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CONTENTS

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Named authors must fit the following three criteria:

1. Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data;
2. Drafting the article or revising it critically for important intellectual content; and
3. Final approval of the version to be published.

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

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

ACKNOWLEDGEMENTS

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

REFERENCES

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

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Identifying details of patients should be omitted. Identifying information, including patients' names, initials, or hospital numbers, should not be published unless the the patient (or parent or guardian) gives written informed consent for publication. When informed consent has been obtained it should be indicated in the published article.

TULAREMIA. CURRENT STATE AND INVESTIGATIONS IN BULGARIA

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ABSTRACT

Tularemia is a very serious zoonotic infectious disease that can be transmitted to humans and cause outbreaks. Control and proper treatment of this disease require early and precise diagnosis. This review summarizes the current state of the problem in Bulgaria and describes worldwide trends in ecology, prevention, diagnosis and therapy. In the last 5 years only few cases of tularemia are reported in our country. However, increased international travel and mobility in the last decades could pose a risk of bringing the disease back.

Key words: *Francisella tularensis*, tularemia, molecular methods, diagnosis

Tularemia was first described in 1911 during an outbreak of a plague-like disease in rodents in the area of Tulare Lake, California. The isolated causative agent was a small gram-negative bacterium which was named *Bacterium tularensis* (1). Subsequently, infections with *Francisella tularensis* are observed in mammals, birds, amphibians, fish, and invertebrates (2). Tularemia is a disease of the northern hemisphere, most often occurring in Scandinavia, northern America, Japan, and Russia (3, 4, 5, 6, **7**). Tularemia has been reported also from Bulgaria, Turkey, Serbia, Spain, and Switzerland (9, 10, 11, 12). Various colloquial names are associated with this disease, including „rabbit fever", „hare fever", „deerfly fever", „lemming fever" (2).

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The etiological agent of tularemia*, F.tularensis,* comprises four subspecies: 1) *F.tularensis* biovar *tularensis* (or type A) is found almost exclusively in North America and as the most virulent of all known subspecies it is associated with lethal pulmonary infections; 2) *F.tularensis* biovar *palearctica* (also known as biovar *holarctica* or type B) is found in Euroasia and rarely leads to fatal disease outcome. This biovar does not demonstrate citrulline ureidase activity and ability to produce acid from glucose; 3) *F.tularensis subspecies novicida* (former *F.novicida*) is characterized as a relatively nonvirulent. Only two tularemia cases in North America have been attributed to *novicida* and these were only in severely immunocompromised individuals; 4) *F.tularensis* biovar *mediasiatica* is found primarily in Central Asia and still, little is known about its ability to infect humans (27).

F.tularensis is considered as a biological threat agent. Bioweapons based on *F.tularensis* were designed during the Cold War in the USA and the former USSR. In the last decades the emphasis has shifted towards defence against biological terrorism (28). Appropriate methods for rapid and precise detection are therefore essential to warrant public safety.

The γ-proteobacterial family *Francisellaceae* contains only the genus *Francisella* and lacks close relatives that are pathogenic to humans. 16S rRNA data suggest that the ciliate endosymbiont *Caedibacter taeniospiralis* (29) (87% sequence similarity of the 16S rRNA molecule to that in *F.tularensis*) is a member of a sister clade. The fish pathogen *Piscirickettsia salmonis* (30) is also a phylogenetic relative, although more distant. Two species of the genus *Francisella* are accepted according to current taxonomy. The second species is *F.philomiragia*, which rarely causes disease and only in immunocompromised individuals or near-drowning victims (31). The genus *Francisella* will certainly gain additional taxonomic members in the near future. Different genetic clades of *Francisella*-like bacteria have emerged recently: pathogens capable of enzootic disease in several species of fish; tick endosymbionts; and bacteria in soils and sediment. According to phylogenetic analysis there is a

major split deep within the *Francisellaceae*, dividing the lineages that contain the species *F.tularensis* and *F.philomiragia*. Thus, the taxonomic division between these two accepted species is supported by phylogeny. As previously reported (32), the strains of *F.philomiragia* appear to closely resemble isolates that have been found to cause disease in several species of fish. Species reported to be affected by *Francisella* infections include tilapia (*Orechromis* spp.) (33), hybrid striped bass (*Morone saxatilis*) (34), three-line grunt (*Parapristipoma trilineatum*) (35), and Atlantic cod (*Gadus morhua*) (36). Thus, both freshwater and saltwater fish can be affected, and these infections may be of considerable economic importance.

