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PROBLEMS

of Infectious and Parasitic Diseases

NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 45, NUMBER 1/2017

ISSN 0204-9155

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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 45, NUMBER 1/2017

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BRUCELLOSIS - AN UNKNOWN AND UNDERDIAGNOSED INFECTION IN BULGARIA

R. Nenova, I. Tomova

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ABSTRACT

Brucellosis is still the most common zoonosis worldwide. Despite this, it is unknown and underdiagnosed infection in non-endemic areas. For some parts of the world it became a re-emerging infection. After several decades brucellosis re-emerged in Bulgaria. In 2005 an outbreak of imported human cases was detected and soon after, two autochthonous outbreaks occurred (2006 and 2015) with a total of 161 persons diagnosed.

Key words: brucellosis, re-emergence, outbreak, autochthonous, microbiological diagnosis.

Brucellosis, also known as Maltese fever, “undulant fever”, Gibraltar fever, Bang’s disease, is a zoonotic infection caused by microorganisms from the genus Brucella. It is a chronic relapsing disease known for millennia. Nowadays it still remains an important economic and medical problem, especially in endemic areas, where significant losses of livestock and high morbidity rate among human population are reported. In some other regions the disease remains unknown and underdiagnosed. After several decades brucellosis became a re-emerging infection in Bulgaria with the occurrence of two autochthonous outbreaks in 2006 and 2015 with a total of 161 diagnosed persons.

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THE CAUSATIVE AGENT

Genus *Brucella* is named in honour of David Bruce, who isolated *Brucella melitensis* (Table 1) in 1887 from a British soldier in Malta (1). A second species, *B. abortus* was isolated from cases of epizootic abortion in cows (2), and both agents were put together in the genus *Brucella* due to their similarity (3). Later Huddlestone described *B. suis*, which causes infectious abortions in pigs (4). In the 50s of the last century two other species have been identified: *B. ovis,* causing reproductive problems in sheep and *B. neotomae*, isolated from rats in the United States (5, 6). Further, the causative agent of abortion in dogs, *B. canis* was described in 1968 (7). Due to the very close genetic similarity (>90%) between the members of genus *Brucella* it is considered as monospecific genus, but different species are classified as biovars of *B. melitensis* (8). From a practical point of view (main reservoir) it is adopted that the traditional names of the species could be used for non-taxonomic purposes (9). In the 1990s some newly isolated strains, pathogenic for marine mammals and phenotypically different from the first six species were categorised as *Brucella* spp. (10, 11). They were named depending on the animals they affect: *Brucella ceti* sp. nov. (cetaceans as preferred hosts) and *B. pinnipedialis* sp. nov. (seals as preferred hosts) (12). The ninth described species, *B. microti,* was isolated from wild common voles and red foxes during epizootics in Central Europe, but was also recognized as a soil contaminant (13). Based on phenotypic and genotypic tests it was incorporated in genus *Brucella* and so far, there is no evidence for its pathogenicity for humans. Furthermore, another two strains (BO1 and BO2), similar to *B. ovis* were isolated from human clinical samples. Based on phenotypic and molecular analyses they were considered as a novel species, named *B. inopinata* (14, 15). The last described species, *B. vulpis* sp. nov., was isolated from Austrian red foxes and carries the *Brucella* specific IS711 and *bcsp*31, but 5% of its genome was acquired from different soil bacteria (16, 17).

Table 1. *Brucella* species according to their natural host and pathogenicity for humans.

***Brucella* spp.**	**Biovar**	**Natural host**	**Pathogenicity for humans**
B. melitensis	1-3	goats, sheep, camels	high
B. abortus	1-7, 9	cattle	variable
B. suis	1, 3 2 4 5	pigs pigs, hares Canadian deer and reindeer rodents	high low variable high
B. canis	-	dogs	low
B. ovis	-	sheep	not reported
B. neotome	-	rodents	not reported
B. ceti	-	cetaceans, dolphins, whales	human cases are reported
B. pinnipedialis	-	seals	human cases are reported
B. microti	-	common voles, red foxes	not reported
B inopinata	-	unknown	human cases are reported
B. vulpis		red foxes	not reported

Brucellae are small Gram-negative coccobacilli or short rods, which are facultative intracellular parasites. They are non-motile, non-spore forming, without true capsules, pili and natural plasmids. Although brucellae are aerobes, some types require additionally CO_2, especially for primary isolation. All members are fastidious and need rich peptone media supplemented with blood and/or serum. The lipopolysaccharide (LPS) of the cell wall is the immunodominant antigen. It cross-reacts with other Gram-negative bacteria such as *Yersinia enterocolitica* O9, *Escherichia coli* O157, *E. hermannii*, *Salmonella enterica* O:30, *Vibrio cholerae* O1 and *Stenotrophomonas maltophilia* (18). The protein antigens (outer membrane and cytoplasmic) have a protective effect against brucella by stimulating the cellular and humoral immune response in infected individuals. They are common for both - smooth and rough types and do not cross-react with other Gram-negative bacteria (19).

SOURCES AND ROUTES OF INFECTION

The main source of infection for humans are domestic animals. Their signifcance is determined by the most common host species (Table 1). Different types of *Brucella* spp. have different geographical distribution. The most widespread, in the Mediterranean region and the Arabian Peninsula is *B. melitensis*, which causes the majority of outbreaks. *B.abortus* is usually causative agent of sporadic cases or small outbreaks. *B. canis* is less pathogenic, mainly for immunodefcient persons, while *B. ovis* and *B. neotome* are non-pathogenic for humans. (19, 20, 21). There are reports that marine representatives can cause serious infections in humans, including laboratory acquired (22, 23, 24). Brucellae are released in large quantity with the fetus, placenta, and amniotic fuid during abortion and stillbirth, as well as with milk, urine, and vaginal secretions.

Brucellosis is mainly an occupational disease. Infection can occur via skin lesions and healthy mucosa (incl. conjunctiva) during breeding of infected animals. Quite often another way of human infection is consumption of unpasteurised dairy products and poorly heat-treated meat. Contamination of hands is also important for the oral route. Air-powder mode of infection is performed by inhalation of dust containing brucellae while working with leather, wool, and soil. Laboratory acquired infections occur via several mechanisms: production of infectious aerosols, accidental inoculation through skin and mucous membranes, or ingestion of infectious materials. (25). Other, less common routes are: blood transfusion (26), via placenta (27), breast feeding (28), or possible sexual transition (29). In endemic areas the infection rates are the same in children and adults and most often the disease occurred after consumption of contaminated food. In non-endemic areas, such as Bulgaria, mainly adults are infected (occupational or imported cases) (20, 30).

PATHOGENESIS

Brucellae tend to invade and survive in the host. They manage to avoid intracellular killing in phagocytic cells and multiply in macrophages. Through the circulatory system they reach the regional lymph nodes and afterwards -

various organs, mainly the reticuloendothelial system. These processes determine the diverse clinical manifestations of the disease (31). The intracellular survival of bacteria is facilitated by virulence factors such as LPS components, superoxide dismutase and some outer membrane proteins (19, 32). Protection of the macroorganism is carried out by anti-LPS antibodies and T cell-mediated activation of macrophages by the protein antigens (19). The antibody production by B lymphocytes is of small importance for the immune protection, but has a great diagnostic value.

CLINICAL PRESENTATION

Brucellosis has a variety of nonspecif c symptoms and can mimic other infectious and non-infectious diseases. The clinical presentation depends mainly on the stage of the disease. The early symptoms include: fever, sweats, malaise, anorexia, headache, joint and muscle pains, depression. The so called "undulant fever" could appear. Physical abnormalities are generally few and include lymphadenopathy (10-20%), splenomegaly, and/or hepatomegaly (20-30%). Because of the untypical presentation of the disease, it often remains unrecognised or misdiagnosed, especially in non-endemic areas (33, 34). Chronic brucellosis (duration more than 12 months) is presented with recurrent relapses of the above-mentioned symptoms or focal infections. Antibiotic therapy in this phase is less effective. The most common complications (92%) are musculoskeletal: sacroiliitis, peripheral arthritis, spondylitis, bursitis, incl. in Bulgarian patients (34), followed by affection of reproductive, nervous and cardiovascular systems, and more rarely lung, kidney, eye and skin manifestations (33). Serious complications include meningitis, endocarditis, osteomyelitis (35, 36, 37). Relapses are very typical for brucellosis, especially after inadequate and/or delayed treatment (38). The rate of clinical relapses among cluster of imported cases in Bulgaria was also high (38%), because of the aforementioned reasons (34). Mortality is generally low (1-2%) and is due to life-threatening complications.

TREATMENT AND CONTROL

Antimicrobial susceptibility testing for *Brucella* spp. is not routinely performed, because of: rare antimicrobial resistance; discrepancy between *in vitro* and *in vivo* results; high risk of laboratory acquired infections and lack of clear interpretive criteria (39). In special circumstances minimal inhibitory concentration (MIC) by serial dilutions in Cation-Adjusted Mueller-Hinton Broth and E-test on enriched Mueller-Hinton agar are performed (40, 41). Treatment is diff cult because of the intracellular location of the bacteria. Prolonged therapy and multiple antibiotics are imperative for achieving a cure (42, 43, 44). Several regiments are recommended: doxycycline 6 weeks + rifampicin 6 weeks or doxycycline 6 weeks + streptomycin for 2-3 weeks (gentamicin 1 week). The use of ciprof oxacin and co-trimoxazole is optional (esp. pregnant women and children younger than 8 years), but without any priority over the above-mentioned antimicrobial agents. Therapeutic failure or relapses are generally not caused by resistance, but by premature discontinuation of the treatment. Patients with relapse are usually retreated with the same antimicrobial agents, but often the result is not satisfactory. Recurrence of brucellosis may occur from a persistent focus of infection that requires additional treatment, for example surgical drainage.

Due to serious side effects human vaccines are not applicable. Decrease of the incidence is achieved through control of the infection in animals and the contamination of dairy and meat products.

Because of the low infectious dose in aerosols (10 to 10,000 cells depending on the species) *B. melitensis, B. abortus,* and *B. suis* are listed as category B potential bioterrorism agents (45, 46).

DIAGNOSIS

Brucellosis is a disease with wide range of symptoms and multiple clinical forms. Diagnosis is diff cult and complex. Conf rmed diagnosis is based on laboratory data interpreted along with the clinical and epidemiological ones.

LABORATORY EXAMINATION

Culturing is performed mainly on blood samples. Other more rarely used clinical materials are bone marrow, cerebrospinal f uid (CSF), pleural and synovial f uids, urine and tissue or abscess materials. It is extremely important to avoid contamination of the samples, because of the prolonged time for cultivation. Serum or less commonly CSF, are used for serological

testing. PCR is performed mainly on whole blood or serum. Clinical samples should be handled very carefully, although human tissues do not contain high numbers of brucellae (47). Serological testing should be performed without using special precautions, other than personal protective equipment (PPE). In a case of positive culture, strict precautions are required due to the dangerous numbers of organisms presented. Subculturing and other manipulations with living brucellae must be performed by using practices and procedures required for Biosafety level 3.

Direct detection of brucellae. It could be performed both with direct immunofuorescent microscopy (DIFM) and molecular methods. DIFM could be used for examination of clinical samples, as well as for identifcation of bacterial isolates. It is also a valuable method for preliminary diagnosis in case of suspected bioterrorism. PCR tests for direct detection of *Brucella* DNA use inactivated samples, which is a great advantage considering the low infectious dose. However, the specifcity and sensitivity of PCR vary between laboratories due to the lack of standardisation regarding the sample type and processing, the target genes, the visualisation of products and others (48). Blood and serum are most commonly tested with PCR, but various other clinical materials also could be used (49). Some authors propose serum as a more suitable for PCR, because of the lower quantity of inhibitors in it, as well as its easier processing (50). A number of genus specifc primers targeting *bscp31,* omp-2, 16S rRNA, IS711, and other genetic elements are used (Table 2).

Table 2. PCR methods for detection of *Brucella spp.*[a)]

Primers	Target	Amplicon length	Sensitivity	References
Not specifed	43 D OMP [b)]	635 bp [c)]	0.1 pg [d)]	Fekete et al. 1990
B4/B5	*bscp31*	223 bp	10-100 fg [e)]	Bailey et al. 1992
JPF/JPR	omp-2	193 bp	0.025 fg	Leal-Klevezas et al. 1995
Ba148-167F/ Ba928-948R	16S rRNA	800 bp	Not reported	Herman et al. 1992
F4/R2	16S rRNA	905 bp	80 fg	Romero et al. 1995
O1/O2; I1/I2	IS711	325 bp 52 bp	70 fg	Al Nakas et al. 2002

[a)]Ivanov, I. 2010. Molecular methods for detection, identifcation and typing of highly dangerous bacterial pathogens. PhD Thesis.; [b)]OMP-outer membrane protein.; [c)]bp- base pair.; [d)]pg- picogram.; [e)]fg- femtogram

Species-specifc primers are applied rarely, mainly for epidemiological and scientifc purposes, since in general the particular type of *Brucella* is irrelevant for the therapy (48). Real-time PCR techniques for detection of *Brucella* spp. are also developed (51, 52, 53, 54). These methods have a high sensitivity (<10 cells/reaction) and specifcity (99-100%), due to the usage of more than one marker for detection of *Brucella* DNA in clinical specimens. In recent years a number of multiplex methods based on real-time PCR are designed (55).

Cultivation. Isolation of the causative agent remains "gold standard" for confrmed diagnosis. Blood culture is leading in the bacteriological examination for brucellosis, but some data indicates that the amount of bacteria is low (1.3 -1000 CFU/ml), even in the acute phase of the disease (56). Bone marrow is the most suitable material in sub-acute brucellosis, after a negative blood culture and/or previous antibiotic treatment. Other samples are tested rarely. Due to the possibility for contamination of the blood cultures during their prolonged cultivation, as well as the high risk for the laboratory personnel, biphasic culture media are recommended for isolation of *Brucella* spp. (57). The bottles are incubated at 35-37°C, in humid atmosphere, with 5-10% CO_2 (mainly for *B. abortus*). They are inspected daily until visible growth on the agar phase is observed. *Brucella* colonies appear not earlier than the fourth day, but the majority of positives occur between the 7th and 21st day. Therefore, conventional culture methods require 21 days to 6 weeks. Before discharging the bottle, a blind subculture is performed (57, 58). Lysis-centrifugation method is faster (2-4 days), but increases the risk for laboratory accidents, as well as for contamination of the blood culture (59). The automated systems for continuous monitoring of samples, such as

BACTEC and BacT/Alert greatly increase the sensitivity of the method and reduce the time for detection of brucellae (57, 60). For other materials like bone marrow, CSF, synovial f uid, and various tissue homogenates biphasic media, as well as blood and chocolate agar could be used. Because *Brucella* spp. are fastidious, enriched media such as Trypticase soy agar, Heart infusion agar, Brucella agar, and Columbia agar should be used. Addition of blood in different concentrations and 5-10% horse serum enables the growth of the demanding species. The plates are incubated at the above-mentioned conditions for 10 days. For materials with small concentration of bacteria (urine, milk), enrichment could be achieved via inoculation of guinea pigs. Recovering of brucellae from contaminated specimens requires media with different antibiotic supplements such as selective Brucella agar, Farrell medium and others (61).

