Clinical features, diagnostic tools, and treatment regimens for visceral leishmaniasis in Bulgaria

Rumen N. Harizanov, Iskren T. Kaftandjiev, Diana P. Jordanova, Irina B. Marinova, Nina D. Tsvetkova

Department of Parasitology and Tropical Medicine, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

Purpose: Visceral leishmaniasis (VL), caused by the parasite *Leishmania infantum*, which was once largely confined to Southern Europe is now being diagnosed throughout Northern Europe, including Great Britain. In an effort to help EU clinicians improve their diagnosis and management of VL, this paper defines clinical features typical of the disease as it presents in Bulgaria, where VL is endemic.

Methods: The list of clinical symptoms presented here was gleaned from the medical records (patient histories, epidemiological survey cards, laboratory data) of 59 Bulgarian patients with VL. This study also includes microscopic, serological, and molecular laboratory techniques.

Results: Described and analyzed are the clinical features, diagnostic techniques, and therapeutic regimens of 59 cases – part of the total number of VL case histories ($P=120$, 116 Bulgarian and 4 not Bulgarian) collected in Bulgaria over the past 24 years (1988–2011). Although all of the studied 59 cases presented with classical symptoms of VL, only in three occasions, the initial diagnosis was correct.

Conclusions: Left untreated, zoonanthroponotic VL leads to debilitating chronic disease and even death. Yet, because VL is hard to recognize and relatively new to Northern Europe, misdiagnosis is common and treatment too often inappropriate and delayed.

Keywords: Visceral leishmaniasis, Clinical symptoms, Diagnostic techniques, Therapeutic regimens

Introduction

Visceral leishmaniasis (VL) is an emerging zoonotic parasitosis of significant medical and social importance whose spread has been considerably facilitated by increased international travel and the rising number of individuals infected with HIV.¹ The spread of VL within Europe is monitored under the EU Parliament Directive 2003/99,² and its mechanisms and management are being studied by the WHO's program of Tropical Diseases Research.³ Following its unification in 1993, the European Union experienced an influx of immigrants so massive that today, Eurostat estimates some 6.5% of people living in the EU were born outside its borders.⁴ With its substantial foreign-born population and a rising number of tourists, Europe is now seeing parasitic diseases earlier found only in far-off lands.⁵ One such disease is VL, caused by the parasite *Leishmania infantum*, which was once largely confined to Southern Europe and is now being

diagnosed throughout Northern Europe, including Great Britain.^{5,6}

Today, VL is endemic in Bulgaria but relatively rare. Mollov described the first clinical case of endemic VL in 1937. A total of 177 cases have been registered in the country since the first description of the disease. A great majority of these reports or 120 cases were made between 1988 and 2011.^{7–13} According to Bulgarian law, VL is a notifiable case-based disease, and the Department of Parasitology and Tropical Medicine (DPTM) of the Bulgarian National Center of Infectious and Parasitic Diseases (NCIPD) verifies each case diagnosed by testing smear samples for VL. In presenting typical clinical features of VL as it presents in Bulgaria, this paper seeks to improve diagnosis and treatment of the disease worldwide.

Patients and methods

This study is a retrospective analysis of data gathered from 59 cases of VL diagnosed and treated in Bulgaria. These data come largely from patient examinations, both initially and during the follow-up period,

Correspondence to: Rumen Nenkov Harizanov, Department of Parasitology and Tropical Medicine, National Center of Infectious and Parasitic Diseases, 26, Yanko Sakazov Blvd., 1504 Sofia, Bulgaria. Email: harizanov@ncipd.org

performed by the DPTM of Bulgaria's NCIPD. In addition to evidence provided by these DPTM examinations and related tests, our study includes information from other medical records and laboratory test results.

Patient sample

This study includes 59 cases of VL diagnosed and treated in Bulgaria. Our sample therefore represents roughly half (50.86%) of the 116 autochthonous cases registered in different areas of Bulgaria since 1988.

Clinical examination

DPTM clinicians who examined the 59 study subjects diagnosed with VL reported clinical features of the disease. Clinical reports were further supplemented by patient questionnaires, and ultrasonography provided detailed evidence of splenohepatomegaly. This study also includes data from other imaging techniques and from tests and investigations performed outside the DPTM.