F.tularensis is maintained in the environment, terrestrial and aquatic mammals such as ground squirrels, rabbits, hares, voles, muskrats, and water rats (37, 38, 39, 40). In regions where tularemia is endemic, antibodies to *F.tularensis* are frequently detected in the sera of wild animals (41, 42, 43, 44). Outbreaks of disease in humans and wild animals are often related. In Sweden a clear correlation between peaks in vole and hare populations and outbreaks of tularemia in humans have been reported (45) but it is not clear whether these animals are a real reservoir of the bacterium in the environment. Many arthropod vectors are involved in the transmission of tularemia between mammalian hosts. In Central Europe the ticks *Dermacentor reticulatus* and *Ixodes ricinus* are the most important vectors. In Czech Republic and Austria 2.1-2.8% of *D.reticulatus* contain *F.tularensis* (46). In the USA biting flies are the most common vectors in Utah, Nevada and California (38, 47), while ticks are the most important vectors east of the Rocky Mountains. In the former USSR the bacterium is transmitted by both mosquitoes and *Ixodes* ticks (47). These arthropod vectors are transmitting the disease within wild animal populations and from animals to humans (48, 49, 50). Other reservoirs of *F.tularensis* are some free-living amoebas and protozoa such as *Tetrahymena pyriformes* (26).

The first tularemia outbreak in Bulgaria occurred near the Srebarna reserve, in northeastern Bulgaria in 1963. The disease seemed to be eradicated during the following 34 years but in 1997 a second outbreak was reported from western Bulgaria close to the Serbian border. In 2003 incidence increased in the same region. In our country a total of 262 tularemia cases were reported during the period 1998–2003 (23).

Two new *Francisella*-like endosymbionts (FLEs) are found in the ticks *Hyalomma marginatum marginatum, Hyalomma aegyptium*, and *Rhipicephalus sanguineus* in Bulgaria. These FLEs are characterized by 16S rRNA and *tul4* gene sequencing. They lack the molecular marker RD1 and are considered facultative secondary endosymbionts of ticks (14).

The buzzard *Buteo buteo* and other birds can also function as vectors of infection. Such case is reported in Bulgaria in 2010 (15).

Tularemia has six characteristic clinical presentations: ulceroglandular (75% of all forms), glandular, oropharyngeal, pneumonic, oculoglandular, and typhoidal (27).

The incubation period for tularemia is 1-14 days. The onset of most human infections is after 3-5 days (51). Clinical symptoms observed in infected mammals include fever, lethargy, anorexia, septicemia. The lethality is high. Non-human mammals do not develop skin lesions as seen in people. Subclinical infections are common and animals often develop specific antibodies to the organism. Fever is moderate or very high and at this stage tularemia bacilli can be isolated from blood cultures. The face and eyes redden and become inflamed. Inflammation spreads to the lymph nodes, which enlarge and may suppurate. Lymph node involvement is accompanied by a high fever. Death occurs in less than 1% of cases with prompt initiation of adequate treatment.

Prophylaxis and appropriate therapy of tularemia rely on correct diagnosis. *F.tularensis* is a fastidious bacterium requiring enriched growth media and cysteine glucose blood agar is the most commonly used. It can also be recovered on enriched chocolate agar and nonselective buffered charcoal yeast extract agar (28, 52). Because *F.tularensis* is difficult to culture, most cases of tularemia were diagnosed on the basis of clinical manifestations and/or serology (53, 54).

Serological tests for the diagnosis of *F.tularensis* infections were attractive because diagnostic work involving culture procedures carries a risk of infection for the laboratory staff (55, 56). The diagnosis of human cases of tularemia was often accomplished by estimation of an antibody response to *F.tularensis* by tube- or micro-agglutination assay and enzyme-linked immunosorbent assay (ELISA) (57, 58, 59). Specific antibody response in patient serum is detectable from 4 to 7 days after the onset of the disease by micro-agglutination assay. However, these immunological assays can be confounded by serum cross-reactivity with antigens of *Brucella, Haemophilus* and *Yersinia* (60). The diagnosis of patients with tularemia was confirmed by the demonstration of an antibody response to *F.tularensis*, which occurs about 2 weeks after the onset of the disease. Serological methods are not always sensitive enough. Therefore in the last decade a number of molecular techniques for early and correct identification of the different *F.tularensis* subspecies and biovars are being developed.

Multiple Locus Variation Assay (MLVA) for *F.tularensis* utilizes Ft-M3 and Ft-M10 markers for amplifying samples which test positive for both fopA and PPI-helicase genes. The sequence size from Ft-M3 varies between 270-369 bp and the repeating units form 7-18 copies. Based on the number of copies of these loci seven genotypes are identified.