***Identification*.** Suspected colonies are subcultured on blood or chocolate agar. *Brucella* spp. are small Gram-negative coccobacilli that form non-haemolytic colonies on blood agar, and do not grow on MacConkey agar. The latter is useful for differentiation from other small Gram-negative coccobacilli that could be isolated from the same clinical materials (Table 3).

Differentiation of *Brucella* species from other Gram-negative coccobacilli.

Character-istic	*Brucella* spp.	*Acinetobacter* spp.	*Bordetella bronchiseptica*	*Haemophilus* spp.	*Francisella tularensis*
Specimens	blood; bone marrow	various	various	blood; CSF	wound secretion; blood; aspirates
Gram stain	faintly staining small coccobacilli	coccobacilli/ short rods	coccobacilli / small rods	small coccobacilli	very small coccobacilli
Catalase	+[a)]	+	+	V [b)]	+ (weak)
Oxidase	+	-[c)]	+	V	-
Urease	+	-	+	V	-
Motility	-	-	+	-	-
and/or V factor requirement	-	-	-	+	-
Growth on blood agar	+	+	haemolysis	satellite growth	poor requires cysteine
Growth on MacConkey agar (48 h.)	-	+	+	-	-

[a)]+ positive; [b)] V - variable; [c)] – negative

Brucellae are slow-growing and therefore visible growth of subcultures could be seen not earlier than 48 hours. Colonies are small, smooth, raised, transparent, with regular edge and shiny surface. Nonsmooth variants occur, especially after longer subcultivation. Only *B. ovis* and *B. canis* have stable nonsmooth (R) form. The presumptive identif cation on genus level is based on morphological, biochemical and serological criteria. Brucellae are oxidase, catalase and urease positive, and show positive slide agglutination reaction with specif c *B. abortus* and *B. melitensis* antisera (19). The use of commercial identif cation systems is not reliable, if *Brucella* spp. is not included in the database. As mentioned above, identif cation to genus level is suff cient for the etiological treatment. PCR can be used for screening after therapy when *Brucella* DNA is not detectable if the treatment is successful (62, 63). In the cases of relapse PCR tests could become positive again, which is helpful for evaluation of the patient, as specif c IgG antibodies persist long after completion of therapy. However, the conf rmation of relapse is bacteriological (64).

Typing is performed in highly specialised and well equipped laboratories. Phenotypic methods include: sensitivity to dyes (growth in the presence of methionine and basic fuchsin); speed of urea hydrolysis; production of H_2S; phage sensitivity (Tb, Wb); reaction with monospecif c (A and M) sera; determination of the S/R morphology.

Based on these phenotypic characteristics *B. suis, B. abortus* and *B. melitensis* are divided into biovars (Table 1). For reasons mentioned above, molecular techniques have certain advantages over phenotypic methods. For outbreak investigations and phylogenetic studies molecular typing of *Brucella* isolates to subspecies and strain level could be made by Bruce-Ladder and multilocus variable-number tandem-repeat analysis of 21 loci (MLVA-21) and 16 loci (MLVA-16) (65, 66). For example, MLVA-16 performed on 162 human *Brucella* isolates from Turkey indicate that they are most closely related to the neighbouring countries' isolates included in the "East Mediterranean" group (67)
Using MALDI-TOF MS for investigation of 131 *Brucella* human isolates (*B. abortus*, *B. melitensis*, *B. suis*) a 100% identification at genus level was obtained (68). The discrimination to the species level was not reliable. While comparing data obtained from MLVA on 152 *Brucella* isolates with those from MALDI-TOF MS, other authors concluded that the latter could indeed discriminate between different species and biovars (69).

SEROLOGICAL TESTS

Brucellae are fastidious and highly pathogenic bacteria. Therefore, serological methods are essential. Proper detection of all stages of the disease and differentiation between active infection and convalescent period demands the usage of several serologic tests. In general, two types of antigens are used - whole cells and antigen extracts. The first group demonstrates antibodies to cell surface antigens, mainly LPS which is responsible for the cross-reactivity with other Gram-negative bacteria. Such tests are: Rose Bengal slide test (RBST), serum agglutination test (SAT, Wright), complement fixation test (CFT), anti-globulin tests (Coombs' test, Brucellacapt) and indirect immunofluorescence microscopy (IFA). They are not suitable for *B. canis* and *B. ovis*, whose LPS is incomplete (R form). The second group of tests is based on purified LPS or protein extracts, used mainly for ELISA or different precipitation reactions (70, 71). RBST is fast and very sensitive. It is strongly positive in the initial acute phase of the disease, but cross reactions occur with sera from patients infected with *Y. enterocolitica* O9, so the result should be confirmed with the other tests. Positive SAT indicates active infection and together with 2-mercaptoethanol enables the monitoring of brucellosis with long duration. Single titre ≥1:160 or seroconversion is indicative of brucellosis. Lower titres (1:80) should not be overlooked, especially in the onset of the disease (64) as well as in non-endemic areas, like Bulgaria. Such results should be interpreted according to the clinical and epidemiological data. Retesting after 1-2 weeks is reasonable, as it was demonstrated in our experience during the Bulgarian outbreaks 2006-2008 and 2015 (unpublished data). Human anti-globulin test (Coombs') detects blocking (non-agglutinating) antibodies in the chronic stage of the infection, but this method is laborious and requires 48 hours. In such cases Brucellacapt (Vircell, Spain), a one-step test detecting both agglutinating and non - agglutinating IgA and IgG antibodies, is more suitable. It has good sensitivity and specificity comparable with those of the Coombs' test (72). ELISA could detect the different classes of immunoglobulins and is useful for patients' follow-up. It was found that all three classes immunoglobulins appear quickly after the onset of the infection. With time IgM levels tend to decrease, while IgG and IgA persist for longer periods (64). Quick and easy immuno-chromatographic tests for screening, especially for field testing during outbreak have been developed and implemented (73). Monitoring of treatment in patients with brucellosis requires continuous tracking with serological tests. Decrease in titres indicates good prognosis, while long lasting high values point out persistence of the disease and drift to chronification. During relapse IgG and IgA antibodies but not IgM, could be detected in patients' serum. Serological results, especially single titres should be interpreted according to the clinical and epidemiological data.

BRUCELLOSIS AROUND THE WORLD

Brucellosis is one of the most common zoonosis in the world (74). It remains a major problem in the Middle East, especially in Syria (above 100%$_{000}$ per year**).** Turkey has annual incidence of 8-50 %$_{000}$ (20, 75). In the rest of Asia, the incidence is still high in Mongolia (50-100%$_{000}$), but the rate of brucellosis in some former Soviet republics (Kyrgyzstan, Kazakhstan, Tajikistan, Azerbaijan) significantly increases and new foci of the disease appear. The incidence in Australia, Canada, and

the USA is low (<2 cases per million). According to data from the Centre for Disease Control and Prevention about 80 new cases are diagnosed annually in the USA (76). Mexico is the main source for importation of human brucellosis in USA. In Latin America, the incidence is generally low, with the exception of Mexico and Peru (10-50 cases per million) and to a lesser extent for Argentina (2-10 cases per million) (20). The Mediterranean basin is one of the major endemic regions for brucellosis (77, 18). In the European Mediterranean countries, the incidence has been reduced signifcantly through overall control of animal brucellosis. Annual numbers of the cases in Spain, Portugal, Italy and Greece have been dropping according to the latest reports from the European Centre for Disease Prevention and Control (78). Most of the EU Member States, especially in Western Europe have brucellosis-free status. They report annually a small number of imported cases. In 2011, a total of 332 confrmed cases of brucellosis were reported by 28 EU/EEA countries. The notifcation rate was $0.07\%_{000}$. The majority of all confrmed cases (68%) are still reported from Greece, Spain and Portugal. On the Balkan Peninsula brucellosis is a main problem in Albania where the disease is underestimated. High incidence is observed also in the Former Yugoslav Republic of Macedonia, Kosovo, and Bosnia-Herzegovina. (20,79,80). Three outbreaks of bovine and 17 of ovine/carpine brucellosis were detected and 26 humans were infected in Serbia during 2014. According to the Public Health Institute of Republic of Macedonia 299 animals for 2014 and 36 ($1.8\%_{000}$) humans for 2013 were proved as positive for brucellosis. In Greece 8.64% of the sheep and goat focks and 0.97% of the cattle herds were infected with brucellosis in 2012 (81). The highest incidence of human brucellosis in the EU ($1.44\%_{000}$) for 2013 was also registered in Greece.

BRUCELLOSIS IN BULGARIA

The frst cases of human brucellosis in Bulgarian citizens were described by foreign authors (82). In 1903 Neusser demonstrated a resident of town Lom infected with *B. melitensis*, and later on Praussnitz described another patient with brucellosis caused by *B abortus*. Beiling reported several Bulgarian human isolates of *B. melitensis*, originating from Svishtov and Ihtiman regions, which were sent to him during 1914 and 1918. Mollov was the frst Bulgarian author who diagnosed fve cases of human brucellosis in our country. In 1913 Andreev, detected the disease in a 7-year-old boy from Plovdiv and published the case for the frst time. Until 1948 more human cases were described by Detchev, Dobrev, and Ganov. The latter reported an infected veterinarian in the city of Silistra.

Ovine and carpine brucellosis, caused by *B. mellitensis* occurred naturally in Bulgaria in the past, but the country has been considered as free since 1941 (19). In the following more than flthy years few epizootics due to importation of infected animals were registered (83, 84). Bovine brucellosis in Bulgaria was frst reported by Acad. Stefan Angelov in 1924. He described 188 cases in cows for a period of ten years (1924-1933). Later on, Toshkov, Kuyumdjiev, Iliev, Ivanov and other authors reported brucellosis with different frequency in cattle, pigs, buffaloes, sheep, goats, and horses, mostly around Sofa and Samokov (82). Autochthonous animal cases of brucellosis caused by *B. abortus* were still reported during 1953-1954 (84). There was also evidence for sheep, horses, and dogs infected with *B. abortus* infection after contact with sick cows (84, 85). From 1922 to1980 bovine brucellosis was introduced several times in Bulgaria as a result of importation of infected cattle from Central and Western Europe (84, 86). During one of these outbreaks, caused by cows infected with *B. abortus,* 32 persons with brucellosis (breeders and veterinarians) were diagnosed by the National Diagnostic Research Veterinary Medical Institute. Cases of brucellosis in veterinary specialists during 1948-1950 were described by Angelov and Kuyumdjiev. Laboratory acquired infections were also reported, frst by Ganov, in a 29-year-old technician and later (from 1966 to 1968) brucellosis was detected in 14 other laboratory workers in a Research Institute.

As a result of the strict measures undertaken by the National Veterinary Service (NVS) autochthonous cases of brucellosis in cattle, sheep, and goats were not registered after 1958 (19). For the period 1958-2006 epizootics with non-pathogenic or low pathogenic for human species (*B. suis, B. ovis, B. canis)* were reported. Infections with the non-pathogenic for humans *B. suis,* v. Danika (biotype 2) occurred among pigs from the East Balkan breed (86, 87, 88). From 1987, when frst reported offcially, brucellosis in

dogs caused by *B canis* is widely spread among domestic and free-ranging dogs (86, 89, 90).

Nowadays brucellosis is a re-emerging disease in Bulgaria (91). Thirty-seven persons with brucellosis were diagnosed by the National Reference Laboratory for High Medical Risk Infections (NRL HMRI) in 2005. Most of them were from the region of Sliven. All patients, except one who worked in Cyprus, were animal breeders in sheep farms or resided in Greece. Data from NVS for 2005 as well as for the previous years showed no evidence of *B. melitensis* and *B. abortus* positive animals in Bulgaria. Based on these and data obtained from the patients' epidemiological investigation, all 37 cases were classified as imported (81, 30).

In 2006, after a period of more than 40 years during which Bulgaria was brucellosis-free, the first autochthonous cases were detected by the NRL HMRI. This was the start of an outbreak, caused by *B. melitensis,* which occurred after illegal import of goats from Greece. By the end of 2008 several regions were affected (81). In four of them (Smolian, Haskovo, Yambol, and Stara Zagora) an epidemiological link between human and animal cases was established. The highest incidence of the disease was registered in 2007 when 58 new cases were detected ($0,74\%_{000}$). The majority of them occurred in Haskovo region. All infected persons have had contact with *Brucella*-positive animals and/or consumed dairy products from their milk. The epidemiological investigation showed that sale of infected animals without certificates led to the spread of the disease in 11 villages of three districts. By the end of 2008 a total of 88 autochthonous human cases were registered. All *Brucella*-positive animals (496 goats, 117 sheep and 7 cattle) were destroyed. The stringent measures undertaken by the medical and veterinary authorities led to a significant decrease in human case numbers during 2009-2014 with 0 to 4 reports per year without any epidemiological link.

In July 2015 a patient was diagnosed with brucellosis. He was resident of Kyustendil district and without any connection with the regions affected in 2006-2008. By the middle of August an outbreak focus was found with 31 newly registered cases (92). *B. melitensis* was identified as the causative agent. A total of 36 patients were diagnosed until October 2015 (93). Based on data obtained during the investigation, breeding of animals and consumption of unpasteurised milk and homemade soft cheese appeared as the main risk factors for transmission of the disease. All infected animals were destroyed (94).

The investigation conducted by different governmental authorities pointed out that importation of infected animals from a neighbouring country, where the disease is endemic, was the reason for the occurrence of both recent brucellosis outbreaks in Bulgaria. As stated previously, free movement of goods and of people between EU Member States is a fundamental policy of the Community but has some negative epizootological and epidemiological effects, one of which is the re-emergence of brucellosis caused by *B. melitensis* in Bulgaria (81).