Biological samples

To identify leishmaniasis antibodies, the DPTM used ELISA and indirect fluorescence assay (IFA) tests on sera from 46 (78%) of the VL patients enrolled in this study. The DPTM also did Giemsa staining ($n=58$) and polymerase chain reaction (PCR) analysis ($n=13$) of bone marrow extracted by hematologists at various hospitals, either by sternal puncture or trepan biopsy, from 58 (98%) of our study subjects. Different clinical laboratories around the country performed complete blood count (CBC) tests on all 59 patients with VL, both before treatment and during the follow-up period.

Parasitology

A number of different techniques were used to test for the presence and effects of the *Leishmania* parasite, which causes VL: (a) Giemsa staining ($n=58$). Bone marrow samples were stained for cytological and histopathological analysis. (b) Indirect fluorescence assay ($n=14$). In this laboratory test developed by the DPTM, promastigote stages of *Leishmania* spp. are cultivated on Novy–MacNeal–Nicolle blood agar in tubes and used as an antigen. When the titer is $\geq 1:40$, the reaction is considered positive.¹⁴ (c) ELISA tests ($n=32$). Tests were performed using a commercial ELISA kit (Ridascreen R-Biopharm, AG, Darmstadt, Germany) in accordance with manufacturer's instructions. (d) Conventional PCR test ($n=13$). The DNeasy Tissue Kit (Qiagen) was used to extract DNA in accordance with manufacturer's recommendations. Then, PCR was carried out using primers designed to detect a repetitive nuclear sequence from 'Old World' *Leishmania* – forward T2 (5'-CGG CTT CGC ACC ATG CGG TG-3') and reverse B4 (5'-ACA TCC CTG CCC ACA TAC GC-3') primers – which generates an amplicon of 260 bp.¹⁵

Treatment

Once they were correctly diagnosed, the 59 patients in this study underwent WHO-recommended treatment regimens.³ Patients treated with meglumine antimoniate (glucantime) alone ($n=51$) or in combination ($n=5$) received it at a dosage of 20 mg/kg body weight (b.w.) Sb⁵⁺ every 24 hours separated in two intravenous (i.v.) or intramuscular (i.m.) applications for 20–28 consecutive days. Patients treated with miltefosine ($n=2$) received it *per os* (p.o.) at a dosage of 2.5 mg/kg b.w. a day for 28 days. Patients treated with ambisome as monotherapy ($n=1$) or in combination after glucantime treatment ($n=2$) received it at a dosage of 3 mg/kg b.w. – i.v. every 24 hours for 5 consecutive days followed by two additional applications at day 14 and 21 from the beginning of the treatment with ambisome. In the patient treated with pentamidine after glucantime ($n=1$), pentamidine was dosed at 4 mg/kg b.w. – i.m. every 24 hours for 20 days. In the cases treated with combination therapy of glucantime and allopurinol ($n=2$) the dose of allopurinol was 20 mg/kg b.w. p.o. every 24 hours for 20–28 days.

Statistical analyses

Data were analyzed using the Fisher's exact test, Van der Waerden test, and chi-square test as needed.¹⁶

Results

For demographic features (age, sex, location) of the 59 Bulgarians with VL whose data are analyzed in this study, see Fig. 1. Differences between genders (chi-square test 20.763, $p<0.0001$) and patients from urban and rural areas (8.966, $p<0.05>0.0001$) are both statistically significant. The gender difference among those contracting VL in Bulgaria, moreover, is similar to that reported by other international sources.¹⁷ By contrast, in our study sample, distribution of affected individuals by age is not significant (chi-square test 1.373, $p>0.5$), and VL was reported in only one patient with HIV.

As can be seen in Table 1, all of the VL patients we studied exhibited fever and splenomegaly. Other common VL symptoms include, in order of frequency: anemia, leucopenia, hepatomegaly, and weight loss. Far less common was lymphadenitis.

All 59 VL patients (confidence interval 95–95.1, Van der Waerden test, $p<0.05$) experienced fevers, however, the trajectory of these fevers varied. Only 2 patients experienced fevers of 38°C, 29 patients had fevers of 38–39°C, and 28 patients had fevers $>39^\circ\text{C}$. According to the daily temperature peaks recorded, 41 cases had one temperature peak, while 18 experienced two or more.