A developed Real-time PCR assay uses the LightCycler (LC) system for detection of *Francisella*-specific sequence of the outer membrane protein (*fopA* gene). *F.tularensis* strains subjected to this LC-PCR assay test positive, whereas *F.philomiragia* and other bacterial species do not show fluorescent signal. A linear response is observed when using *F.tularensis* genomic DNAs of between 20 fg and 2 ng, corresponding to 1.2 to 1.2×10^5 bacteria. This technique allows specific, sensitive and rapid detection of *F.tularensis* (61).

Antibiotics used to treat tularemia (8) include fluoroquinolones (e.g., ciprofloxacin), tetracyclines (e.g., doxycycline), and aminoglycosides (streptomycin and gentamicin). Because no effective and safe vaccine is currently available, tularemia prophylaxis following proven exposure to *F.tularensis* also relies on administration of antibiotics. New therapeutic alternatives have to be developed for several reasons: potential toxicity of first-line drugs, especially in children and pregnant women; high rate of treatment relapses and failures, especially for severe and/or suppurated forms of the disease; the possible use of antibiotic-resistant strains in the context of a biological threat.

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PREVALENCE OF CARBAPENEMASE GENES AMONG 16S RRNA METHYLTRANSFERASE-PRODUCING ENTEROBACTERIACEAE ISOLATED FROM CANCER PATIENTS

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ABSTRACT

The co-existence of carbapenemase and 16S rRNA methyltransferase can cause serious difficulty in treating infections with multidrug-resistant Gram-negative pathogens. In this study we investigated the prevalence of carbapenemase genes among 16S rRNA methyltransferase-producing enterobacteria isolated from cancer patients. A total of 100 non-duplicate clinical isolates of *Enterobacteriaceae* harbouring methyltransferase genes were studied. Multiplex polymerase chain reaction (PCR) using nine sets of carbapenemase specific primers (VIM, IMP, SIM, GIM, SPM, NDM-1, KPC, GES, OXA-48) followed by sequence analysis of PCR amplicons were used to identify carbapenemase genes. Screening for associated ESBL and AmpC-type genes was carried out by PCR-based assays and PCR products were sequenced. Genotyping, by pulse-field gel electrophoresis (PFGE) of genomic DNA was performed to determine genetic relatedness of carbapenemase-producing isolates. Among the one hundred 16S rRNA methyltransferase-producing enterobacterial isolates, 11 were positive for carbapenemase genes. Of these, 10 ArmA-positive *Proteus mirabilis* isolates were identified with VIM-1 carbapenemase gene and co-existence of SHV-12 and CMY-99 beta-lactamases. In addition, the NDM-1 carbapenemase gene was identified in one *E. coli* strain harbouring *rtmB* methyltransferase gene. The remaining 89 enterobacterial isolates, which were ArmA-positive, did not possess any of the carbapenemase genes studied. This study reports on the low prevalence (11%) of carbapenemase genes among 16S rRNA methyltransferase-producing enterobacteria at the cancer hospital in Sofia, and on the co-existence of VIM-1 carbapenemase gene and ArmA methylase gene in *Proteus mirabilis* isolates.

Key words: *Enterobacteriaceae*, 16S rRNA methyltransferase, carbapenemase

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INTRODUCTION

Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life threatening infections caused by Gram-negative bacteria (1). They act by binding to the A-site of 16S rRNA within the prokaryotic 30S ribosomal subunits, thereby inhibiting translation resulting in cell death (2). Resistance is frequently mediated by aminoglycoside-modifying enzymes that are able to acetylate, phosphorylate or adenylate the antibiotic molecule (3). Recently, methylation of the aminoacyl site of the 16S rRNA has been described as an acquired high-level resistance mechanism to clinically important aminoglycosides such as amikacin, tobramycin and gentamicin (4). Since 2003, eight 16S rRNA methyltransferase genes - *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF* and *npmA* - have been identified in several species of pathogenic bacteria worldwide (5). The genes encoding these determinants are usually borne by mobile genetic elements and have been associated with

mechanisms conferring resistance to other antibiotic classes, such as Qnr or acquired beta-lactamases (5). Recently these methyltransferases have been found in association with carbapenemases (6). Emergence of multidrug-resistant bacteria with carbapenemases and other currently identified resistance determinants such as the 16S rRNA methyltransferases is becoming an increasing clinical and public health threat.

This study was designed to determine the prevalence of carbapenemases among 16S rRNA methyltransferase-producing *Enterobacteriaceae* isolated from cancer patients.