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INFECTION AND COMPLICATIONS AFTER DERMATOLOGICAL SURGERY

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ABSTRACT

The purpose of this article is to present the possible complications occurring as a result of dermatological surgical interventions performed on patients of different age with existing formations of benign and malignant nature. Bleeding during surgical intervention can be due to blood factor disorders, some drugs or foods and connected with specifc localisations. When the localisation of the skin formation is located near nerves and salivary glands, it is possible to affect them while using a deeper cut. In addition, some side effects are due to medical products used during the surgical intervention. Allergic reactions to the materials used for closure of the surgical wound are rare complications. Contact dermatitis that results from adhesive bandage use is common. Infection of the surgical wound poses a serious risk for the patients. Patients at high risk of wound infection should receive antibiotics. Patients must be informed of all possible risks and educated in the early recognition and reporting of adverse events.

INTRODUCTION

Complications due to post-operative infection following dermatologic surgery have to be anticipated. Preventive actions are mandatory in each surgical case. Early diagnosis and appropriate intervention are needed to avert progression to a dangerous situation. Patient education and appropriate follow-up care are also crucial.

The skin surgery includes biopsy or excision of skin lesions aiming to clarify the histological diagnosis. The material is sent to a pathologist. Recovery of the skin defect takes about 2 to 3 weeks, with the fnal recuperation of the wound taking from 6 to 12 months after the surgery. This process can be delayed because of complications occurring during the surgical intervention itself, complications during the healing process, or complications occurring later (1,4).

The complications arising during the surgical intervention are bleeding, disturbance of the integrity of important structures, side effects of drugs, and diffculties in closing the wound. Delayed wound recovery after surgical intervention happens after closure of the operating defect and may include local infammation, reduced blood fow, opening of the surgical wound, and local swelling after surgical intervention. The late complications are unsatisfactory cosmetic results, pigmentary changes, hypertrophic/atrophic scar, or the occurrence of keloid. Atumour process in the place of excision is also possible.

The purpose of this article is to present the possible complications occurring as a result of dermatological surgical interventions performed on patients of different age with existing formations of benign and malignant nature.

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METHODS AND MEDICATIONS

Elliptical (fusiform) excision is a basic technique widely used in dermatosurgical practice. The variants of the elliptical excision are easy to outline and can be adapted to different situations. They are effective, elegant, and fexible in surgical skin interventions (2). The edges of this technique are sharp, unobtrusive, allowing excellent cosmetic results and minimising tissue removal, allowing skin mobility and reducing the length of excision (3).The main indications for this technique are related to disease processes that engage the tissues in depth.

It is recommended that patients stop taking anticoagulants and anti-aggregates before surgery to reduce the risk of haematoma. Some medications such as corticosteroids and alcohol intake may suppress the recovery phase, which requires their use to be prohibited two days before and fve days after surgery.

BLEEDING DURING SURGICAL INTERVENTION

Various factors contribute to bleeding during surgical intervention. Blood factor disorders

such as low platelet counts, blood clotting factors (manifestation of hepatic failure), haemophilia and others. Some drugs (aspirin, clopidogrel, warfarin, dipyridamole, or heparin) and foods (fsh oil, garlic, ginkgo biloba, ginseng, vitamin E, or resveratrol) also play an important role (13,17). Some skin localisations of the body are rich in blood supply and, in violation of its integrity, bleed more. Such are the forehead, scalp, and eyelids. The occurrence of longer registered haematoma is characteristic of these sites.

IMPAIRMENT OF THE INTEGRITY OF IMPORTANT STRUCTURES

During surgical intervention the integrity of the skin, which covers the epidermis, the dermis, and the hypodermis, is impaired. When the localisation of the skin formation is located near nerves and salivary glands, it is possible to affect them while using a deeper cut. The damage to the sensory nerves leads to anaesthesia of the innervation, paraesthesia, or neuropathic pain. It is possible to restore the sensation to a small surface within 12 months.

SIDE EFFECTS OF MEDICINAL PRODUCTS USED DURING SURGICAL INTERVENTION

Local anaesthetics and analgesics are used to reduce the pain of the procedure during the operation. The mode of action of the local anaesthetics occurs by blocking sodium channels in the nerve cell membranes, resulting in blockage of nerve impulse transmission. There are two different main types of local anaesthetics: esters and amides. Their use is exceptionally safe, but some side effects may occur when large amount of the drug isinjected or when it comes directly into a blood vessel. It has been observed tingling of the mouth, appearance of metallic taste, dizziness, speech diffculties, double vision, confusion, cramps, arrhythmia, and cardiac arrest. A real allergy to local anaesthetics is very rare and, if occurring, is most likely to para-aminobenzoic acid or PABA. There is a very low risk of developing allergic reaction to amides. Local anaesthetics are often applied incombination with adrenaline (epinephrine). Adrenaline has vasoconstrictor activity andless systemic absorption. Overdose with adrenaline may result in: headache, tremor, tachycardia, chest pain and/or increased blood pressure.

REACTIONS TO THE MATERIALS USED FOR CLOSURE OF THE SURGICAL WOUND

Sutures made of synthetic or natural fbre materials (cotton, silk, etc.) are used, some of which are resorbable and others require removal after wound healing (12). An allergic reaction to suture material is a rare complication. Hypersensitivity to chromic catgut suture is the most commonly reported reaction (15). Allergies to silk and nylon sutures have also been reported (14). Patients suspected of suture allergy should be patch tested to guide future treatment.

CONTACT DERMATITIS AND HYPERSENSITIVITY

Differential diagnosis of contact dermatitis is wound infection or suture reaction as both of which cause erythema around the wound. An erythematous plaque in the shape of a bandage is typical for contact dermatitis. The presence of vesicles and pruritus may follow.

Dermatitis may result from irritation or delayed-type hypersensitivity to any of the topical agents used in wound care. Neomycin represents the most common allergen in this group. The frequency of allergic contact dermatitis due to neomycin was 5.3% (19). The incidence of bacitracin allergy has been increasing in recent years (16).

Irritant contact dermatitis that results from adhesive bandage use is common. However, true allergic contact dermatitis is rare (20). Paper tape may be used as an alternative in patients who experience dermatitis caused by bandage adhesive. Allergic contact dermatitis to povidone-iodine solution and chlorhexidine has been reported, but is rare.

LATE COMPLICATIONS AFTER SKIN SURGERY

Such complications may occur hours, days, or weeks after surgery.The postoperative wound infection rate in dermatologic surgery is low. Reported rates are 0.7-2.29% (11). It depends on thetype of surgery, type and localisation of the tumour, and patientfactors.

INFECTION OF THE WOUND

Infection of the surgical wound shows redness, swelling, and pain around the wound, presence or absence of pus, fever, bacteraemia (5). The factors increasing the risk of infections are:

ulceration of the skin lesion, increased skin tension at the site of the wound, poor blood supply to the area, smoking, immune deficiency, unsatisfactory control of patients with diabetes mellitus, and taking of certain medications (systemic corticosteroids, chemotherapeutics, and others). If one of the earlier mentioned factors is present, oral antibiotic therapy is recommended as a prevention of possible infection (7,8,9). The most frequently used antibiotic groups include penicillins, macrolides, tetracyclines, or cephalosporins.

Patients at high risk of wound infection should receive antibiotics to cover the organism most likely to cause infection. *Staphylococcus aureus* is the most common etiologic agent of skin wound infections. Viridans group streptococciare common residents of the oral mucosa, and *Escherichia coli* is present near the GI and GU tracts. *Pseudomonas* species are common pathogens of the external ear.

Prophylactic antibiotics should be administered prior to surgery to allow systemic absorption and incorporation into the coagulum, which seals the wound. The coagulum is formed within the first 3 hours of wounding. Recent recommendations in dermatologic literature advise a single dose given 1 hour before surgery (21). In cases of lengthy procedures or in those involving high-risk areas, a second dose should be given 6 hours after surgery.

Patients should be closely monitored to evaluate the clinical response. If the patient continues to worsen, despite appropriate treatment, possible concomitant infection with less common pathogens, such as *Candida*, fungal, and mycobacterial organisms, should be considered.

CONCLUSION

Serious postoperative complications arising from dermatologic surgery are uncommon. Many complications may be prevented by preoperative measures, appropriate surgical technique and follow-up care. Communication with patients and their feed-back are of significant importance. Patients must be informed of all possible risks and educated in the early recognition and reporting of adverse events.

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GIARDIA DUODENALIS: THE MOST COMMON ENTERIC PROTOZOAN ORGANISM IN THE WORLD

(Review)

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ABSTRACT:
Giardia duodenalis (syn. *Giardia intestinalis, Giardia lamblia*) is the most common enteric protozoan organism in the world. The organism is considered to be an important causative agent of waterborne, and rarely, foodborne infectious outbreaks of gastroenteritis. The parasite can infect not only humans but many other different species of mammals, birds, and reptiles. The life cycle of *G. duodenalis* includes two distinct morphological forms-infectious cyst and trophozoite (proliferating form). The transmission of the parasite is supported by both zoonotic and anthroponotic cycle. *G. duodenalis* is considered to be a complex of species that show small morphological differences between themselves but have remarkable genetic diversity. This species is divided into eight different genetic assemblages (A to H) but only the representatives of assemblages A and B infect humans.
Keywords**:** *Giardia duodenalis,* diagnostics, assemblages

Giardia duodenalis (syn. *Giardia intestinalis, Giardia lamblia*), is the most common enteric protozoan organism in the world (1, 2, 3, 4). The estimated annual incidence rate of giardiasis is 2.8 x 108(5). In developed countries the prevalence of infection among humans is between 2 and 7%, andover 50% in developing ones. Asymptomatic excretion of the parasite is common in some populations, such as children in organised children's collectives, where a prevalence of 21-26% is reported (6). For the last nine years the spread of giardiasis in Bulgaria varies from 0.49% to 0.25% among the examined persons, with a tendency to decrease (7, 8, 9, 10, 11). For the same period our country`s rate per 100000 population ranks first in the European Union

surface of the front two-thirds of the ventral surface (also called striated or ventral disc), and four pairs of flagella. Two nuclei that look identical, lysosomal vacuoles, glycogen and ribosomal granules are present. Although the concept of classification of *Giardia* is as a primitive eukaryotic organism (19, 21) many of the common eukaryotic organelles, including mitochondria, peroxisomes, smooth endoplasmic reticulum, and nucleoli are not found. Trophozoites move by swirling undulating movements pathognomonic for the species (20).

The cyst is the infectious form of *Giardia* with size about 5 to 8 μm surrounded by a 0.3 μm wall. It is relatively resistant to environmental conditions and gastric acid in the stomach of the infected host (Fig. 1 - C, D) (21).

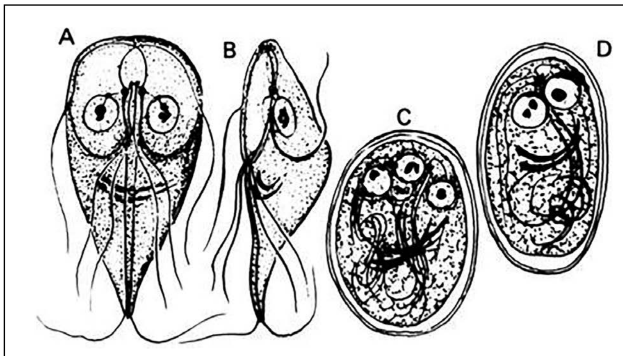

Figure 1. Trophozoites of *Giardia duodenalis* (A and B). Cysts of *G. duodenalis* (C and D).
(Source: McPhee SJ, Papadakis MA: *Current Medical Diagnosis and Treatment* 2011, 50th Edition: http://www.accessmedicine.com)

BRIEF HISTORICAL OVERVIEW

The first description of *Giardia* was by Antonie van Leeuwenhoek in 1681 when he examined his own loose stools under a microscope (22). In 1859, the organism was described more thoroughly by Lambl. Some researchers named the genus after him, while others used the name only for species occurring in the human population (i.e., *G. lamblia*). In 1882 and 1883 Kunstler described an organism in tadpoles, which he called *Giardia agilis*, thus for the first time *Giardia* was used as a generic name (1). In 1888, Blanchard offered the name of the parasite to be *Lamblia intestinalis*, which Stiles in 1902 changed to *G. duodenalis* (23). Subsequently Kofoid and Christiansen offered the names *G. lamblia* in 1915 and *G. enterica* in 1920 (1). Controversy over the number of species in the genus *Giardia* continued for years and some researchers offered the species to be named based on the host, in whom it was found, but others focused on morphology. More than 40 species names based on the host origin are proposed. In 1952, Filice published detailed morphological description of *Giardia* and proposed three names for species based on the morphology of the medial body: *G. duodenalis, G. muris,* and *G. agilis*. The species name *G. lamblia* became widely accepted during the 70s of the 20th century. In the 80s, some researchers promoted the use of the name *G. duodenalis*, and in the 90s the name of *G. intestinalis* gained popularity among others. At this time there was not enough reason to abandon the term *G. lamblia,* which was widely accepted in the medical and scientific literature (1). Nowadays, the names *Giardia duodenalis, Giardia intestinalis,* and *Giardia lamblia* are used as synonyms (1, 2, 3, 4). According to the International Code of Zoological Nomenclature, for forms of *Giardia* occurring in humans and other mammals, “duodenalis” has priority over “intestinalis” (24).

LIFE CYCLE

The life cycle of *G. duodenalis* includes two distinct morphological forms-infectious cyst and trophozoite (proliferating form) which colonises the intestinal lumen, but does not go into the mucosa (25). Parasitic cysts are shed in the stools. They are the source of faecal-oral transmission of the parasite. Contaminated water and food are also effective carriers of infection. Giardiasis is caused by ingestion of cysts from the environment, where they maintain their viability for a long period of time. *Giardia* prefers damp and cool conditions, for example, in water with a temperature of 4-10°C cysts can survive for several months. After ingestion, acid-resistant cyst passes through the stomach to the duodenum. There, each cyst releases two trophozoites that rapidly multiply asexually and colonise

the upper small intestine (26). These two processes - excystation and colonisation of the jejunum are facilitated by gastric acid and bile salts. Trophozoites attach to the epithelial cells of the surface of the wall of the upper part of the small intestine with its ventral disc, thereby creating a barrier to the absorption of nutrients from the host. The large number of trophozoites may cause symptoms of malabsorption directly through this barrier effect, and/or indirectly by interfering with the metabolism of bile salts (27). Finally, under the influence of bile salts, fatty acids, and other factors originating from the small intestine (28), trophozoites undergo encystation within the lumen of the small intestine, pass through gastrointestinal tract and are excreted with faeces. Trophozoites may be shed during the acute diarrheal phase of infection but it is less likely to infect another host, since they do not remain viable for long time period outside the body and also are sensitive to gastric acid (26). Ingestion of 10-25 cysts is enough for infection to develop. An average of 900 million cysts per day is shed with faeces with pauses from 1 to 17 days (20).