Anemia, another near-universal VL symptom, was determined in 57 of our 59 study cases, and lack of anemia in the remaining two patients may be an artifact of early diagnosis. Leucopenia was present in

Distribution of the diseased by age, sex and residence.

According to our statistics, in Bulgaria VL most commonly occurs in adults (57.6%), most of whom are males (70.6%) living in urban areas (69.4%).

Figure 1 Epidemiological distribution of visceral leishmaniasis (VL) in Bulgaria.

50 patients, hepatomegaly in 49, weight loss in 46, and lymphadenitis in 10. Our results show that all of our study subjects developed classical VL. While all of our subjects presented with fever and splenomegaly, reports of other symptoms varied (Table 1). Changes in hemoglobin (Hb), white blood cells (WBC), platelets (PLT), and transaminases (Aspartate Aminotransferase [ASAT], Alanine Aminotransferase [ALAT]) in the 59 patients are presented in Table 2.

In 58 of our 59 cases, the VL diagnosis was confirmed by different laboratory methods (Giemsa stain, IFA, ELISA, and PCR). In the one patient with classic clinical symptoms of VL but a negative

result on the ELISA test, the VL diagnosis was determined *ex juvantibus* when the patient, having refused sternal puncture, was treated with miltefosine and recovered completely (Table 3).

Fifty eight out of 59 patients were tested by Giemsa staining of bone marrow smears, and in 48 of these cases, detection of amastigotes confirmed the diagnosis of VL (Fig. 2). In 46 cases of VL of our patient sample, in fact, Giemsa staining and other serological tests – including ELISA (32 cases) and IFA (14 cases) – were undertaken to confirm an earlier VL diagnosis. These confirmatory tests showed positive results as follows: serological analysis was positive in 39 of the

Table 1 Frequency of the major clinical symptoms in 59 patients with visceral leishmaniasis

Symptom		Number of cases	Relative share (%)	Confidence interval
Fever	Up to 38°C	2	3.4	0.3–9.6
	Up to 39°C	29	49.2	36.4–62.1
	Above 39°C	28	47.5	34.7–60.4
Temperature curve	With one peak	41	69.5	57.0–80.7
	With two peaks	4	6.8	1.8–14.7
	>Two peaks	14	23.7	13.7–35.5
Anemia		57	96.6	90.4–99.7
Leucopenia		50	84.7	74.3–92.8
Splenomegaly		59	100.0	95.1–100.0
Hepatomegaly		49	83.1	72.4–91.6
Lymphadenitis		10	16.9	8.4–27.6
Weight loss		46	78.0	66.4–87.7

Table 2 Clinical laboratory data for the values of hemoglobin (Hb), white blood cells (WBC), platelets (PLT), ASAT, ALAT of the 59 patients with visceral leishmaniasis

Patient	Hb	WBC	PLT	ASAT	ALAT
1	100	1.3	111	100	102
2	117	3.7	149	204	71
3	69	3.8	58	85	51
4	81	2.2	47	2073	520
5	99	3.1	102	42	58
6	66	2.1	93	47	42
7	94	1.9	166	127	120
8	73	1.8	310	69	67
9	74	2.3	150	36	15
10	96	3.0	84	408	389
11	68	5.8	70	81	82
12	89	1.6	80	545	480
13	94	2.9	145	132	263
14	103	3.3	110	259	300
15	84	2.1	78	263	256
16	60	3.0	60	74	61
17	88	2.6	300	64	128
18	104	3.0	112	132	115
19	108	1.5	85	93	76
20	133	1.2	57	47	58
21	98	2.8	115	148	158
22	85	3.9	122	35	23
23	87	5.0	100	56	63
24	109	5.8	131	45	41
25	107	3.8	228	802	571
26	125	3.9	145	43	115
27	97	2.1	130	110	113
28	90	7.6	116	52	48
29	78	2.4	96	525	352
30	59	0.7	40	40	18
31	68	3.1	96	48	54
32	65	2.8	132	65	58
33	77	2.7	101	76	111
34	57	1.3	61	43	161
35	87	3.1	110	185	144
36	85	3.2	114	55	29
37	87	2.6	205	25	21
38	75	2.9	160	49	22
39	87	3.0	225	45	10
40	90	3.4	207	33	31
41	61	1.5	49	11	42
42	101	2.5	103	46	53
43	115	2.7	193	23	24
44	116	3.2	272	79	41
45	49	3.2	28	51	56
46	111	3.3	124	520	242
47	66	3.1	140	55	53
48	111	3.4	148	167	247
49	95	2.8	125	135	64
50	101	3.2	115	175	84
51	115	2.7	143	27	33
52	95	2.9	83	33	37
53	84	2.6	87	81	57
54	94	3.1	208	26	47
55	84	2.1	127	38	42
56	119	3.2	100	201	152
57	92	2.8	47	95	33
58	84	2.7	108	76	83
59	83	1.3	97	83	112

