005 – 65 from 712 (9.12%); 2006 – 64 from 829 (7.72%); 2007 – 33 from 942(3.50%). It is noteworthy that the number of people with ascariasis in social institutions represent a significant proportion of the total number of infected in the country (Fig. 5).

Data for the next 5-year period (2008-2012) display some decrease in the number of reported cases - 108 out of 3382 for the country (3.19% of all cases).

Even more clearly the trend of relatively high proportion of infected people to be in social facilities is observed in trichuriasis. In recent years this STH primarily affects residents of care facilities. The ratio between the absolute number of infected in the country and in social institutions by year shows that a significant proportion of people with trihuriasis are residents of care facilities: in 2003 out of 262 infected, 78 (29.77%) were residents of care facilities; in 2004 – 93 from 126 (73.81%); in 2005 – 79 from 192 (41.14%); in 2006 – 159 from 211 (75.35%); in 2007 – 117 from 142 (82.39%); in 2008 – 108 from 129 (83.72%); in 2009 – 78 from 109 (71.56%); in 2010 – 109 from 109 (100%); in 2011 – 76 from 118 (64.41%); and in 2012 – 42 from 54 (77.78%) (Fig. 6).

As with ascariasis, the average prevalence of trihuriasis in care facilities exceeds the national average. The annual registration of cases in the social facilities reveal their importance as foci for STH transmission and the need of corresponding measures for their prevention and control.

DISCUSSION. After 2006 is observed a consistent trend of decline in the prevalence of ascariasis and trichuriasis. However, this is due to a significant increase in the number of persons covered by prophylactic laboratory tests, while the absolute number of infected people remained at relatively constant levels (Fig. 7).

For example in 2001 the recorded prevalence for ascariasis is 0.71% (996 persons infected from 139719 surveyed) and in 2007 is more than 3 times lower - 0.20%, but the number of infected persons is almost identical - 942, and tested were 462 576 people. This is a reason for our opinion that for countries with low levels of prevalence of STH, such as Bulgaria, the absolute number of reported cases and incidence per 100,000 are more useful indices for monitoring the local transmission among the population.

It is noteworthy that a large percentage of infected persons are residents of care facilities, which requires measures for control and surveillance of STH in these institutions.

CONCLUSION. STH are problem diseases in Bulgaria, as in the past and now, and cases are registered in both endemic and non-endemic regions in the country. Despite some fluctuations, the number of infected with ascariasis and trihuriasis in the last twenty years has been relatively stable. Their prevalence among the Bulgarian population is low, but cases of ascariasis and trichuriasis are registered annually in the country and their social and health significance can not be ignored as is the need of measures for their surveillance, control and the need for health education of the population.

References

1. Crompton, D.W. 1999. "How Much Helminthiasis Is There in the World?"Journal of Parasitology 85: 397–403.
2. Montresor, A., D. W. T. Crompton, T. W. Gyorkos, and L. Savioli. Helminth Control in School-Age Children: A Guide for Managers of Control Programmes. Geneva:World Health Organization. 2002.
3. WHO (World Health Organization). 2002. Prevention and Control of Schistosomiasis and Soil-Transmitted Helminthiasis. WHO Technical Series Report 912. Geneva: WHO.
4. Genov, G. Epidemiological study of the most important helminthic diseases among the population of the People`s Republic of Bulgaria and measures for their decrement. D. Sc. Thesis, 1974, 57 pp. (in Bulgarian)
5. Genov, G. Practical parasitology. Znanie, 1998, 310 pp. (in Bulgarian)
6. Kurdova, R., D. Jordanova, D. Vuchev, et al. Local and important parasitic diseases in Bulgaria and their surveillance and control in 2002. Inf. Journal NCIPD, 2003, 5:31 pp. (in Bulgarian)
7. Kurdova, R., D. Jordanova, D. Vuchev, et al. Current situation of the parasitic diseases among people in Bulgaria 2003 – 2005. Inf. Journal NCIPD, 2006, 3:24 pp. (in Bulgarian)
8. Kurdova, R., P. Petrov, D. Jordanova, et al. Parasitic morbidity in Bulgaria 2006 – current state, epidemiological control and prognosis. Inf. Journal NCIPD, 3:30 pp. (in Bulgarian)
9. Kurdova, R., P. Petrov, D. Jordanova, et al. Parasitic morbidity in Bulgaria for 2007 – 2008. Current situation and prognosis. Inf. Journal NCIPD, 2010, 5:30 pp. (in Bulgarian)
10. Kurdova, R., I. Rainova, D. Jordanova, et al. Current state of the local and important parasitic diseases in Bulgaria in 2009. Inf. Journal NCIPD, 2011, 1:32 pp. (in Bulgaria)
11. Kurdova, R., I. Rainova, D. Jordanova, et al. Characteristics of the parasitic incidence in Bulgaria for 2010. Inf. Journal NCIPD, 2011, 2:31 pp. (in Bulgarian)
12. Rainova, I., D. Jordanova, R. Harizanov, et al. Current state, surveillance and control of parasitic diseases in Bulgaria for 2011. Science Infectology Parasitology, 2013, 1(6):35-40 (in Bulgarian)
13. Harizanov, R., R. Kurdova, I. Rainova, et al. Intestinal parasites in the social facilities in Bulgaria for the period 2003 – 2007. Infectology, 2009, 4:28-31 (in Bulgarian)