MATERIALS AND METHODS

A total of one hundred 16S rRNA methyltransferase-producing clinical *Enterobacteriaceae* strains recovered at the Cancer hospital in Sofia, Bulgaria were screened for the presence of carbapenemase genes. These strains were collected consecutively between 2004 and 2013. Clinical samples were urine, pus, sputum and blood. The MICs of gentamicin, amikacin, meropenem and imipenem were determined using the Etest (AB BioMerieux, Solna, Sweden). Susceptibility to trimethoprim-sulfamethoxazole, ciprofloxacin, nalidixic acid, tetracycline, tigecycline, fosfomycin and chloramphenicol was tested by disk diffusion method on Mueller-Hinton II agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines with disks supplied by Becton Dickinson (Sparks, MD). The results were interpreted following CLSI-2013 recommendations (7). *Escherichia coli* ATCC 25922 was used as antibiotic-susceptible control strain.

Bacterial DNA was isolated by PrepMan Ultra reagent (Life Technologies Inc.). Multiplex EvaGreen Real-time PCR was performed for the detection of methyltransferase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, and *npmA*) by combining previously published primers (8, 9). Amplicons from positive samples were confirmed on a high-resolution capillary electrophoresis QiAxcel (Qiagen). Similarly, Multiplex EvaGreen Real-time PCR was conducted to detect nine carbapenemase genes encoding VIM, IMP, SIM, GIM, SPM, NDM-1, KPC, GES and OXA-48 (10-15). A multiplex PCR was performed for the detection of acquired AmpC genes as previously described (16). The detection of genes encoding CTX-M, SHV and TEM ESBLs was carried out according to Rodriguez-Villalobos (17). DNA sequencing was performed with primers amplifying the complete genes. Molecular typing, by pulse-field gel electrophoresis (PFGE) of *Apa*I digested genomic DNA was performed to determine genetic relatedness of VIM-1-producing *Proteus mirabilis* isolates (13).

RESULTS

Among the one hundred 16S rRNA methyltransferase-producing enterobacterial isolates, 11 were positive for carbapenemase genes. Of these, 10 ArmA-positive *Proteus mirabilis* isolates were identified with VIM-1 carbapenemase gene and co-existence of SHV-12 and CMY-99 beta-lactamases. In addition, the NDM-1 carbapenemase gene was identified in one *E. coli* uropathogenic strain harbouring *rtmB* methyltransferase gene and CTX-M-15 ESBL. The remaining 89 enterobacterial isolates, which were ArmA-positive, did not possess any of the carbapenemase genes studied. The VIM-1-producing *P. mirabilis* isolates were recovered from urine or pus, and based on clinical data they were epidemiologically unrelated (Table 1). These isolates were found in five distinct wards between 2007 and 2012. Based on genotyping data, however, VIM-1-producing *P. mirabilis* isolates yielded identical PFGE patterns suggesting probable nosocomial persistence of VIM-1 and ArmA-positive strain.

DISCUSSION

The 16S rRNA methyltransferases pose a public health threat because they confer broad and high-level resistance to most clinically available aminoglycosides. Some of the 16S rRNA methyltransferases presumably originated as intrinsic, chromosomally encoded enzymes in environmental Actinomycetes, serving to protect these bacteria from the aminoglycosides that they produce (18). In recent years, however, diverse 16S rRNA methyltransferases have emerged as acquired resistance determinants in several genera of the *Enterobacteriaceae* and have

Table 1. Characteristics of VIM-1 and ArmA-producing Proteus mirabilis clinical isolates from the cancer hospital in Sofia

				Resistance evaluation		
Strain	Specimen	Date of isolation	Ward	Genotypic	Phenotypic	PFGE pattern
PM1421	urine	2007-04-04	Radiotherapy	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM1228	urine	2009-04-07	Abdominal surgery	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM1468	urine	2009-04-23	Abdominal surgery	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM4127	urine	2009-09-23	Intensive care unit	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM4255	urine	2009-12-03	Abdominal surgery	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM2966	urine	2011-08-03	Gynaecology	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM3866	urine	2011-10-06	Gynaecology	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM902	urine	2012-02-02	Urology	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM3014	urine	2012-07-26	Urology	CMY-99, SHV-12	Sxt Nx Cp Cm	A
PM5529	pus swab	2012-11-13	Abdominal surgery	CMY-99, SHV-12	Sxt Nx Cp Cm	A

Resistance abbreviations: Sxt, trimethoprim-sulfamethoxazole; Nx, nalidixic acid; Cp, ciprofloxacin; Cm, chloramphenicol.

been detected not only in humans, but also in pets (19) and food (20). The genes encoding these determinants are usually associated with ESBLs (21, 22), but recently they have been found in association with carbapenemases (6).