46 patients tested, with ELISA showing a positive result in 28 patients and IFA in 11 patients. Giemsa staining tests, moreover, showed that seven patients with negative serology results were morphologically positive for amastigotes. A positive correlation between morphological (Giemsa staining) and immunological test results was found in 29 cases of the 39 patients who underwent both procedures. Finally, in

10 of the 39 patients where the morphological examination of Giemsa-stained samples was negative, VL diagnosis was confirmed by positive serology and the presence of typical clinical symptoms.

DPTM clinicians used a conventional PCR method for the diagnosis of leishmania infection. All 13 patients whose bone marrow was analyzed using this PCR method tested positive for VL (Fig. 3).

Because of its rarity and obscure nature, VL is often misdiagnosed initially and proper treatment delayed. Our research reveals that even in Bulgaria, only 3 of the 59 patients studied were sent immediately to the parasitic diseases clinic with a diagnosis of 'suspected VL'. This means that most of the cases of VL in our sample, including the one where it occurred in combination with HIV and 'HIV syndrome' was the first diagnosis, were initially misdiagnosed and therefore treated incorrectly in ambulatory or hospital settings. In these cases, the most common working diagnosis was 'fever of unknown origin', and only after initial treatment proved unsuccessful and time had elapsed was the correct diagnosis of 'suspected VL' proposed (Tables 4 and 5). The study we conducted on these 59 cases of VL in Bulgaria reveals that etiologic diagnosis was established in different periods of time after the onset of the first clinical symptoms: within a month ($n=37$), within 2 months ($n=7$), within 3 months ($n=6$), within 4 months ($n=1$), within 5 months ($n=4$), and after more than 5 months ($n=4$).

All patients were treated etiologically and symptomatically. Most of the cases were treated with meglumine antimoniate and rarely with other medications or combinations (Table 6). Complete recovery was observed in 54 cases. Three cases (one child and two adults) suffered a relapse before being treated successfully. In five cases the disease was lethal due to complications and additional severe pathology: Patient 1 was a 65 year old man, who had accompanying ischemic heart disease, steroid diabetes, and non-Hodgkin lymphoma. Patient 2 was a 67 year old man, who in the course of the disease developed hepatorenal syndrome and sepsis. Patient 3 was a 64 year old man, who at the end of the treatment developed sudden cardiac arrest and died. Patient 4 was a 40 year old man with accompanying chronic myelofibrosis, who developed hemorrhagic diathesis and died. Patient 5 was a 43 year old woman with accompanying thyrotoxicosis, she also developed hemorrhagic diathesis before death. All of the five patients who died were treated etiologically with meglumine antimoniate (glucantime).

Discussion

The clinical features and laboratory findings described here are based on data from the case

Arrows in panel A show two extracellular amastigotes. The arrowhead in panel B shows a single amastigote with kinetoplast, an independent organelle of *Leishmania* sp. Giemsa stain, scale bar=10 μ m.

Figure 2 Detection of amastigotes in Giemsa-stained smears of bone marrow aspirates obtained from patients with visceral leishmaniasis.

histories of 59 Bulgarian patients diagnosed with VL. From these patients, we determined that VL is typically characterized by fever, anemia, splenomegaly, hepatomegaly, leucopenia, and weight loss. Deviations of the laboratory values of Hb, WBC, PLT, ASAT, and ALAT are characteristic of the disease, but are not pathognomonic, and etiological diagnosis was placed only after focused parasitological investigations. Time elapsed since the onset of the first clinical symptoms and establishment of etiological diagnosis varied from a month to more than 5 months, but most cases ($n=37$) were diagnosed within the first month of the disease.