ACTIVITY OF NITROFURANTOIN AGAINST UROPATHOGENIC ENTEROCOCCI

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ABSTRACT

Nitrofurantoin is an oral broad-spectrum antimicrobial agent recommended for treatment of urinary tract infections (UTIs). The drug is not used in the clinical practice in Bulgaria and local data is not available. The aim of the study was to assess the activities of nitrofurantoin and comparative, commonly used antimicrobials against uropathogenic isolates of enterococci. A total of 88 non-duplicate enetrococcal strains, isolated from urine of patients hospitalized in University Hospital Pleven, Bulgaria during 2012, were investigated. Identification of enterococci to the species level was based on series of conventional physiological tests. Susceptibility to antimicrobial agents was determined by using the disc diffusion method according to the recommendations of Clinical Laboratory Standards Institute (CLSI). Nitrofurantoin demonstrated higher activity (88.64%) against uropathogenic isolates of enterococci, compared to ciprofloxacin and ampicillin (34.09 and 67.05%), respectively. It was more active against *Enterococcus faecalis* (100%) than against *E. faecium* (67.74%). Nitrofurantoin retained its activity against 60% of multidrug-resistant *E.faecium* strains with concomitant resistance to ciprofloxacin and ampicillin. On the base of its superior activity we recommend this cheap antimicrobial agent for treatment and prevention of lower UTI.

Key words: *urinary tract infections, enterococci, nitrofurantoin*

ABBREVIATIONS USED IN THIS PAPER:

UTIs - Urinary tract infections,
CLSI - Clinical Laboratory Standards Institute

INTRODUCTION

Urinary tract infections (UTIs) are one of the most common bacterial infections. They are substantial medical and financial problem. According to the 1997 National Ambulatory Medical Care Survey and National Hospital Ambulatory Medical Care Survey, UTIs accounted for nearly 7 million office visits and 1 million emergency department visits, resulting in 100,000 hospitalizations with a total annual cost of approximately $2 billion [11, 22].

Although not being the most common etiologic agent, enterococci have been recognized as important uropathogens in community as well as in hospitals [3, 12, 20]. Over the last decade enterococcal UTIs have become a serious clinical challenge in our hospital. They have been documented as the second most frequent cause of nosocomial UTIs in our institution [17].

Enterococcal UTIs occur most frequently in patients below 10 and above 60 years of age [23] and affect patients with genitourinary anomalies [2] and obstructive uropathy[1]. Such UTIs are frequently associated with urinary catheterization and/or instrumentation as well as with preceding antimicrobial therapy [7]. Enterococcal urinary tract infection are often difficult to treat, due to specific characteristics of patient population and a limited number of antimicrobials with anti-enterococcal activity. *Enterococcus* species are known to have innate resistance to cephalosporins and trimethoprim-sulfamethoxazole, which are important drugs in UTI therapy. Furthermore, many of *E.faecium* strains have emerging multiple-resistance to other antimicrobials such as beta-lactams, aminoglycosides, and fluoroquinolones. This poses a serious challenge to clinicians, due to lack of therapeutic options.