GIARDIA IN THE ENVIRONMENT

G.duodenalis is among the main causative agents of diarrhoea, with worldwide distribution among humans and animals, and may even lead to reduced life expectancy in immune compromised persons. The transmission of the parasite is supported by both zoonotic and anthroponotic cycle (16, 29). The infected host, whether human or animal, sheds very large number of transmissible elements - cysts in their faeces, resulting in increased environmental contamination. Moreover, the cysts are very resistant to adverse environmental conditions and disinfectants at concentrations typically used in sewage treatment plants to reduce bacterial contamination. Water resources for human consumption can be contaminated when faeces containing the parasite fall in the water source (29). The cysts of *Giardia* can flow into surface waters from urban and agricultural runoff, disposal of waste water, flowing septic systems, direct faecal contamination from wildlife, and many more. The small size and ubiquity of these pathogens have caused many outbreaks affecting public health with drinking water and water for recreation (30). Drinking water, even from a well-managed treatment plants, can lead to an illness if it contains a sufficient number of viable and infective cysts of *G. duodenalis* (29). More than 100 waterborne giardiasis outbreaks have been reported worldwide since the beginning of the 20th century. The largest outbreak associated with drinking water, as described so far, originated in Norway in 2004 and affected about 1,500 people (31).

Various methods have been developed to determine the viability of the cysts or assemblages, but there is no method that is a combination of both (32). Deficiencies in the process of purification of drinking water are among the most frequently cited reasons for outbreaks of giardiasis, including insufficient barriers, inadequate or poorly administered treatment. The risk of infection with the parasite increases with the duration of the exposure to the pathogen, particularly in areas where concentrations of *Giardia* in the environment are high (32).

DIAGNOSTICS

Microscopic methods

Microscopy of faecal smear of fresh and/ or concentrated sample is native (for trophozoites) or stained with Lugol solution (for cysts). In order to detect trophozoites, faecal sample is examined microscopically extempore or after preservation with polyvinyl alcohol or formalin and subsequent staining with trichrome or iron haematoxylin. For detection of cysts, faecal smear is examined by microscopy after staining with Lugol solution or trichrome (20).

Concentration methods for cysts (formalin-ether method - FEM, zinc sulphate method, sucrose) increase the efficiency of the examination. FEM is three times more effective than a survey of fresh sample. The intermittent excretion of cysts in the faeces and the sensitivity of trophozoites to atmospheric conditions suggest that persons suspected of giardiasis should be tested three to four times at intervals of three to four days after the first negative result. In a single

examination, cysts are found in 50-70% of cases, and rises to 97% when tested three times (20).

Cultivation methods

Culture diagnosis of giardiasis is used mainly in laboratory research for axenic propagation of trophozoites and isolation of antigen (20).

Immunological methods

Immunological methods (antigen identification in faeces by ELISA and immunochromatographic tests, and tests for serological detection of antibodies in blood serum (for epidemiological screening)) have different sensitivity and specificity (20).

Imaging techniques for diagnosis

Various techniques such as ultrasonography, radiography, and endoscopy are used. Data from ultrasonography and radiography is not specific – the gallbladder is often S-shaped and curved. Mucosal edema, spasm of the pylorus, fragmentation of the column is revealedby fibrogastroduodenoscopy and contrast radiography of the upper gastrointestinal tract. Endoscopy with duodenal drilling and small bowel biopsy in positive for *Giardia* individuals is the recommended approach in patients with immunosuppression, HIV-positives, and others (20).

Biomolecular methods

Biomolecular methods are applied in modern clinical and epidemiological studies, mainly genotyping of isolates of *G.duodenalis* from the external environment and faecal samples (20).

PCR-based methods for diagnostics and/or genetic analysis

The polymerase chain reaction (PCR) assay enables specific amplification of DNA regions of complex genomes. Different versions of PCR are developed. Reliable techniques for nucleic acid isolation from biological or environmental samples, effectively removing the PCR reaction inhibitors, are essential for the effective implementation of the PCR. A variety of methods were evaluated (3, 5, 33, 34, 35), including the use of sonication, freeze/thawing, extraction with glass beads and/or phenol/chloroform, followed by ethanol precipitation, and/or commercial extraction kits. However, the isolation of DNA and treatment methods require critical evaluation of each application and biological matrix which is tested to ensure that the inhibition of PCR was minimised. PCR inhibitors originate from the reference sample or sample preparation prior to PCR, or both. The most common inhibitors and the mechanisms by which they can affect, are divided into three categories - inactivation of the thermostable DNA polymerase, degradation or capture of the nucleic acids, and interference in the step of cell lysis. Various components were proved to be inhibitors of PCR – bile salts, complex polysaccharides in faeces, collagen in food samples, haem in the blood, humic substances in soil, proteinases in milk, urea in the urine, etc. The thermostable DNA polymerase is perhaps the most important target location for PCR-inhibitory substances (35).

PCR "fingerprinting" methods rely on screening the genome for variations in the organisation and the length of the sequences. The advantage of some of them is that they do not require prior knowledge of genome or genes to characterise the parasite. The disadvantage of "fingerprinting" is that it presents the isolate from a cyst or a trophozoite of *Giardia* as a population of organisms, and not as individuals (35).

RFLP (Restriction Fragment Length Polymorphism), specific PCR and PCR sequencing techniques, using specific primer pairs for the selective amplification of different genetic loci, followed by enzyme restriction or sequencing, are used to characterise and classify the types, the assemblages and/or sub-assemblages of *Giardia* (33, 37). Some of the key genetic markers (loci) are the genes coding for -giardin (bg), elongation factor 1 alpha (ef1), glutamate dehydrogenase (gdh), triose phosphate isomerase (tpi), variable surface protein (vsp), *G. duodenalis* open reading frame C4 (GLORF-C4), nuclear ribosomal RNA genes and DNA spacers. Genes encoding the small ribosomal subunit provide useful genetic markers for the specific identification of *Giardia*, which are with relatively low intraspecific and high

interspecific sequence variations (33, 37). Additional markers achieving assemblage or sub-assemblage identification based on specific PCR or sequencing are tpi, gdh, and bg genes (2, 33, 37, 38, 39, 40, 41).

Two methods - multiplex tandem PCR (MT-PCR) and Luminex system have a significant potential for making medium and large-scale epidemiological studies of *Giardia* and other aquatic pathogens (35).

ASSEMBLAGES OF *G. DUODENALIS*

Molecular classification tools are important for understanding the pathogenesis and host range of *Giardia* isolates derived from humans and a variety of other mammals. The first study of the molecular differences between isolates of *G. duodenalis* was a zymodeme (zymodeme - a group of parasites with the same isoenzyme profiles) analysis of five axenised isolates, three from people, one from a guinea pig, and one from a cat using six metabolic enzymes (1). A RFLP analysis of 15 isolates of random samples was performed in 1985. These studies have led to the description of three groups of which group 3 is so different from groups 1 and 2, that leads to the conclusion that isolates belong to different species. Subsequently, by using the RFLP and zymodeme assays a number of other studies for molecular classification were carried out. Pulsed-field gel electrophoresis (PFGE) analysis was also applied for chromosome models, but proved to be of limited significance for the classification because of the frequent occurrence of chromosomal rearrangements (1). These studies were very helpful but the conclusions that can be drawn out of them are limited by the semi-quantitative nature of the data. To allow quantitative comparison of *Giardia* isolates, in a number of subsequent studies the sequences of genes for rRNA of the small ribosomal subunit, triose phosphate isomerase (*tim*), and glutamate dehydrogenase (*gdh*) were compared (1, 38, 39).

G. duodenalis is considered to be a complex of species that show small morphological differences between themselves but have remarkable genetic diversity (42). This species is divided into eight different genetic assemblages (A to H) but only the representatives of assemblages A and B infect humans. Assemblage A is divided into three sub-assemblages: AI is predominantly found in animals; AII is common in humans, although in several studies it has been reported in animals; and AIII is found exclusively in animals. Assemblage B is divided into two sub-assemblages - BIII and BIV, hosts are mostly humans and very rarely animals (41, 43). Mixed assemblages are often seen in individual isolates, but the frequency of mixed infections may be underestimated due to the use of a single marker in the genetic research (43).

The application of the assemblage–specific primers, coupled with the use of more than one molecular marker was used to make a more accurate assessment of the incidence of mixed infections in clinical samples, and to improve the detection and determination of different assemblages (33, 40). Until now, the molecular analysis of the genes encoding -giardin (*bg*), glutamate dehydrogenase (*gdh*), and triose phosphate isomerase (*tpi*) from samples of *G. duodenalis* confirms the high genetic diversity in assemblage A and B (37). Assemblages A and B are considered genetic variants of the same species. However, recent studies have shown that the genomic differences between assemblages A and B are sufficient to classify them as two different species (42). Other assemblages are likely to be specific to their host, because assemblages C and D mainly occur in dogs and other members of the family Canidae, assemblage E occurs in ungulate animals, assemblage F in cats, assemblage G in rats, and assemblage H in marine mammals (39, 44).

Canine isolates of *Giardia* are extremely difficult for axenisation compared to isolates from humans. Based on this fact, it is proposed that they differ from cats or human isolates. However, certain canine isolates can be characterised and axenised (45). For further evaluation of the zoonotic potential, mice have been successfully infected with canine isolates of *Giardia* from 11 dogs. Based on sequence analysis, they were divided into

assemblages C and D, which are quite different from assemblages A and B. PCR-based study of nine faecal samples from dogs found that one of the nine isolates is similar to the human ones, while the remaining eight are different. These results show that most canine isolates were genetically different from those found in humans and have little potential for zoonotic transmission (1). Assemblages E to G are found in isolates from hooved livestock, cats, and rats (39). Further studies of *Giardia* isolates derived from bovine animals, have shown that some of the isolates belong to assemblage E, while others belong to assemblage A, and therefore may have the potential to infect humans (46). Assemblages from C to G have not yet been isolated from humans, suggesting that there is a possibility that some assemblages of *G. duodenalis* have a wide range of hosts, including humans, while others are with more limited host range and cannot pose a risk of zoonotic transmission (1).

A study on the genotypes of *G. duodenalis* has been carried out only in one district in Bulgaria so far and the results showed prevalence of genotype B (15). Our study is a pilot one and further genotype identif cation of samples obtained from across the country will be conducted. Analysis of the samples processed until now conf rmed the assemblages of *G.duodenalis* common for human population with prevalence of assemblage B.

Conclusion

G. duodenalis is a major causative agent of diarrhoea in humans and animals worldwide. Molecular techniques are especially useful for studying the taxonomy, population structure, zoonotic potential of isolates from animals or humans, as well as the correlation between the genetic diversity of the parasite and the extent of clinical symptoms observed in humans.

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RAPID MOLECULAR DIAGNOSIS OF PNEUMOCOCCAL BACTERAEMIA IN MINIMAL BLOOD SAMPLE VOLUME

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ABSTRACT

In patients with bacteraemia the concentration of pathogens in the blood is extremely low. Currently in Bulgaria, the diagnosis of invasive pneumococcal diseases is based on culture methods. They are time-consuming and have low sensitivity, especially when performed after antibiotic treatment. Molecular methods are highly sensitive and are not affected by antibiotics in the frst 1-2 days as they do not require viable bacteria. In this study, 140 children between 6 months and 17 years of age were tested for the presence of pneumococcal DNA in the blood. For this purpose, we used real-time PCR targeting the LytA gene. Out of 140 children with fever up to 40˚C, pneumonia, pleural empyema, pulmonary abscess, hydrothorax, and other diagnoses (sepsis, otitis media, tonsillitis, rhinitis), *Streptococcus pneumoniae* was detected in 22. In conclusion, real-time PCR is sensitive, specifc, and can be used in routine diagnosis alongside standard culture methods.

INTRODUCTION

Bacteraemia is a condition in which presence of bacteria is detected in the bloodstream. In certain cases, depending on patients` symptoms, blood culture is performed to determine the agent causing disease. In most patients, treatment starts empirically with a broad-spectrum antibiotic as blood cultures give results within 2-3 days. It is estimated that there is 5-10% increase in mortality with each passing hour before initiation of effective antimicrobial treatment (1). Time reduction between onset of symptoms and appropriate antibiotic administration is imperative to improve treatment of the patient. In the present study we used polymerase chain reaction (PCR) in real time, which can provide results within 3-5 hours and requires very small volume of blood.

Streptococcus pneumoniae is a leading cause of bacterial infections and associated bacteraemia in children. Worldwide around 10.6 million children under 5 years of age suffer from pneumococcal disease annually. Other community groups at risk are the elderly and immunocompromised patients. Overall, 1.6 million deaths each year are caused by pneumococcal infections (2, 3). Rapid and accurate microbiological diagnosis is important for correct treatment and epidemiological analysis.

Microbiological culture methods are considered the golden standard for diagnosing invasive pneumococcal disease. Nevertheless, these methods lack sensitivity especially when performed following antibiotic treatment (4, 5). Another factor is the common for the paediatrics practice of handling small volume material for analysis. These obstacles could be overcome by using molecular biology methods such as real-time PCR.

Molecular methods for detecting bacterial DNA in blood, cerebrospinal fuid (CSF), or other samples, usually sterile body fuids, may be used for effective diagnosis and determination of invasive pneumococcal serotypes (6, 7, 8, 9, 10, 11, 12, 13). They do not require viable bacteria, small sample quantities are used, and are more precise than culture methods. This makes them potentially useful tools for diagnosis and serotype determination of *S. pneumoniae*. Many clinical laboratories all over the world already use molecular methods as part of their routine work. It is important to choose the appropriate gene that does not give

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any false positive results as in previous years when assays were conducted with primers for the streptococcal pneumolysin (*ply*). This gene gives false positive results with blood taken from healthy individuals even if *S. pneumoniae* colonisation is not present (14).

The current study introduces a method using the pneumococcal autolysin (LytA) gene, which is reported to be more specif c than other genes, such as the *ply* gene (15, 16).

MATERIAL AND METHODS

In this study 140 children between 6 months and 17 years of age were included. Among them, there were patients of UMBAL ER „N. I. Pirogov" and UMBAL "Alexandrovska", suffering from pneumonia, empyema, and lung abscess (Table 1). For most of the cases antibiotic was administered on the f rst or on the second day. Part of the samples had positive cultures with *S. pneumoniae.*

Table 1.