All five cases with fatal outcome of the disease were diagnosed within the first month and that is why we believe in these cases that the fatal outcome was not due to delayed diagnosis and treatment, but rather due to the accompanying pathology and complicated course of the disease.

Visceral leishmaniasis diagnosis

Our findings indicate that microscopic examination of biological samples taken from a patient suspected of having the disease is the surest and most effective way to diagnose VL. Today, the gold standard for diagnosing VL is the microscopic examination of Giemsa-stained spleen or bone marrow aspirated samples.¹⁸ In Bulgaria, bone marrow is preferred, because spleen punctures – sometimes cited as a relatively good diagnostic tool¹⁸ – carry the risk of rupture and are therefore considered too dangerous.

(Run on 2.5% agarose-gel electrophoresis and stained with ethidium bromide.) The positive band (K+) is visible on the 260 base-pair MW DNA ladder. Lane M is a DNA ladder. Lane (K-) is a negative control. Lanes 1–2 contain 10 and 20 μ l samples of bone marrow DNA. Lanes 3–4 represent *in vitro* 1 and 2 μ l samples of DNA.

Figure 3 PCR products obtained with primers amplifying a repetitive DNA sequence from Old World *Leishmania*.

Although the literature indicates that splenic aspirates are more informative, Bulgarian clinicians do not use this technique for diagnosis of VL. Bone marrow aspirate, moreover, retains high sensitivity under microscopic examination and is therefore the preferred choice for laboratory diagnosis of VL in Bulgaria.

In our research, 36 of the 46 patients tested with both Giemsa staining and ELISA techniques were

Table 3 Applied laboratory methods for diagnosis of visceral leishmaniasis and results

Used method	Number of cases (%)	Result + (sensitivity%)	Confidence interval
Giemsa stain	58 (98.31%)	48 (82.8%)	71.8–91.5
Indirect fluorescence assay	14 (23.7%)	11 (78.6%)	51.9–96.3
ELISA	32 (54.2%)	28 (87.5%)	73.4–96.8
Polymerase chain reaction	13 (22.0%)	13 (100.0%)	79.7–100

Table 4 Differential diagnostic difficulties in the cases of visceral leishmaniasis (VL)

Initial diagnosis	Number of cases	Relative share (%)	Confidence interval
Fever of unknown origin	32	54.2	41.3–66.9
Acute viral infection	5	8.5	2.7–17.1
Bronchopneumonia	10	16.9	8.4–27.6
Sepsis	1	1.7	0.1–6.6
Hematological disease	5	8.5	2.7–17.1
Collagenosis	2	3.4	0.3–9.6
HIV	1	1.7	0.1–6.6
Under observation for VL	3	5.1	0.9–12.1

positive for amastigote morphology, and 39 had positive serological tests. As to effectiveness, we found no statistically significant difference between Giemsa staining and ELISA techniques (Fisher's exact test, $p > 0.05$).

Treatment

In our practice for treatment of VL we most often used meglumine antimoniate (glucantime). It was used in 56 patients, and in three of the patients due to a lack of therapeutic effect it was necessary to hold a new course of therapy with different medications – two patients were treated with ambisome and one with pentamidine. In two other patients because of insufficient response after the first week of therapy with glucantime alone combined therapy with allopurinol was administered. Only three cases were treated with other preparations – two with miltefosine and one with ambisome. Five of the 59 patients died due to complications in the course of treatment.

Algorithm for diagnosis, treatment, and prophylaxis of VL

Visceral leishmaniasis is a disease with sporadic occurrence in Bulgaria and probably because of this it is omitted from the diagnostic plans of the general practitioners. To bring the attention of the broad medical community to this insufficiently known parasitic disease we have structured a short diagnostic algorithm. The proposed algorithm is based on data from international experience, as well as our research and experience from the daily routine in the National consulting office for parasitic and tropical diseases at NCIPD. The algorithm stands on correlation of clinical, laboratory, and epidemiological criteria that if implemented in a specific sequence facilitates the accurate diagnosis.