In this study we found that 11% of consecutive 16S rRNA methyltransferase-producing *Enterobacteriaceae* from a cancer hospital were positive for VIM-1 and NDM-1 carbapenemase genes. In addition, theses isolates also contained ESBLs and CMY-99 AmpC-type beta-lactamase. All of these genes have been recently reported in Bulgaria suggesting their rapid spread (23,24).

Multidrug-resistant organisms, including those expressing resistance to carbapenems, are becoming increasingly prevalent worldwide. The identification of 16S methyltransferase and carbapenemase genes co-resident in strains harbouring ESBLs, acquired AmpC enzymes and fluoroquinolone-resistance mechanisms not only leads to the potential for co-selection and maintenance of resistance by the use of other antibiotics, but also seriously compromises the treatment of life-threatening infections caused by Gram-negative organisms. Although the level of aminoglycoside consumption in Bulgarian hospitals is second to beta-lactams and fluoroquinolones, the overuse of cephalosporins in the recent years might lead to the co-selection of resistance plasmids harbouring beta-lactamases and 16S rRNA methylases (25).

CONCLUSION

In conclusion, we identified the emergence of NDM-1 and VIM-1 carbapenemase genes associated with RtmB and ArmA methyltransferase, respectively, in *Enterobacteriaceae* isolated from cancer patients. Collaborative multinational programmes are crucial if further development of antimicrobial resistance is to be delayed.

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EXTENDED-SPECTRUM BETA-LACTAMASE SURVEILLANCE IN BULGARIA IN VIEW OF ANTIBIOTIC CONSUMPTION

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ABSTRACT

The objective of the present study was to assess the relationship between consumption of ceftriaxone and incidence of extended-spectrum beta-lactamase-producing (ESBL) *Escherichia coli* and *Klebsiella pneumoniae* in Bulgarian hospitals between 2007-2013. Cephalosporins and carbapenems on average account for 50% of total hospital antibiotic use, of which ceftriaxone use gradually increases up to 70% during the monitored time period. National surveillance database BulSTAR shows evident increase in ESBL producers from 8% in 2007 to 14% in 2013. Linear regression was used to analyze trends in the annual consumption of ceftriaxone and rates of ESBL confirmed hospital isolates *E.coli* and *K.pneumoniae*. We demonstrated statistically significant positive correlation (r=0,87; P=0,01) between these two variables. On average 70 hospitals participated with a total of 25957 isolates yearly. Inferring causal associations between antibiotic use and dynamics in relevant resistance traits is important for identifying possible risk factors for multidrug-resistant infections and outbreaks.

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INTRODUCTION

Many studies establish the relationship between prevalence of resistance to antibiotics and their use as one of the factors determining this process. In European countries there is a well-defined north-south gradient with respect to antibiotic consumption that is also applicable to observed resistance rates in bacterial pathogens prevalent in the community (1, 2).

In the early 1980s a major acclaim in clinical practice is the introduction of third-generation cephalosporins, which at this time proved vital against the emerging β-lactamase-mediated bacterial resistance to antibiotics. Shortly after this breakthrough, in 1983 followed the first report describing plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins. Since then extended-spectrum beta-lactamases (ESBLs) have evolved greatly more than 20 years (3). ESBL-producing pathogens gained successful spread worldwide and their emergence continuously provokes challenges in managing patients` outcome, i.e. complicated therapy and limited treatment options, predisposing infected patients to higher mortality and longer length of hospital stay, and causing serious consequences for infection control. One of the risk factors associated with the acquisition of ESBL-producing organisms, which is still undergoing further studies, is the consumption of third-generation cephalosporins, other beta-lactams and fluoroquinolones (4).

The objective of the present study was to describe relationships existing between antibiotic use, extended-spectrum cephalosporins in particular, and the incidence of ESBL-producing pathogens in hospitals in Bulgaria for a 7 year period. This study aims to launch the first attempts in inferring the causal associations between antibiotic use and resistance in Bulgaria.

MATERIALS AND METHODS

ESBL annual rates of hospital isolates *E.coli* and *K.pneumoniae* were extract-

ed from the National surveillance system BulSTAR for the period 2007-2013. ESBL testing results are reported from participating hospitals with the utilization of the Double Disc Synergy Test (DDST) which uses extended-spectrum cephalosporins. The average number of hospital participants (multiprofile and specialized) in the surveillance network per year is 70 and the average total number of *E.coli* and *K.pneumoniae* isolates is 25957.