DIAGNOSIS	NUMBER OF POSITIVE PATIENTS DETERMINED WITH PCR	NUMBER OF NEGATIVE PATIENTS DETERMINED WITH PCR
Pneumonia	5	58
Pleural empyema	9	31
Pulmonary abscess	1	5
Hydrothorax	0	14
Fever up to 40˚C	3	4
Other (sepsis, otitis media, tonsillitis, rhinitis)	4	6
Total	22	118

DNA was extracted from 100 µl blood samples using the AmpliPraimRIBO-prep kit, NextBio, Russia, following the manufacturer`s instructions.

The abundance of *S. pneumoniae* DNA in blood was measured with PCR in real time, as already described (7). The primers used in the reaction were LytA gene sequences published earlier (9). The end concentration for primers was 200 n and 200 n for the probe marked with carboxyf uorescein (Fam). Negative and positive control was applied for each PCR reaction. PCR in real time was conducted with *LightCycler® 480 Instrument II* with the following parameters:

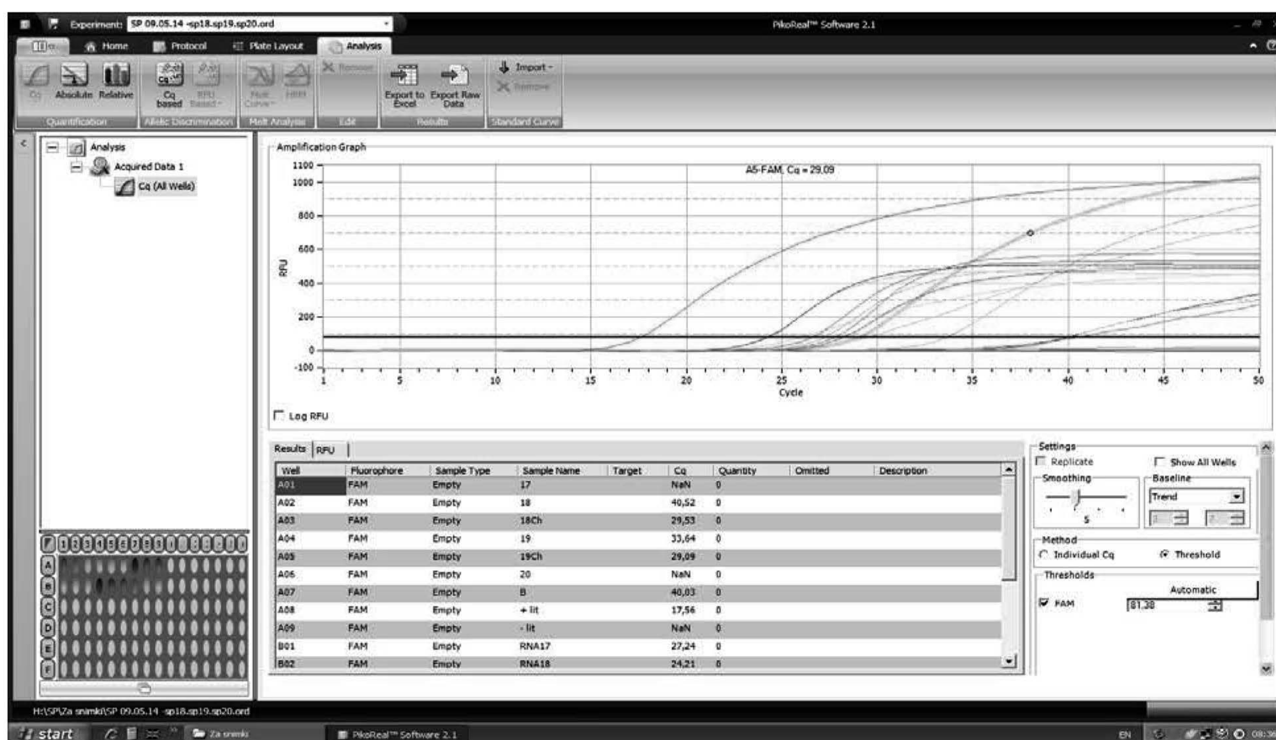

Figure 1. Analysis of the blood samples carried out with real-time PCR.

Denaturation: 95° for 10 minutes, followed by 50 cycles at 95° for 15 seconds and 60° for 1 minute. The samples were considered negative if there was no increase in the fuorescent signal after the fortieth cycle.

RESULTS AND DISCUSSION

The low sensitivity of culture methods for detection of pneumococci may be partially attributed to antibiotic treatment infuence. PCR is considered a more sensitive technique (6, 7, 8, 9, 10, 11, 12, 13).

When the pneumolysin gene (*ply*) is used to detect *S. pneumoniae* from bronchoalveolar lavage or throat secretions, the abundance of bacteria such as *S. mitis* or *S. oralis* could be the cause of false positive results (15).

Not long ago, the identifcation of the lytA gene sequence was proposed as more specifc for the diagnosis of pneumococcal diseases (9, 15).

Real-time PCR is a rapid, more sensitive, and specifc method in the microbiological analysis.

A total of 22 blood samples from 140 patients with pneumonia were positive for *S. pneumoniae* using PCR in real time.

In conclusion, the usage of real-time PCR analysis for detection of the *lyt*A gene sequence is both sensitive and specifc method, and may be a useful tool in routine diagnosis of invasive pneumococcal diseases. Sensitivity exceeds that of other methods but not at the expense of specifcity. As molecular techniques do not provide antimicrobial susceptibility testing of viable microbes, they must be performed alongside the time-consuming conventional methods.

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SEROPREVALENCE OF TICK-BORNE ENCEPHALITIS VIRUS IN DOMESTIC ANIMALS IN BULGARIA

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ABSTRACT

Serum samples from 732 cattle, 130 sheep, and 88 goats from all districts of Bulgaria were examined by ELISA method for IgG specif c antibodies against Tick-borne encephalitis virus (TBEV). The seroprevalence rate was low - 0.42% (varying 0%-6.67% in different districts), which is in accordance with the seroprevalence rates among the healthy population of the country. Antibodies were found in Pernik, where most of the recent cases were reported, and Razgrad (with no history of TBE cases) districts. Additional 7 animal sera exhibited borderline reactions in the ELISA. These animals originated from west and northeast regions of the country. This study ref ects the current seroprevalence rates of TBEV among domestic animals in Bulgaria.

Keywords: .
Bulgaria, seroprevalence

INTRODUCTION

Tick-borne encephalitis (TBE) is considered the most important tick-borne viral disease in Europe (Suss 2011). It is characterised by severe acute and chronic neurological infections in humans. TBE is caused by Tick-borne encephalitis virus (TBEV), a *Flavivirus* member of the *Flaviviridae* family. There are at least three subtypes of the virus: the European, the Siberian, and the Far-Eastern subtype (Dobler et al, 2012).

In Europe, the principal vector of TBEV is the hard tick *Ixodes ricinus*, which also serves as a reservoir to the virus and is the most common tick in Central Europe. TBEV can be transmitted not only through tick bite but also by raw milk (or its products) consumption (Holzmann et al, 2009). Depending on their life stage, ticks feed on different hosts. The larvae prefer small animals like rodents, which also serve as a reservoir to the virus, while nymphs feed on squirrels, hedgehogs, birds, and others. The adult ticks prefer large animals like deer and boars and also domestic animals such as cattle, sheep, and goats. Both wild game and domestic animals develop antibodies against TBEV after infection and the viremia is short-lived. That makes them useful sentinels for TBEV.

Investigations on TBE in Bulgaria have started in 1953. Since then only a few cases have been conf rmed (Mohareb et al, 2013). Considering the increasing number of cases of TBE both in Central Europe and in Bulgaria in recent years, and the fact that most TBEV infections (70-98%) are asymptomatic (Dumpis et al, 1999) we conducted a serological study to determine the distribution of the virus in the country. Furthermore, in Bulgaria a similar study was conducted more than 20 years ago, where anti-TBEV antibodies were found in domestic animals, wild game, and birds (Kamarinchev, 1996). Therefore, the collection of current data was necessary.

MATERIAL AND METHODS

A total of 950 serum samples from livestock (732 cattle, 130 sheep, and 88 goats, originating from all districts of Bulgaria) were tested for the presence of specif c anti-TBEV IgG antibodies. The samples were collected by veterinary personnel during 2014-2016, transported, and stored at -20°C until examination.

All samples were tested using the “Immunozym FSME IgG All Species ELISA kit®” (Progen, Heidelberg, Germany) according to the manufacturer’s instructions. Results were expressed as Vienna units per ml (VIEU/ml): <63 VIEU/ml were considered as negative, between 63 and 126 VIEU/ml as borderline, and

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over 126 VIEU/ml as positive according to the manufacturer's recommendations.

RESULTS

Specifc anti-TBEV IgG antibodies were detected in 4 animal sera (cattle), originating from 2 districts of Bulgaria – Pernik and Razgrad. That gives a total seroprevalence rate of 0.42% and a rate of 6.67% and 6.25% in Pernik and Razgrad districts, respectively. Pernik has a history of a number of TBE cases, while interestingly Razgrad has no history of TBE cases.

Another 6 cattle sera and one sheep serum, respectively, exhibited borderline reactions in the ELISA.Thus, the overall seropositivity, counting all positive and borderline results, was 1.15%. Three of the animals originated again from Pernik district, one from Razgrad district, while the other three were from Pazardzhik, Veliko Tarnovo, and Targovishte districts (Figure 1).

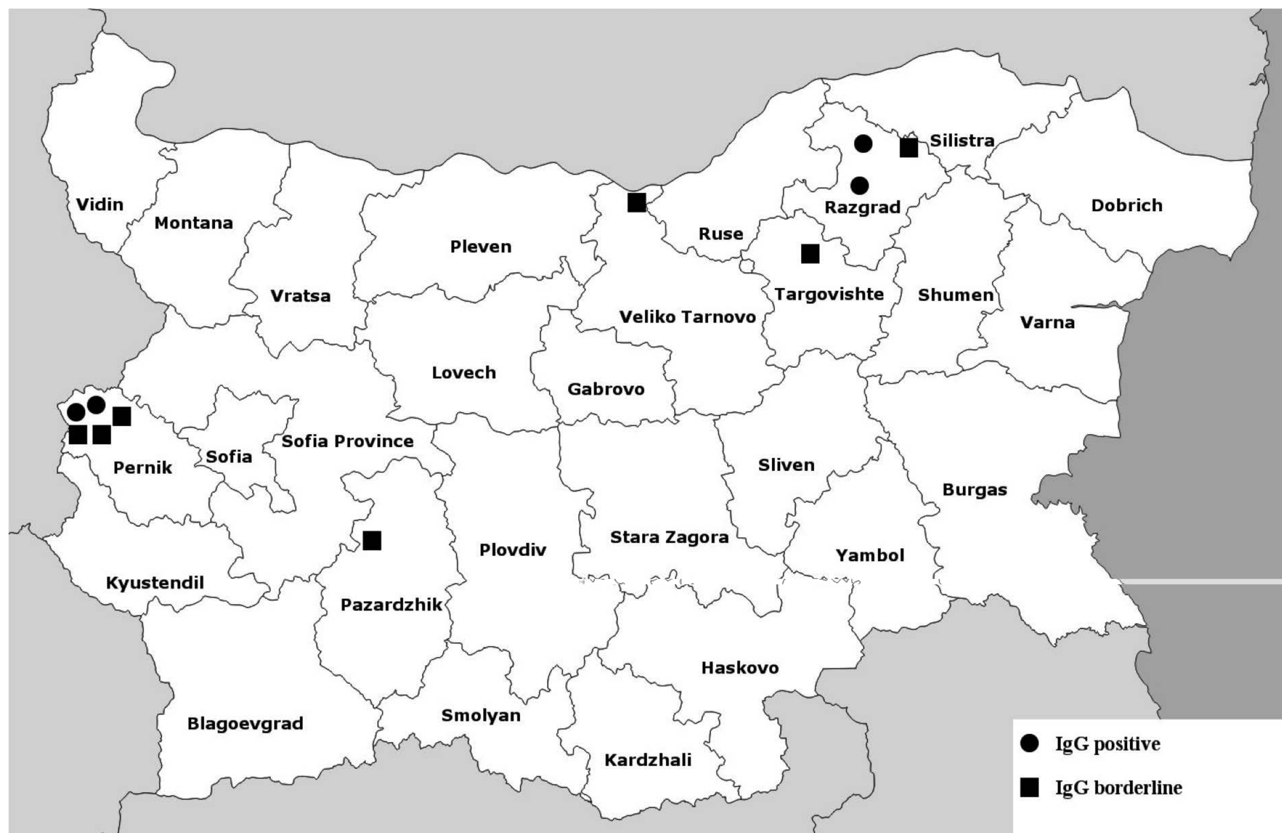

Figure 1. Seroprevalence of TBEV in domestic animals in Bulgaria, geographic distribution.

DISCUSSION

The seroprevalence rate of TBEV among domestic livestock is low. Especially compared to endemic Central European countries, i.e. Hungary reports seroprevalence rateof 26.5% among domestic animals (Sikutova et al, 2010); Poland – varying between 4.1% and 16.8% in both domestic animals and wild game (Cisak et al, 2012). Nevertheless, even such low rates could be of epidemiological importance, especially since the virus can occur in the milk of the animals and thus pose a threat to human health after raw milk consumption. Furthermore, Lithuania showsseroprevalence rate of 1.38% among domestic animals (Juceviciene et al, 2005) even though it is one of the countries in Europe with the most reported cases.

Highest seroprevalence rates were detected in Pernik district, which is no surprise as for the past two years 4 cases of TBE have been reported in Pernik. In Razgrad district, where anti-TBEV antibodies were also found, no cases have been reported so far. Probably this could be considered as evidence that some viral encephalitis casesare not diagnosed or not reported.

The results correlate with another study we conducted - on seroprevalence of TBEV among the healthy population in Bulgaria (Christova et al, 2017), where specifc anti-TBEV antibodies were found in 0.6% of the tested human sera. They originated from geographically close regions to those in which we found antibodies in animals: west and northeast Bulgaria.

All positive samples were cattle sera, which is probably due to the majority of test samples being from cattle. Nevertheless, since cow milk and its products are highly consumed throughout the country, raw milk consumption could be of epidemiological importance.

CONCLUSIONS

This study represents the current seroprevalence rates of TBEV among domestic animals in all districts of Bulgaria. The results show overall low seroprevalence rates and two active centres. Additional risk assessment and studies should be considered.

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CHARACTERISTICS OF CURRENT BACTERIAL NEUROINFECTIONS IN PLOVDIV REGION,

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ABSTRACT

Introduction: Bacterial meningitis and meningoencephalitis are severe diseases with frequent complications, residual neurological damages, and high mortality rate. The aim of this work is to examine the etiological structure of current bacterial neuroinfections, age characteristics of patients, clinical and therapeutic approaches, and outcomes with the purpose of early diagnosis and appropriate treatment.