Leading criteria can be summarized in the following succession:

1. Clinical criteria: protracted and irregular fever, a gradual reduction of formed blood elements as most frequently anemia is leading, enlarged spleen and liver, body weight reduction.
2. Laboratory criteria: detection of amastigote stages of the parasite in Giemsa-stained biopsy material from bone marrow, spleen, liver, or lymph nodes; detection of promastigote stages of the parasite in culture medium after inoculation with specimens from the patients; establishing the presence of a specific antigen by immunological methods; establishing the presence of a specific antibody using immunodiagnostic methods (ELISA, Direct agglutination test [DAT], IFA, Western blot, etc); detection of DNA/RNA of the parasite by PCR methods.
3. Epidemiological criteria: living in or coming from a region or country endemic for VL.

In Bulgaria the patients diagnosed with VL are directed to hospital admission and the treatment takes place in hospital settings.

Prophylaxis consists of follow-up clinical and laboratory exams of the patients in months 3 and 12 after treatment. Required tests are: blood test for at least eight indicators: Hb, erythrocytes (Er), Haematocrit (Hct), WBC, PLT, Erythrocyte sedimentation rate (ESR), ASAT, ALAT; serological test for the presence of anti-leishmanial antibodies.

Our study shows that the clinical manifestations of the disease in Bulgaria do not differ significantly from those described in the literature.^{3,19,20} The diagnosis in the 59 cases was established using widely applied methods for diagnosis of VL.^{20,21}

In the absence of national guidelines and official treatment policy for VL in Bulgaria, as in some other countries,^{3,22} for treatment we applied mainly pentavalent antimonials (meglumine antimoniate) which according to some authors are drugs of choice for treatment of all types of leishmaniasis,^{3,23} and also are not costly and are more affordable than the lipid formulations of amphotericin B.

Conclusion

Our research analyzed data on 59 cases of VL in Bulgaria. From these combined data, we were able to describe Bulgarian VL's major clinical, diagnostic, and therapeutic aspects. We further found that, even in a country where VL is endemic and relatively common, it is frequently misdiagnosed. Because VL

Table 5 Initial treatment and used medications

Used medications	Number of cases	Relative share (%)
Corticosteroids	1	1.7
Antibiotics	46	78.0
Corticosteroids and antibiotics	10	16.9
Not conducted	2	3.4

Table 6 Etiological therapy

Medication	Number of cases	Relative share (%)	Dosage for children and adults	Administration schedule and duration
Glucantime	51	86.4	20 mg/kg body weight (b.w.) every 24 h. Antimony (Sb) base	20–28 days, applied twice daily (intramuscular (i.m.)/intravenous (i.v.))
Ambisome	1	1.7	3 mg/kg b.w. every 24 h	Five consecutive days, once a day (i.v.) followed by two applications at day 14 and day 21
Miltefosine	2	3.4	2.5 mg/kg b.w. every 24 h	Twice daily (<i>per os</i> (p.o.)) for 28 days
Glucantime followed by ambisome	2	3.4	The two medications are dosed as it is shown above	The two medications are scheduled as it is shown above
Glucantime followed by pentamidine	1	1.7	As above (for glucantime) and 4 mg/kg b.w. every 24 h (for pentamidine)	As above (for glucantime) and 20–28 days, once a day (i.m./i.v.) (for pentamidine)
Glucantime+allopurinol	2	3.4	As above (for glucantime) and 20 mg/kg b.w. every 24 h (for allopurinol)	20–28 days (i.m./i.v) glucantime and 20–28 days for allopurinol (p.o.)

is hard to recognize and relatively new to Northern Europe, misdiagnosis is common and treatment too often inappropriate and delayed. Because this disease is lethal if left untreated, and is now spreading in parts of Europe where it was hitherto unknown, VL poses significant new medical and social risks. We therefore humbly offer these observations – gleaned over many years from the Bulgarian experience – on how best to recognize, diagnose, and treat VL.

Conflict of interest

The authors declare they have no conflict of interest.