Information on expended antimicrobials for systemic use as numbers of packages is collected annually from the hospital and ambulatory care sector and these data are obtained from the Intercontinental Medical Statistics (IMS Health). The Anatomical Therapeutic Chemical (ATC) classification and the unified Defined Daily Dose (DDD) measurement unit were assigned to the data (ATC/DDD version 2012) (5). The amount in grams for an antimicrobial agent was converted to a number of defined daily doses (DDD). Consumption, i.e., antimicrobial usage density, was expressed as daily defined doses and normalized per 1,000 inhabitants per day, instead of hospital-days, since our data on antibiotic use is aggregated for the hospital sector of the whole country. One DDD is the standard adult daily dose of an antimicrobial agent for a 1 day's treatment, as defined by the World Health Organization (5).

Linear regression was used to analyze the trend in the annual consumption of the most used cephalosporin agent ceftriaxone and the trend in the rate of ESBL confirmed hospital isolates *E.coli* and *K.pneumoniae*. Pearson's correlation coefficient was used to determine the relationship between the monitored antibiotic consumption and trends in monitored resistance (ESBL producers). A P value <0.05 was considered statistically significant.

RESULTS

In Bulgarian hospitals highlights of antibiotic consumption form a characteristic structure. During the 7 year period 2007-2013 almost half of the total hospital consumption is in favor of the cephalosporins with measured values around 0.8 DDDs/1000 inhabitants per day (6). Considering these values they occupy between 46-54% of