In searching for therapeutic alternatives we take a look at an old, cheap antimicrobial agent with oral uptake. Nitrofurantoin is not sold in Bulgaria, so it is not routinely used in the clinical practice, thus local susceptibility data is not available. The present study was carried out to compare and assess the effect of nitrofurantoin and other commonly used antimicrobial agents on uropathogenic isolates of enterococci.

MATERIAL AND METHODS

A total of 88 non-duplicate enetrococcal strains were investigated. They were isolated from urine of patients with urinary tract infections, treated in University Hospital Pleven, Bulgaria during 2012. Identification of enterococci to the species level was based on series of conventional physiological tests [9, 10] and Vitek 2 automated system (bioMerieux, France). Susceptibility to ampicillin, gentamicin, ciprofloxacin, nitrofutantoin, vancomycin, and teicoplanin (BBL, Becton Dickinson, USA) was determined by using the disc diffusion method according to the recommendations of Clinical Laboratory Standards Institute (CLSI, 2012). Screening tests for high-level aminoglycoside resistance and vancomycin resistance were performed according to the CLSI standards [6]. Isolates were tested for beta-lactamase production with the chromogenic nitrocefin disc test (BBL, Becton Dickinson, USA). Multidrug resistance was defined as resistance to two or more classes of antimicrobial agents.

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Tabl.1. Susceptibility of urinary enterococcal isolates towards 6 antimicrobial agents.

Antimicrobial agent	Number (%) of strains:		
	S	I	R
Ampiciclin	59 (67.05)	-	29 (32.95)
Gentamicin	41 (46.59)	-	47 (53.40)
Ciprofloxacin	30 (34.09)	3 (3.41)	55 (62.50)
Vancomycin	88 (100.00)	0	0
Teicoplanin	88 (100.00)	0	0
Nitrofurantion	78 (88.64)	2 (2.27)	8 (9.09)

RESULTS

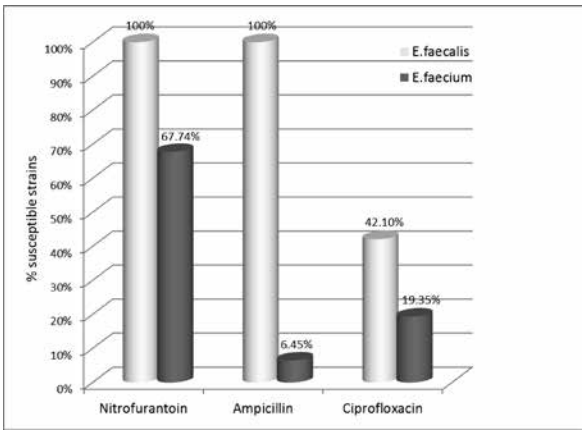
Among 88 urinary enterococcal isolates, 57 (65%) were identified as *Enterococcus faecalis* and 31 (35%) were identified as *Enterococcus faecium.*

The results from susceptibility testing of urinary enterococcal isolates towards 6 antimicrobial agents are presented in Table 1.

All enterococcal isolates were susceptible to vancomycin and teicoplanin and 88.64% of them were susceptible to nitrofurantoin. Uropathogenic enterococcal strains displayed moderate susceptibility to ampicillin (67.05%), but lower to gentamicin and ciprofloxacin (46.59 and 34.09%) respectively. None of the ampicillin resistant isolates produced beta lactamase.

Comparative data about the susceptibility of *E. faecalis* and *E. faecium* strains towards nitrofurantoin, ampicillin and ciprofloxacin are shown on the Figure 1.

Fig.1. Comparative data about the susceptibility of *E. faecalis* and *E. faecium* strains towards 3 selected antimicrobial agents.



E. faecalis strains were significantly more susceptible to antimicrobials recommended for UTI, than *E. faecium* strains. *E. faecalis* demonstrated uniform susceptibility to both antimicrobial agents: nitrofurantoin and ampicillin, and moderate (42.10%) to ciprofloxacin. On contrast *E. faecium* strains demonstrated low level of susceptibility to ciprofloxacin (19.35 %) and negligible susceptibility to ampicillin (6.45%), and only nitrofurantoin had encouraging results with susceptibility levels of approximately 68%.