Material and methods: The study included 66 patients with bacterial neuroinfections (meningitis – 31, meningoencephalitis - 35) hospitalised in the Clinic of Infectious Diseases, University Hospital "St. George" Plovdiv, from 1 January 2013 to 31 December 2015. The following methods were used: clinical and epidemiological analysis, haematological, biochemical, microbiological, PCR tests, and cranial imaging.

Results: The etiological structure was established in 55% of the patients as: *Streptococcus pneumoniae* - 14 (21%), *Listeria monocytogenes* - 7 (11%), *Staphylococcus aureus* - 6 (9%), *Neisseria meningitidis* - 4 (6%), *Mycobacterium tuberculosis* - 3 (5%), other *Streptococcus* spp. - 2 (3%). Disease incidence was highest in children under 1 year of age. 50% of the patients had comorbidities. The main clinical symptoms were: fever - 82%, vomiting - 72%, headache - 69%, hyperaesthesia - 25%, meningoradicular irritation - 90%, disturbances of consciousness - 57%. In 15% of patients the meningoradicular syndrome was with incomplete presentation. The most common CSF constellation was: 1000.10^6 cells /l (71%), protein up to 3 g/l (47%), decreased glucose level (58%). In the initial antibiotic combination therapy mainly vancomycin, ceftriaxone or cefotaxime, and amikacin were included. Outcome: 62.1 % of the patients were cured and 18.2% had residual neurological damages (motor and cognitive). The mortality rate in our patients was 19.7%, especially high in *L. monocytogenes* meningitis cases - 71%.

Conclusions: The proportion of bacterial neuroinfections with unspeci f ed etiology remains unacceptably high, which requires optimisation of the diagnostic process. The high frequency and lethality in *L. monocytogenes* meningitis cases demands improvement of the empirical therapy.

Keywords: *meningitis, meningoencephalitis, etiology, cerebrospinal (CSF)*

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INTRODUCTION

Bacterial meningitis and meningoencephalitis are severe diseases, with frequent complications, residual neurological damages, and high mortality rate. Data for community-acquired acute bacterial meningitis suggest that almost 50% of cases are due to *Streptococcus pneumoniae*, 25% to *Neisseria* meningitidis, 13% to group B streptococci, 8% to *Listeria* monocytoneges, and 7% to *Haemophilus* (1). In contrast, over 33% of nosocomial meningitis cases are associated with Gram-negative bacteria with the most common agents in adults *Escherichia coli, Klebsiella* spp., and *Enterobacter* spp (1). Despite the availability of potent antibacterial agents, mortality from bacterial meningitis in adults is 20% to 40% in patients over 60 years. In 10-20% of the survivors there are residual damages, such as epilepsy, mental retardation, deafness, and others. *H.* affects unvaccinated children between the age of 3-6 months and 6 years. *N. meningitidis* strikes children and young adults. *S. pneumoniae* usually affects children and morbidity decreases with age.

Recent studies report a dramatic reduction in cases of meningitis in developed countries due to the introduction of vaccines against bacterial agents such as *S. pneumoniae, H.* type b, and *N. meningitidis* (1).

According to a survey of Viale et al., 2015, involving 70 patients the etiological agent was determined in 51 (72.8%) as follows: 34 *S. pneumoniae*, 11 *N. meningitidis*, 3 *H.* , 2 *L. monocytogenes*, and 1 *S. salivarius*. 25 patients (35.7%) were treated in the ICU. Lethality rate was 4.3% (3 patients) and permanent neurological def cits were observed in 15.7% (11 patients). The average duration for stabilisation of the condition of surviving patients was 5 days (IQR 1-15) (2).

The aim of this work is to examine the etiological structure of contemporary bacterial neuroinfections, age characteristics of patients, clinical and therapeutic approaches, and outcomes with the purpose of early diagnosis and appropriate treatment.

MATERIAL AND METHODS

The study included 66 patients with bacterial neuroinfections (meningitis - 31, meningoencephalitis - 35) treated at the Clinic of Infectious Diseases, University Hospital "St. George" Plovdiv, from 1 January 2013 to 31 December 2015.

The following methods of clinical analysis were used - history and epidemiological history; physical examination; haematological tests - complete blood count with differential count, indicators of inf ammation - ESR, CRP, f brinogen; biochemistry; blood-gas analysis; microbiological studies involving CSF culture (conventional or BACTEC), blood culture, urine culture, throat, and nasal discharge; PCR for one patient; imaging - CT for all and MRI for some of the patients.

RESULTS AND DISCUSSION

The distribution of patients over the study period was as follows: 2013 - 26 (39% of the total), 2014 – 24 (36%), 2015 - 17 patients (25%).

Etiological diagnosis was established in 55% of the patients. 45% of the neuroinfections remain with unspecif ed bacterial cause. The isolated etiological agents were: *S. pneumoniae* - 14 (21%), *L. monocytogenes* - 7 (11%), *Staphylococcus aureus* - 6 (9%), *N. meningitidis* - 4 (6%), *Mycobacterium tuberculosis* - 3 (5%), other *Streptococcus* spp. - 2 (3%) identif ed as group B *Streptococcus* and the -haemolytic *S. parasanguinis* (Table 1).

Table 1. Main characteristics of the observed neuroinfections.

Etiological agent	Clinical forms		Period (year)			Patient	
	Meningitis	Meningo-encephalitis	2013	2014	2015	Number	%
N.meningitidis	3	1	1	3	0	4	6%
S.pneumoniae	6	8	4	3	7	14	21%
Streptococcus spp.	2	0	0	0	2	2	3%
S.aureus	2	4	4	1	1	6	9%
L.monocytogenes	2	5	1	4	2	7	11%
M. tuberculosis	0	3	1	2	0	3	5%
Bacterial, unspecif ed	16	14	15	10	5	30	45%
Total number	31	35	26	23	17	66	100%
%	47%	53%	39%	36%	25%		

A signif cant proportion of neuroinfections remain with undetected etiological agent, both bacterial and viral (10). The most common etiological agent of bacterial meningitis/ meningoencephalitis in our patients was *S. pneumoniae*, followed by *L. monocytogenes* - an organism with growing health importance. *L. monocytogenes* is related to consumption of dairy products, canned meat, raw vegetables and affects people with impaired immunity (1). Most of our patients with *L. monocytogenes* meningitis/meningoencephalitis were immunocompromised, residents of villages, and over 40 years of age. *N. meningitidis* was the fourth most common etiological agent and affected children of the age group under 1

year and from 1 to 3 years. Depending on the season and age, there was a predominance of various bacterial agents (14, 17).

Our data show signif cant decrease in the number of neuroinfections with unspecif ed etiology during the three years of observation. The reason for this could be associated with improved microbiological diagnostics and organisation of the diagnostic process. The increase in the proportion of pneumococcal meningitis/meningoencephalitis, particularly in the elderly, is worth mentioning. In our country the most commonly isolated serotype is 19F (11). Furthermore, meningitis caused by *H.* type b was not registered during the study period. The diagnosis could be improved by real-time PCR (15, 16).

Seasonal pattern. Most cases of bacterial meningitis/meningoencephalitis occurred in winter as 36% of the patients were in the f rst quarter (January-March). In the second and third quarter there was equal number of patients (21%), and during October to December - 22%. In 2015 50% of the cases occurred from January to March.

The age characteristics of patients with bacterial neuroinfections show that the incidence was highest in children under 1 year of age, followed by the age group 4 to 7 years, and over 60 years of age. The lowest incidence was in the age group 8 to 18 years. Analysed in absolute numbers, over the three years most patients were in the age intervals: over 60 years – 23 (35%) and from 41 to 60 years – 17 (26%) (Fig. 1 and 2).

Clinical characteristics. The most common clinical symptoms observed were: fever 82%, vomiting - 72%, headache - 69%, hyperaesthesia - 25%. Disturbances in consciousness (quantitative and qualitative) were observed in 57% of the patients and seizures in 19%. Symptoms of meningoradicular irritation - nuchal rigidity, symptoms of Kernig, Brudzinski, Lesazh, spinal symptoms, and others were present in 90% of the patients. In 15% of the cases, the course was atypical with incomplete syndrome of meningoradicular irritation. Most often patients had neck stiffness (87%) and positive Kernig`s sign (76%). Rarely they were positive for upper Brudzinski (67%) and lower Brudzinski`s sign (40%). The symptom of Lesazh was particularly signif cant in infants (100%). Abnormal ref exes

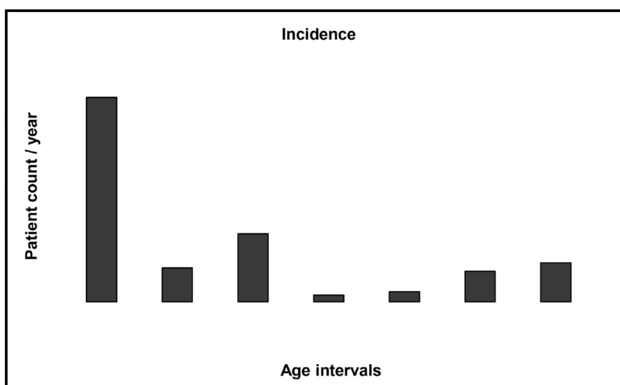

Figure 1. Age characteristics of patients with bacterial neuroinfections

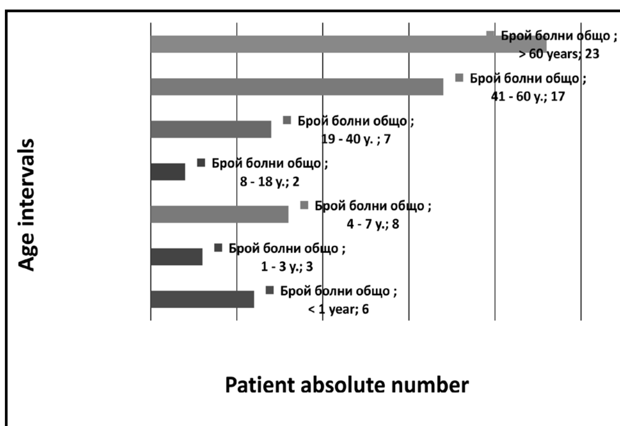

Figure 2. Incidence in the absolute number of patients

from the group of Babinski were observed in 64% of the patients and focal neurological symptoms in 17%. Brisk tendon ref exes in 24%, while twice as many patients (48%) had weakened ref exes. Damages to the cranial nerves were found in small number of patients, mainly n. facialis - 11%. These neurological disorders were reported mainly in patients with meningoencephalitis. Other observed symptoms were: rash, catarrhal angina in 36 patients, and purulent tonsillitis in 2. Rash was found in 12 patients, most commonly maculopapular or petechial. In the course of disease pneumonia was established in 13 patients and 3 other developed acute respiratory distress syndrome (ARDS).

The presence of comorbidities inf uenced the clinical course of neuroinfections and its outcome. The majority of patients had at least one underlying medical condition as their number increased with age. Elderly patients, especially over 60 years, experiencing severe comorbidities – mainly diabetes mellitus, respiratory, and cardiovascular insuff ciency had decompensation during the acute neuroinfection. Infants and children had no comorbidities.

Structure of the accompanying diseases: cardiovascular - 31% (mainly represented by arterial hypertension and rhythm conduction disorders, and one patient with tetralogy of Fallot); diabetes mellitus - 16%; liver diseases - 12%, including cirrhosis and alcoholic hepatitis; lung diseases (COPD) - 10%; haematological diseases - 6%, including leukaemia, Hodgkin and non-Hodgkin's lymphoma; a single case of a solid tumor – astrocytoma; hypothyroidism; epilepsy; prostate adenoma; pyogenic spondylitis, and others.

In about 50% of the adult patients, bacterial meningitis/meningoencephalitis developed along with another illness.

CSF findings (Table 2). The most common CSF constellation was: up to 1000 cells/µl (71%); protein up to 3 g/l (47%); decreased glucose level (58%); prevalence of neutrophils more than 50% (82%). CSF glucose, lactate, and pyruvate values should be interpreted considering their concentration in the blood. Due to elevated levels of blood sugar in the majority of our patients, CSF glucose values were normal, although the a b ove ratio is reduced below 0.4 as a result of the bacterial neuroinfection. According t o literature data, the interpretation of CSF g l ucose should be made in consideration with i ts values in the blood (2).

Table 2. CSF f ndings.

Laboratory f ndings		Results
WBCs (cell/µl)	<1000	71%
	1000 - 10000	24%
	>10000	5%
Predominant cell type	Neu >50%	82%
	Ly >50%	18%
Protein (g/l)	<1	23%
	1 -3	47%
	>3	30%
Glucose (mmol/l)	<1	33%
	1-2	9%
	Normal	58%

The gold standard for etiological diagnosis is microbiological examination of CSF. A major drawback is the duration - usually over 36 hours. Unfortunately, prior antibiotic treatment reduces the chances of isolating the causative agent with 30% (1). 45% of our patients had received an antibiotic before hospitalisation, which may explain the high prevalence of etiologically unspecif ed neuroinfections. According to the rules of good medical practice, empirical antibiotic and pathogenetic therapy should be started within 30-40 minutes after the onset of a suspected neuroinfection. Diagnostic imaging must not delay the treatment. PCR techniques have proved to be rapid, sensitive, and specif c tool for the diagnosis of neuroinfections, for example meningococcal meningitis (12, 13).

The most common initial antibiotic combination therapy was: ceftriaxone/cefotaxime plus vancomycin. When *L. monocytogenes* was suspected, empirical therapy included amikacin or ampicillin. After obtaining the microbiological results, treatment was adjusted to the specif c agent and its sensitivity. Mandatory for the treatment of bacterial meningitis/ meningoencephalitis was administration of a third-generation cephalosporin. Ceftriaxone and cefotaxime were used in patients included in our study. The effectiveness of ceftriaxone has been proven in many clinical studies. It crosses eff ciently the blood-brain barrier and in the majority of patients (88%) it reaches therapeutic levels in the cerebrospinal f uid hours after infusion (4). The most appropriate antibiotic

belonging to the third-generation cephalosporin group is cefotaxime because of the growing resistance to ceftriaxone.

Depending on the duration of hospital stay, patients were divided into 3 groups: up to 10 days - 24%; 10 to 20 days - 47%, and over 20 days - 29%. Our data correlate with these from other studies. According to Turtle et al., the average hospital stay with bacterial meningitis is 16 days, IQR 9 – 21 (5).