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References

- 1 Faucher B, Gaudart J, Faraut F, Pomares C, Mary C, Marty P, *et al.* Heterogeneity of environments associated with transmission of visceral leishmaniasis in South-Eastern France and implication for control strategies. *PLoS Negl Trop Dis.* 2012;6(8):e1765
- 2 European Parliament and Council Directive 2003/99/EC. Nov 17, 2003. On the Monitoring of Zoonoses and Zoonotic agents (Amends Council Decision 90/424/EEC and repeals Council Directive 92/117/EEC. *OJ L 325*, 12.12.2003: 31.
- 3 WHO. Control of leishmaniasis: report of a Meeting of the WHO Expert Committee on the Control of Leishmaniasis. Geneva, 22–26 March. WHO Technical Report no. 949, 2010. p. 202.
- 4 Vasileva K. Population and social conditions. *Eurostat.* 2011;34:1–7.
- 5 Pavli A, Maltezos HC. Leishmaniasis, an emerging infection in travelers. *Int J Infect Dis.* 2010;14:e1032–9.
- 6 Malik AN, John L, Brucson AD, Lockwood DN. Changing pattern of visceral leishmaniasis, United Kingdom, 1985–2004. *Emerg Infect Dis.* 2006;12:1257–9, doi: 10.3201/eid1208.050486
- 7 Gruev T. Contributions to the study of leishmaniasis (kala-azar) in the countryside. *Medical Records.* 1949;8:845–55 [in Bulgarian].
- 8 Bratanov B. First contributions to autochthonous childhood visceral leishmaniasis in Bulgaria. *Scientific Works of Internal and Pediatric Medicine.* ISUL, 1952:113–58 [in Bulgarian].
- 9 Andreev I, Angelov A, Karapanchev B. Observation of cases of kala azar in childhood. *Ped Obst Gyn.* 1958;2:38–46 [in Bulgarian].
- 10 Filipov G, Harizanov R, Jordanova D, Vuchev D, Kurdova R, Dimitrov H. Current state of visceral leishmaniasis in Bulgaria. *Infectology.* 2003;4:29–31 [in Bulgarian].
- 11 Vutova K. Visceral leishmaniasis: clinical and laboratory features and treatment. *Med Rev.* 2007;3:26–31 [in Bulgarian].
- 12 Harizanov R. Visceral leishmaniasis. In: Kurdova R (eds.), *Parasitic zoonosis in humans.* NCIPD; 2008. p. 145–75 [in Bulgarian].
- 13 Boeva-Bangiozova V, Yancheva N. Visceral leishmaniasis. *Physician Pract.* 2009;11(1):21–7 [in Bulgarian].
- 14 Filipov G, Jordanova D, Rainova I. Studies on the diagnostic capabilities of the immunofluorescence reaction in visceral leishmaniasis. *Infectology.* 1999;2:39–41 [in Bulgarian].
- 15 Piarroux R, Fontes M, Perasso R, Gambarelli F, Joblet C, Dumon H, *et al.* Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. *Mol Biochem Parasitol.* 1995;73:249–52.
- 16 Ranchov G. Biostatistics. Sofia, Bulgaria: GorexPress 2004. p.182 [in Bulgarian].
- 17 WHO. Control of the leishmaniasis. Technical Report Series 793. 1990. p. 158.
- 18 Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. *Clin Diagn Lab Immunol.* 2002;9(5):951–8, doi: 10.1128/CDLI.9.5.951-958.2002
- 19 Cruz I, Chicharro C, Nieto J, Bailo D, Canavate C, Figueras M-C, *et al.* Comparison of new diagnostic tools for management of paediatric Mediterranean visceral leishmaniasis. *J Clin Microbiol.* 2006;44(7):2343–7, doi: 10.1128/JCM.02297-05
- 20 Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, Desjeux P, *et al.* Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect Dis.* 2002;2:494–501.
- 21 Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, *et al.* Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol.* 2007;5:7–16, doi:10.1038/nrmicro1748
- 22 Gradoni L, Gramiccia M, Scalone A. Visceral leishmaniasis treatment, Italy. *Emerg Infect Dis.* 2003;9(12):1617–20.
- 23 Monzote L. Current treatment of leishmaniasis: a review. *Open Antimicrob Agents J.* 2009;1:9–19.