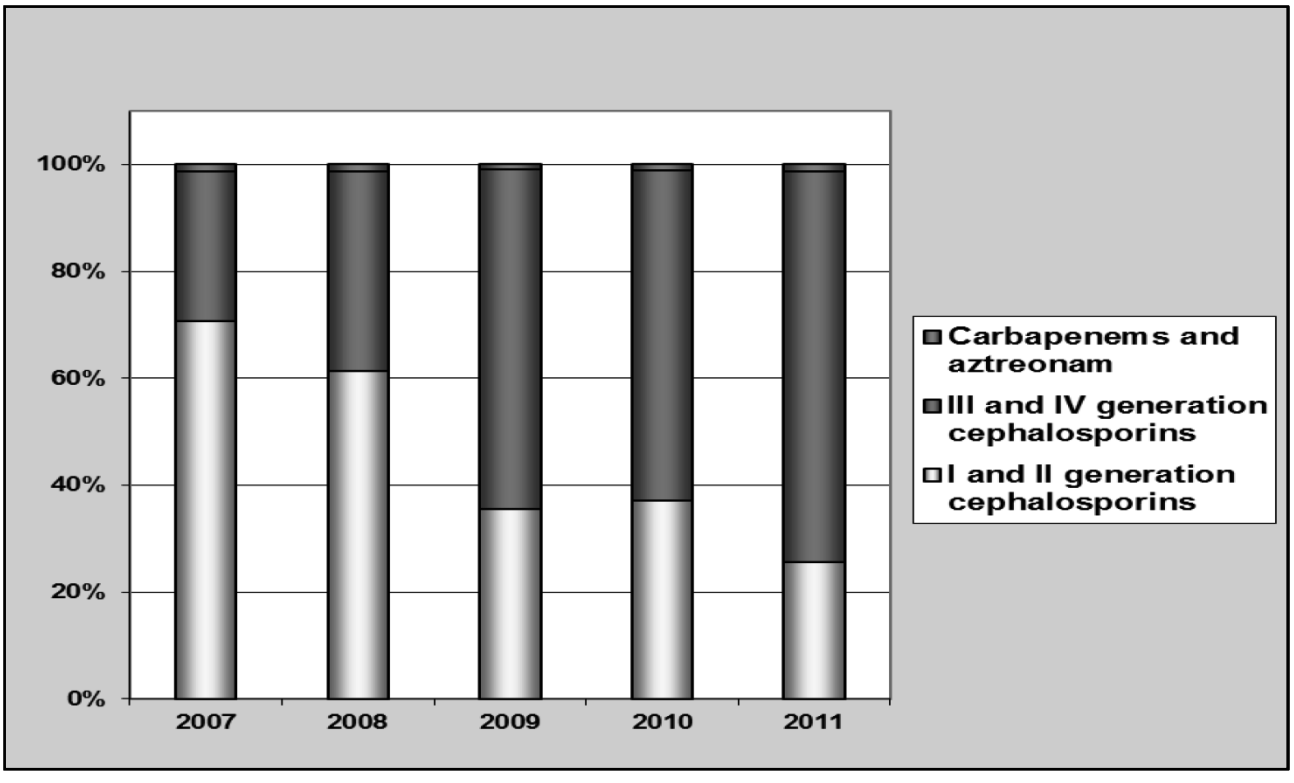

Fig. 1. Percentage values in hospital consumption of the J01D group

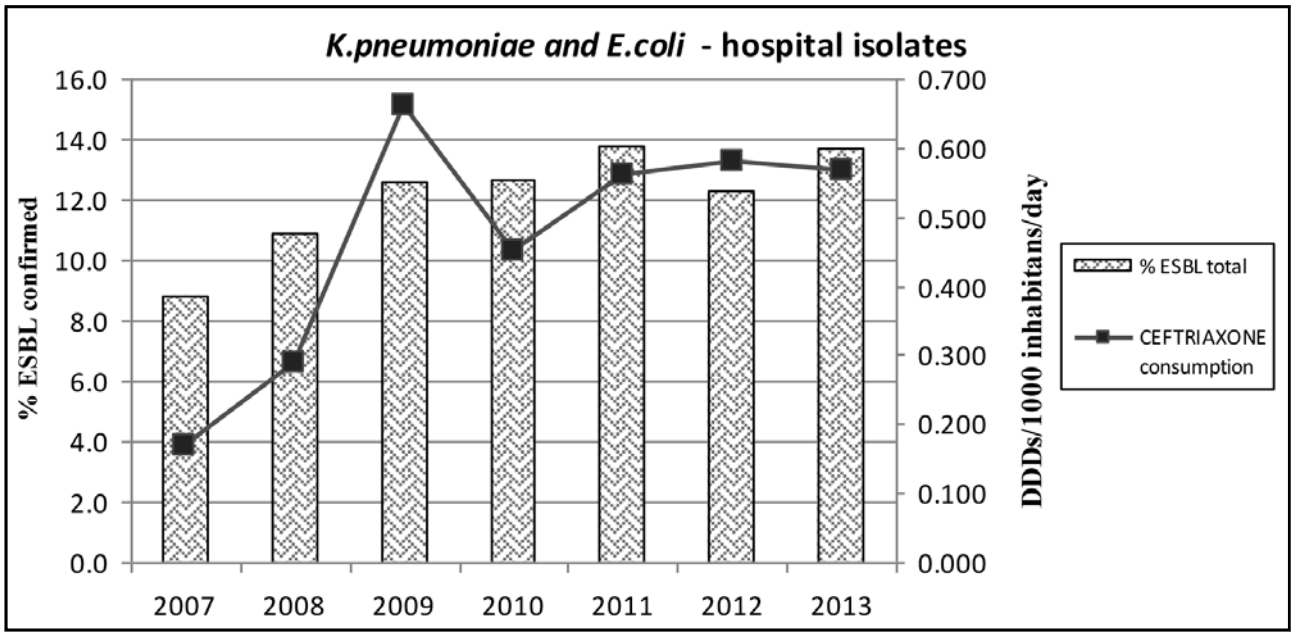

Fig. 2. *K.pneumoniae* and *E.coli* ESBL rates and consumption of the extended-spectrum cephalosporin ceftriaxone