Most of *E.faecium* strains demonstrated multiple- resistance to antimicrobial agents. Twenty-five (80.64 %) of *E.faecium* strains were resistant to ampicillin and ciprofloxacin simultaneously. Nitrofurantoin was found to be effective against 60% of such strains.

DISCUSSION

Nitrofurantoin is a synthetic nitrofuranic antimicrobial agent for oral administration, recommended for treatment of acute lower UTIs. Its low price and oral acceptance makes it convenient for long-term treatment of chronic infections and for the suppression of catheter-associated bacteriuria [8].

At the concentrations achieved in urine nitrofurantoin is bactericidal for many Gram-positive and Gram-negative bacteria. Although nitrofurantoin has been in use for 6 decades in clinical practice, bacterial resistance develops slowly due to its unique and broad-based mechanism of action. After enzymatic activation within the bacterial cell nitrofurantoin derivatives alter ribosomal proteins, DNA and other bacterial macromolecules. This results in inhibition of protein, DNA and cell wall synthesis [14]. There is no cross-resistance between nitrofurantoin and other antimicrobial agents and transferable resistance is a very rare phenomenon [16]. It has generally been reported that resistance to nitrofurantoin is low [5, 19].

According to the recommendations of producers, nitrofurantoin has excellent activity against *E.faecalis*, but the majority of *E.faecium* isolates are not susceptible [16]. On the other hand several studies [25, 26] have reported high susceptibility of *E.faecalis* and *E.faecium* to nitrofurantoin, including even vancomycin-resistant isolates.

Our results indicate that both enterococcal uropathogens were highly susceptible to nitrofurantoin. Better results were established only for vancomycin and teicoplanin; however, it is well known that glycopeptides are restricted to severe infections, and are not routinely used for treatment of UTIs. Nitrofurantoin was

found more active against our enterococcal uropathogens as compared to other commonly used antimicrobials: ampicillin and ciprofloxacin. This fact is most probably associated with the established high prevalence of *E.faecium* strains among our urinary isolates. Significant correlation between enterococcal species and resistance to nitrofurantoin was observed in our study. Nitrofurantoin was more active against *E.faecalis* than against *E.faecium*. We have documented that isolates of *E.faecalis* were uniformly susceptible to nitrofurantoin, which is in concordance with several studies carried in different parts of the world [5, 15, 19, 26]. However, development of resistance among *E.faecium* isolates has recently been reported. Rudy et al. in 2004 showed that only 50% of *E.faecium* strains isolated from children with urinary tract infection in Poland were susceptible to nitrofurantoin [21]. The rate of susceptibility demonstrated by our strains was more evident (68%) and similar to that reported by Butku et al. in Turkey (60.6%) [4], but lower than that reported by Zhanel et al. Their multi-center study in United States and Canada, investigating antibiotic activity against 697 UTI isolated vancomycin-resistant enterococci (88.4% *E.faecium*), revealed excellent activity of nitrofurantoin with only 0.6% of resistance [26].

On the other hand, the results of this study highlight the increased role of *E.faecium* as uropathogen in our hospital settings. This finding has important clinical impact due to its growing resistance to agents routinely used for treatment of UTIs. Our *E.faecium* strains were highly resistant to ampicillin and ciprofloxacin, and more than 80 % of them have demonstrated concomitant resistance to both drugs, leaving no therapeutic options. The poor activity of fluoroquinolones and ampicillin against multidrug-resistant isolates of *E.faecium* has been reported previously [18]. It is known that this phenomenon appears as good phenotypic marker of hospital-acquired *E.faecium* strains [13, 24]. Dissemination of such strains is a major threat and therapeutic challenge for the clinicians. We have demonstrated that nitrofurantoin retained its activity even against 60% of such problematic isolates, thus it was found to be the only alternative drug against uncomplicated *E.faecium* UTIs.

CONCLUSION

On the base of its superior activity against uropathogenic enterococci, we recommend this cheap antimicrobial agent as an alternative for treatment and prevention of lower UTIs in our hospital settings.

A part of this work was presented at the 11th International Medical Scientific Conference for Students and Young Doctors, Medical University-Pleven, 16-18 October, 2013, Сб. рез., стр.119

CONFLICT OF INTEREST STATEMENT (AUTHORS)

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