The outcome of the disease is determined by timely and appropriate treatment, but depends largely on the type of the bacterial agent. 62.1% of the observed patients were cured. Outcome was lethal in 19.7%. Furthermore, 18.2% of the patients survived with residual deficits - motor (paresis, paralysis) and cognitive impairment. Lethality rate was particularly high in *L. monocytogenes* meningitis - 71%, especially when the inclusion of ampicillin to the therapy is late (6). Mortality in unspecified bacterial meningitis was higher - 17%, due to the inability to conduct targeted antibiotic therapy. Best results were achieved for patients with meningococcal meningitis where the recovery rate was 100% without residual neurological deficits (Table 3).

Bacterial neuroinfections outcomes.

Etiological structure	Patient number	Recovery	Residual damages		Death	
			Motor	Cognitive	Number	%
N. meningitidis	4	4	0	0	0	0%
S. pneumoniae	14	10	3	1	1	7%
Streptococcus spp.	2	1	0	0	1	50%
S. aureus	6	6	0	0	0	0%
L. monocytogenes	7	2	0	0	5	71%
M. tuberculosis	3	1	1	1	1	33%
Bacterial, unspecified	30	17	5	4	5	17%
Total	66	41	9	6	13	
%		62.1%	13.6%	9.1%	19.7%	

CONCLUSIONS

1. The number of etiologically unspecified bacterial neuroinfections remains unacceptably high, which requires optimisation of the diagnostic process. It is necessary specimens to be collected before initiation of antibiotic therapy and to ensure their proper examination.
2. The observed high frequency, severity, and mortality in *L. monocytogenes* meningitis cases require optimisation of the initial therapeutic algorithm. In patients over 55 years of age the starting empirical treatment should include ampicillin or amikacin until listeriosis is excluded from consideration.

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CHEMOTHERAPY OF HUMAN HYDATIDOSIS

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ABSTRACT

Human hydatidosis is a parasitic disease caused by the larval stage of *granulosus.* It is characterised by development of hydatid cysts which can be localised in all tissues and organs, most frequently in the liver and lungs, leading to extensive organ damages and human disability. This determines the health significance and social importance of the disease and the need for effective radical treatment. Surgery was believed to be the only treatment of this parasitic disease for a long time. Since introduction in clinical practice of chemotherapy with benzimidazoles and percutaneous treatment, the therapeutic options have expanded. Conservative chemotherapy of hydatidosis with benzimidazole derivatives is an alternative to surgery in inoperable cases, recurrent disease, multiple hydatid cysts, or multiple organ involvement. Benzimidazoles are administered pre- and postoperatively or along with the percutaneous treatment as chemoprophylaxis to prevent recurrences and secondary echinococcosis. From this drug group albendazole is the drug with better pharmacokinetic profile compared to mebendazole that achieves better therapeutic results. Nevertheless, it was observed that in 20-30% of the cases hydatid cysts do not respond to therapy with albendazole. In order to find alternative treatment options, the search and study for other medications to contribute to a more rapid and effective conservative therapy of hydatidosis are continuing.

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Key words: human hydatidosis, chemotherapy, benzimidazoles, albendazole

INTRODUCTION

Human hydatidosis is a helminthiasis with endemic distribution in some regions, including the Mediterranean and Eastern Europe (1). In Bulgaria incidence rates are the highest in Europe(4.35% , 2015) (2,3,4,5). Hydatidosisis caused by the cestode *granulosus* belonging to the genus *Echinococcus*, family *Teniidae.* Dogs and other canids are its definitive hosts shedding eggs of *E. granulosus* with the faeces. Humans are accidental intermediate host and following oral ingestion of the eggs, the larva stage of the parasite develops into a hydatid cystin different organs and tissues (6). These cysts are filled with fluid and protoscoleces, and consist of an internal germinal layer and an outer laminated layer – both with parasitic origin, surrounded by a fibrous capsule (7). Hydatid cysts have long-term growth and can be localised in all organs but most commonly the liver (70%) and the lungs (20%) are invaded (8). The cysts can reach considerable sizes and can cause serious organ damages (9). Therefore, an accurate diagnosis and adequate treatment of the disease are required. The therapeutic options of hydatid disease include surgery, chemotherapy with benzimidazoles, and percutaneous treatment (PAIR – puncture, aspiration, injection, reaspiration or Modified Catheterisation Techniques for liver cysts) (10). According tothe recommendation of the World Health Organisation (WHO), optimal therapeutic regimen should be chosen individually for each patient considering the stage of cysts (determined by ultrasound classification of hepatic cysts – Fig. 1), their size and location, as well as the presence or absence of complications (1). Surgery is considered to be a radical therapeutic option that can provide complete cure of the disease. Chemotherapy and percutaneous procedures are also indicated and they are first-choice therapy in certain cases of hydatid disease. Chemotherapy with benzimidazoles as a conservative method of treatment has an important role in the therapy of human hydatidosis because of the significant activity of these drugs (1,11).

Fig.1. WHO ultrasound classification of liver hydatid cysts

Chemotherapy and chemoprophylaxis of recurrences with benzimidazoles

Benzimidazoles are used for treatment (chemotherapy) and for prevention of postoperative recurrences or secondary cystic echinococcosis (chemoprophylaxis) (11).

Mebendazole is the first benzimidazole derivative used for the treatment of human hydatidosisas studies on its therapeutic effects began in the 1970s (1). It is poorly soluble in water and has low intestinal absorption. Larger amount can be absorbed with concomitant consumption of high-fat meal. In the liver mebendazole is metabolised to inactive metabolites. Excretion is mainly via urine and to a lesser extent with bile (9). The recommended daily dose is 40-50 mg/kg in three divided doses (1,11). Administration of the drug should continue for at least 3-6 months (11). Currently mebendazole is used only if albendazole is not available (1,12).

Albendazole was introduced in clinical practice in the 1980s. It has advantage over mebendazole as a result of greater *in vitro* activity, greater intestinal absorption, higher plasma levels, and better outcome results from the treatment (13).High-fat meal increases the bioavailability also of albendazole (1). In the liver it is rapidly metabolised to active metabolite albendazole sulfoxide, the maximum concentration of which is achieved 3-4 hours after administration of albendazole in a dose of 400 mg (13). Although the bioavailability of albendazole after oral administration shows individual differences, the amount of the active metabolite that penetrates the hydatid cyst is about 20% of the amount in the serum (9).

Albendazole and mebendazole exert their action by preventing the -tubulin polymerisation of the parasite microtubules. They irreversibly block the glucose uptake by inhibiting the enzymes involved in the Krebs cycle and interfering with its absorption through the wall of the cyst. After that follows depletion of glycogen stores, degenerative changes in the endoplasmic reticulum and mitochondria of the cells of the germinal layer (7,13). As a result, morphological changes of the cyst wall can be observed due to damage of the microvilli, destruction of the germinal membrane and the protoscoleces. Destruction of the germinal membrane and protoscoleces within daughter vesicles of the cysts cannot always be achieved (9).

For chemotherapy of hydatidosis albendazole is administered to children over 2 years and adults at daily doses of 10-15 mg/kg in two divided doses (1,10,11). Treatment consists of 3 to 6 courses. The course usually lasts four weeks, followed by drug-free interval of 10-15 days (11,14). It is believed that treatment with less than three courses does not achieve optimal results (11,12). According to some authors, there are better results with continuous administration of albendazole for 3 to 6 months without drug-free intervals (15).

Patients treated with surgery or PAIR should receive benzimidazoles 4 days before and 1 month after these procedures as chemoprophylaxis to prevent recurrence and development of secondary echinococcosis (1,11,12). Preoperative administration of benzimidazoles leads to reduction in pressure in the hydatid cysts as well as degeneration of the protoscoleces and the germinal membrane. A study found that 72% and 92% of the hydatid cysts were sterile at the time of surgery as a result of preoperative administration of albendazole for 1 or 3 months, respectively (15).

Adverse reactions occur in 5.8 to 20% of patients treated with benzimidazoles (16) and include gastrointestinal symptoms, hepatotoxicity, suppressed bone marrow function, alopecia, embryotoxicity, and teratogenicity (1,11,12). Adverse reactions are usually transient and do not cause significant discomfort (9).Patients undergoing therapy with benzimidazole medicaments are recommended liver enzymes,

complete blood count, bilirubin, and creatinine to be monitored every two weeks during the first 3 months, and then monthly (11,12,17).Therapy is discontinued in 2 to 4.7% of the patients due to side effects (9)which disappear afterwards because are reversible (15).

Benzimidazole carbamates are indicated in inoperable cases of hepatic and pulmonary hydatid disease, multiple organ and peritoneal echinococcosis, multiple hydatid cysts, or recurrent disease (1,11,12).They are contraindicated in infected, superficial or inactive cysts, in large cysts that can rupture, in chronic liver disease, bone marrow suppression, and in early pregnancy (1,11).

In 20-30% of the cases hydatid cysts do not respond to conservative chemotherapy with albendazole (15,16). Reduction of clinical symptoms and changes in the ultrasound image of the liver cysts (reduction in the cyst size with more than 25%, detachment of the germinal membrane, or wall calcification) occur in 50-74% of the patients as a result of chemotherapy (7,13,16). With prolonged administration of albendazole the number of patients with a change in the ultrasound image and reduced clinical symptoms increases, but the number of cured patients does not change significantly (1,12). Complete cure with standard doses of albendazole received for 3-6 months is observed in 20-30% to 50% of the patients (1,13). Relapses of the disease occur in 3-30% of the cases as they are sensitive to retreatment (15). More frequently relapse occurs when the cysts are in the liver and in elderly patients (7,16). According to literature data, 1.5-2 years after initial response to therapy with benzimidazoles, in approximately 25% of the cases cysts return to the active stage(7), especially those containing daughter vesicles (16).

The effectiveness of chemotherapy depends on the stage and size of the cysts. Albendazole is found to be more effective in younger patients and smaller cyst size (< 5 cm), and in stage CE1 and CE3a localised in the lung and liver (1,12). Lung cysts respond better to chemotherapy compared to liver cysts. There is no sufficient efficacy in cyst stage CE2 and CE3b (1).

Drugs studied as alternative to benzimidazoles

Another drug used in the treatment of human hydatidosis, although with limitation, is praziquantel (13). It is a derivative of isoquinolone. Its action as an antiparasitic agent is associated with increase in the permeability of the cell membrane to calcium, leading to paralysis of the musculature of the adult form of *E. granulosus* located in the gastrointestinal tract of dogs. Praziquantel is also active against the larval forms of the parasite. It has been found that praziquantel destroys protoscoleces *in vitro* and in animal models (13,18).This drug does not affect the growth of hydatid cysts because it does not penetrate the walls of the cysts and has no impact on the germinal membrane (18). The dose is 40 mg/kg once a week or every two weeks as monotherapy or in combination with albendazole (13,18). The combined treatment with benzimidazoles and praziquantel is proved to be more effective than monotherapy with benzimidazoles (8,13,15). Co-administration of albendazole and praziquantel increases 4.5 times the bioavailability of albendazole (18). This leads to a higher concentration of the active metabolite albendazole sulfoxide in cysta are damaging of the germinal membrane and protoscoleces (13,18).The initial damage to the wall as a result of the action of albendazole is possible to favour the penetration and action of praziquantel. Regardless of these data, currently the routine use of praziquantel in prolonged chemotherapy of hydatid disease is not strongly recommended (18).

Hydatidosis is known to cause immunosuppression in humans. For this reason, some researchers study the therapeutic effect of the immunomodulating drug Isoprinosine (inosine pranobex) on this disease (19,20).This is a synthetic purine derivative used preferably in the therapy of a variety of viral diseases. In a study conducted on mice infected intraperitoneally with *E. granulosus* and treated with Isoprinosine at various dose regimens, it has been observed that a course of treatment with 1 mg/kg daily for 5 days, with a second dose on the 13th day results in decrease in the size and number of cysts, disability or destruction of the protoscoleces and partial destruction of the germinal membrane (21). In the literature there are only few reports on the effects of combined therapy with Isoprinosine and albendazole in human hydatidosis. Hence, large randomised trials are needed to determine whether benefits from immunomodulators or their combination with benzimidazoles are significant (20).

CONCLUSION

Hydatidosis is a parasitic disease that considerably affects human health, the optimal treatment of which still remains a challenge. Although treatment options signif cantly improved with the introduction of conservative chemotherapy with benzimidazoles, not all patients respond well and incomplete cure occurs in some cases. This requires the search for alternative or new drugs and drug combinations with higher activity against *E. granulosus*in order to achieve better therapeutic results without invasive intervention.

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POINT PREVALENCE OF INTESTINAL PARASITES IN HOSPITALISED AND OUTPATIENTS WITH DIARRHOEA

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ABSTRACT

Intestinal parasitic infections are generally presumed to affect children in low- and middle-income countries but recent reports expose their role as an actual and undermined problem in industrialised countries and in adult population.

The aim of this study is to evaluate the point prevalence of intestinal parasites (IP) and other pathogens in patients with diarrhoea and other gastrointestinal symptoms and to compare their distribution among hospitalised and ambulatory treated children and adults.

Material and Methods: The study included 360 patients in 3 equivalent groups: hospitalised and ambulatory patients with acute diarrhoea and a control group of patients tested on prophylactic basis. All samples were submitted for morphological identification of IP, viral, and bacterial pathogens.

Results and discussion: 104 (28.9%) of the samples were positive for intestinal pathogens – intestinal parasites diagnosed in 21.7% cases, enteric viruses in 9.2%, and pathogenic bacteria in 2.5%. In hospitalised children younger than 1 year, the Rotavirus was established as a major cause. In children older than 1 year, the highest point prevalence had the intestinal protozoans *Giardia duodenalis* (6.22%), *Blastocystis* spp. (6.0%), and *Cryptosporidium* spp. (2.0%) in similar rates in all of the investigated groups. The only identified intestinal helminth was *Enterobius vermicularis* with highest prevalence of 10.0% in asymptomatic children.

The study demonstrates that the IP's spectrum and prevalence in hospitalised individuals is significant and at least as high as in all other groups. Therefore, testing for IP should be included in the mandatory diagnostic panel for patients with acute intestinal diseases subject for hospitalisation.

Keywords: intestinal parasitic diseases, gastrointestinal pathogens, intestinal parasites' prevalence, diarrhoea

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Abbreviations:

APD - ambulatory patients with diarrhoea
GI – gastrointestinal
HPD - hospitalised patients with diarrhoea
IP – intestinal parasites
STH – soil-transmitted helminthoses

INTRODUCTION

It is estimated that 3.5 billion people are infected with intestinal parasites worldwide and the morbidity is higher than 450 million annually (1). Parasitic infections are generally assumed to affect mostly children in low- and middle-income countries due to poverty, poor sanitary conditions, overcrowding and inadequate water treatment (1–4). Less focus has been placed on the impact of the IP diseases in industrialised countries and in adult population. Recently expanding research confirms that parasitic infections present an actual and undermined problem where the estimation of the IP's prevalence is complicated by a lack of reliable surveillance data and/or underdiagnosis (2, 5, 6).

Not all of the IP infections can be associated with the most recognisable symptom of the gastrointestinal (GI) system – diarrhoea (4, 7). In most of the otherwise healthy individuals the invasion of IP presents with mild symptoms, self-limiting course or asymptomatic carriage. Severe, life-threatening or prolonged diarrhoea due to IP (*Giardia duodenalis, Cryptosporidium* spp., *Blastocystis* spp.) is reported mainly in newborn children and persons with immunosuppression or comorbidities (4, 7–10).

In Bulgaria data for the prevalence of the most common IP can be derived from the official surveillance information and several recent epidemiological studies. The most commonly diagnosed intestinal protozoans are *G. duodenalis* with annual estimated prevalence of 0.44% and *Blastocystis* spp. - 0.26% (11). *Cryptosporidium* spp. is infrequently diagnosed as an opportunistic agent in immunocompromised patients (8, 9, 12), in cases of "traveller's diarrhoea" or amongst healthy individuals living in rural regions and/or with regular contact with animals (13). In all annual reports from the last decade *Enterobius vermicularis* infection is reported with the highest prevalence - 0.6%-0.7% (even higher in children -1.07%) of all intestinal helminths (11, 14). Soil-transmitted helminthoses (STH) - ascariasis (0.1%) and trichuriasis (0.01%) are still a significant problem in some endemic regions (11, 15). The *Cestodes* have lowest prevalence of 0.04% for *Hymenolepis nana* and incidence of $0.38\%_{000}$ for *T. saginatus* (11, 14). Higher than average IP's prevalence is reported in different geographic regions throughout the country and in certain risk groups (children, immunocompromised, institutionalised persons etc.) (10,15–18).

The aim of this study is to estimate the point prevalence of intestinal parasites and other pathogens in patients with diarrhoea and other GI symptoms (recurrent abdominal pain, dyspepsia, bloating, etc.) and to compare their burden in hospitalised and ambulatory treated children and adults.

MATERIAL AND METHODS

The study included 360 patients (age between 1 month up to 69 years) tested for IP between July 1 and November 30, 2016. To maintain patient confidentiality only demographic data (sex, age, residence) were obtained.

The investigation was organised as a "case-control" experiment with 3 groups with equal number of participants (n=120 per group), as follows:

Group 1. Hospitalised patients (HPD): Patients with acute diarrhoea and other GI symptoms as a main or associated cause for hospitalisation in Infectious (n=100) and Paediatrics (n=20) Clinics of University Hospital for Active Treatment "St. Marina"-Varna. The age structure of this group is as follows: 42 children younger than 1 year, 58 children between 1-18 years and 20 adults.

Group 2. Ambulatory patients (APD): Patients with acute diarrhoea and other GI symptoms tested for intestinal pathogens on outpatient basis at the Parasitology and Microbiology Laboratories of SMDL "Status"-Varna. This group includes 4 children younger than 1 year, 86 children between 1-18 years and 30 adults.

Constitutes a random sample of an equivalent number of persons without GI symptoms tested for IP on prophylactic basis in the same laboratories. The age distribution is 2 children <1 year of age, 88 children between 1-18 years and 30 adults. The unintentional selection of the control group was implemented daily in the corresponding of the outpatients' numbers through random election of the samples.

All patients included in the study submitted at least 1 stool and 1 perianal scotch tape specimen for morphological identification of IP. The specimens were collected in labelled plastic vials and transported to the laboratory, and tested or stained on the day of the collection.

The morphological identification of IP was performed by direct wet mount, Lugol's iodine solution, perianal scotch tape microscopy, and sedimentation technique. In addition to the routine IP diagnostic tests, the selective modified Ziehl–Neelsen stain for identification of coccidian parasites was performed on three different slides per patient. The staining was executed according to the reference protocol of the National Reference Laboratory of Parasitology at NCIPD.

All tests for morphological identification of IP were performed at the Parasitology Laboratory of SMDL "Status"-Varna. The routine identification of viral and bacterial pathogens was done at the Virology and Microbiology Laboratories of UMHAT - Varna and SMDL "Status"-Varna.

Results: Of the 360 unique samples studied, 104 (28.9%) were positive for intestinal pathogens (Table 1) and a total number of 256 (71%) remained negative. Of the specimens, 89 (24.7%) had infection with only one etiological agent and in 15 (4.2%) more than one was identified. Intestinal parasites were diagnosed in 78 (21.7%) of the samples, followed by enteric viruses 33 (9.2%), and bacteria 9 (2.5%).

Table 1. Absolute number and point prevalence of identifed intestinal pathogens.

	Group 1. HPD				**Group 2. APD**							
Pathogens	**Children**		**Adults**		**Children**		**Adults**		**Children**		**Adults**	
	n	**Prev. %**	**n**	**Prev. %**	**n**	**Prev. %**	**n**	**Prev. %**	**n**	**Prev. %**	**n**	**Prev. %**
Enterobius vermicularis	2	1.9	-		5	5.6	1	3.3	9	10.0	1	3.3
Giardia duodenalis	4	3.9	-		4	4.4	2	6.7	7	7.8	3	10.0
***Cryptosporidium* spp.**	2	1.9	-		2	2.2	-		1	1.1	-	
***Blastocystis* spp.**	6	5.8	2	10.0	5	5.6	2	6.7	4	4.4	4	13.3
Entamoeba coli	1	1.0	1	5.0	1	1.1	1	3.3	3	3.3	4	13.3
• **Intestinal parasites**		**12.6**		**15.0**	**17**	**18.9**	**6**	**20.0**	**24**	**26.7**	**12**	**40.0**
Escherichia coli (EP)	2	1.9	1	5.0	1	1.1	-		-		-	
Shigella flexnery	1	1.0	-		-		-		-		-	
Salmonella (group D)	-		1	5.0	-		-		-		-	
Campylobacter jejuni	-		1	5.0	-		-		-		-	
Yersinia enterocolitica	-		-		-		2	6.7	-		-	
• **Intestinal bacteria**		**2.9**		**15.0**	**1**	**1.1**	**2**	**6.7**	**-**		**-**	
Rotavirus	22	21.4	-		-		-		-		-	
Norovirus	7	6.8	-		-		-		-		-	
Adenovirus	4	3.9	-		-		-		-		-	
• **Intestinal viruses**			**-**		-		-		-		-	
• **Uniden- patho- gen**	**55**		**14**	**70.0**	**75**	**82.2**	**20**	**66.7**				

In **Group1. HPD** the age structure differentiates from the other two groups because of the leading role of the **enteric viruses** and especially Rotavirus as a major cause of diarrhoea in the newborns. The children under 1 year of age represent 1/3 of the hospitalised individuals in this study and the enteric viruses were diagnosed in 50% of them (Rotavirus-16, Norovirus-5, coinfection of Rota+Norovirus-1). In children younger than 1 year, no other pathogens were identified neither in this, nor in the other groups.
Enteric viruses were identified in 14.8% (Rotavirus-5, Adenovirus-3, Norovirus-1) of the older children. In 1 patient, the Adenovirus infection was found in combination with cryptosporidiosis.
Protozoan **intestinal parasites** were diagnosed in 13 (21.3%) of the hospitalised children older than 1 year. Leading IP were *Blastocystis* spp. (6; 9.8%) and *G. duodenalis* (4; 6.6%) with one coinfection. *Cryptosporidium* spp. was diagnosed in 2 patients (3.3%). In one of the cases the infected was a 3-year-old boy and the other was a 2-year-old girl (in association with Adenovirus). Both patients live in rural villages of Varna district and both families reported close animal contact. Not one of the investigated family member tested positive for *Cryptosporidium* spp.
As *E. vermicularis* infection cannot be associated with acute diarrhoea (7) we believe that our findings of 2 infected hospitalised children are accidental and the clinical symptoms should not be attributed to this pathogen.
In the hospitalised adults, only *Blastocystis* spp. was identified as clinically significant cause of diarrhoea in 2 patients (10%).
Diverse genera of **intestinal bacteria** were identified as a cause in 3 (2.9%) of the children and 3 (15%) of the adults (Table 1).
In **APD** (Group 2) **intestinal parasites** were diagnosed in 17 (18.9%) children and 4 (13.3%) adults. Intestinal protozoa in children older than 1 year show similar to the hospitalised group prevalence. *G. duodenalis* and *Blastocystis hominis* showed equivalent prevalence of 3.3% (per 3 patients with sole infection) and in one case a co-invasion was observed.
Cryptosporidiosis was confirmed in 2 patients (2.2%) (2 and 3 years of age). In one of the cases *Cryptosporidium* spp. was in coinfection with *G. duodenalis* and *B. hominis* as part of a small family outbreak of giardiasis (n=3 cases) but no other family member tested positive for *Cryptosporidium* spp. (in several samples). The other child was a resident of rural outskirts of Varna and its family refused any treatment or additional monitoring.
Adults in the APD group showed similar rates of giardiasis (2; 6.7%) and clinically significant blastocystosis (2; 6.7%).
E. vermicularis was the most frequently diagnosed intestinal helminth in outpatient tests (5 children (5.6%) and 1 adult (3.3%)) mainly in patients with different than diarrhoea GI symptoms – abdominal pain, dyspepsia, etc.
Pathogenic intestinal bacteria were confirmed as a cause of acute or prolonged diarrhoea in only 3 of the APD (Table 1).
In the healthy controls (Group 3) exclusively **intestinal parasites** were identified in 25.6% of the children's samples and in 36.7% of the adults' samples. The higher percentage of positive findings in the control group must be attributed to a significant number (8 cases) of coinfections of pathogenic, or pathogenic and non-pathogenic parasitic species.
Solitary infection of *G. duodenalis* was identified in 4 (7.8%) children and 2 adults (10%). In three other cases *G. duodenalis* was in combination with *Blastocystis* spp., in two cases with *E. coli* and in one with *E. vermicularis.* No one of the other patients diagnosed with *B. hominis* had a clinically significant infection according to laboratory and clinically accepted criteria (17, 19) and were considered as asymptomatic carriers. In the control group, one sample was positive for *Cryptosporidium* spp. with only a few number of oocysts (less than 5 per slide) present. No GI symptoms were stated and the infestation was considered a self-limiting process, confirmed by the consecutive negative controls. In both children and adults, clinically insignificant prevalence (16.6%) of commensal amoebas was observed - *E. coli* - 6, *E. nana* - 1.
The most identified IP in the CG was *E. vermicularis* with point prevalence of 10.0% (n=9) in children and 3.3% (n=1) in adults.

DISCUSSION

The major cause of hospitalisation of children under 1 year with acute diarrhoea in Infections Clinics in Varna are enteric viruses, mainly Rotavirus. These results agree with regional

data which state that Rotaviral gastroenteritis is the cause of 27–44% of the hospitalisations of children between 1-5 years, with 17.8% prevalence in this age group (20, 21).

The absence of IP diagnosed in children under 1 year can be attributed to the lower risk of exposure to contact parasitoses due to proper caring and hygiene of the newborns and the passive immunity provided by breastfeeding in the first 6 months (10, 22).

In children older than 1 year, *G. duodenalis* has the highest point prevalence (average of 6.22%) in all investigated groups. Significant number of *Giardia*-infected adults are diagnosed with children vs. adults ratio of 3:1. Parallel proportions are estimated in our previous studies of IP's epidemiology in Varna region (6, 23) and prevalence of 4.1% in children and 3.8% in adults is reported by Chakarova in a broad epidemiological study in Stara Zagora region (10, 24).

Blastocystis spp. was the most commonly found IP in stool samples (similarly to the majority of epidemiological reports (2, 4, 19, 25)) and therefore requires a differentiation between clinically significant and asymptomatic cases. The estimated total prevalence of blastocystosis in children in all groups is comparable (6%). Even higher rates are established in adults which corresponds to the findings of a large scale study of blastocystosis distribution in Pleven region by Angelov (17, 25).

The estimated point prevalence of both *G. duodenalis* and *Blastocystis* spp. in adults contradicts the common opinion that IP are diseases inherent in childhood. This misconception frequently excludes those pathogens from the differential diagnosis of adult patients with diarrhoea and other GI symptoms leading to misdiagnosis and unnecessary treatment. On the other hand, these patients are epidemiologically relevant but "hidden" sources for distribution of the IP (6, 23).

These are the first published data regarding the prevalence of *Cryptosporidium* spp. in patients from Varna region. Although small in numbers our findings confirm that the infection is mainly diagnosed in small children (all between 2 and 3 years) and with analogous prevalence of approximately 2 % of the symptomatic patients with diarrhoea in the European population (5, 13).

The only identified intestinal helminth was *E. vermicularis,* in 18 patients (5.0%). This confirms the leading role of enterobiosis as an increasing health problem in the region which was stated in our previous research (6, 26). The absence of other intestinal helminths identified here can be ascribed to the lack of endemic distribution of STH (15) in the region and the relatively small numbers of the excerpt.

CONCLUSION

We already have described the spectrum and prevalence of IP amongst symptomatic outpatients and asymptomatic persons in Varna district (6, 23, 26). This study however is the first in the region ever to test for and report on the IP's distribution in hospitalised patients and the results demonstrate that the prevalence of IP is at least as high as in the other groups (Pearson's test for association ^{2} = 0.2; p=0.9). This challenges the observed practice to neglect IP in the differential diagnosis of acute diarrhoea. Therefore, testing for IP should be included in the mandatory diagnostic panel for children, as well as for adults, with acute (and chronic) intestinal diseases subject for hospitalisation.

Acknowledgements: The authors acknowledge the support of Dr. Svetoslav Todorov, head of SMDL "Status" for providing the facilities and reagents necessary for this research.We are grateful to the heads and the staff of all Infectious and Paediatrics Clinics of UHMAT "St. Marina"-Varna for facilitating the patient sampling. We wish to thank the Virology and Microbiology Laboratories of UMHAT "St. Marina"-Varna and Microbiology laboratory of SMDL "Status" for providing the data on the diagnosed viral and bacterial pathogens. Thanks goes to Vanja Yambolova for the technical assistance and to Zvezdalina Yaneva for her assistance in data collection.

No conflict of interest to declare.

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