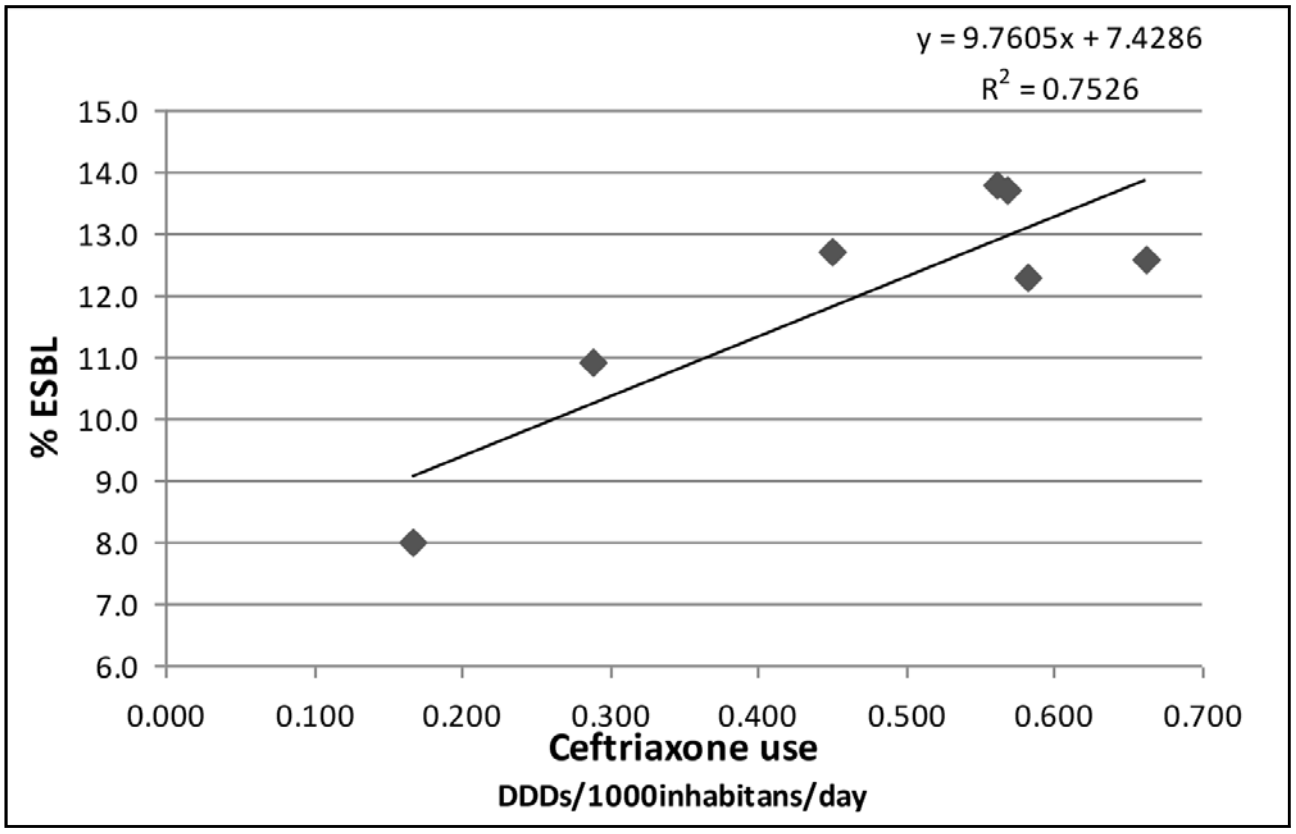

Fig. 3 Linear regression analysis of ceftriaxone use and incidence of ESBL producers among hopspital isolates *E.coli* and *K.pneumoniae*

the consumption compared with all other classes of antimicrobials. Figure 1 shows the progressive increase in the percentage of use of extended-spectrum cephalosporins in hospital settings versus the more narrow-spectrum agents. In 2007 they account for 29% of consumption of the entire group cephalosporins, carbapenems, and aztreonam. In 2011 this value has increased to 71%.

Annual measurements of ESBL confirmed hospital isolates *E.coli* and *K.pneumoniae* on Figure 2 are plotted together with consumption values of ceftriaxone whose usage is the most prevalent. The increment of ESBL producers is evident as a major trend reaching almost 14% in 2013.

Figure 3 represents the scatter plot of ceftriaxone consumption density and rates of ESBL confirmed isolates. This univariate analysis found a significant positive correlation (r=0,87; P=0,01). The coefficient r^2, which can assume values between 0 and 1, shows the proportion of variance explained by this analysis, that is 0,75 or 75%.

DISCUSSION

Introduction of hospital antibiotic stewardship programs follows the purpose of an attempt to reduce the occurrence of antibiotic resistance. Still there is no clear answer or warrant as to whether reducing antibiotic use will result in a parallel reduction in antibiotic resistance (4). The objective of the present study was to study the relationship between hospital antibiotic use of ceftriaxone and the incidence of ESBL-producing pathogens.

Former studies in Bulgarian hospitals (7, 8, 9) identify different families of ESBL enzymes, with CTX-M-like and SHV-like often found as the most prevalent. Our study demonstrated statistically significant relationship between the use of a particular extended spectrum-cephalosporin and the incidence of ESBL-producing pathogens. The hospital consumption of ceftriaxone correlated positively with the observed resistance trait. The consistency of these findings drew a parallel with those reported by others in relation to the contribution of this antibiotic class to high incidence rates of ESBL-producing pathogens in healthcare settings (10, 11, 12, 13), thus following the lines of evidence for a cause-effect relationship between antibiotic use and resistance proposed by McGowan (14). Considering that almost half of the total antibiotic consumption in Bulgarian hospitals is dominated by the cephalosporin group our research intended to conclude the direct relationship between this antibiotic class and the indicator resistance determinant of ESBL production.

In conclusion, the present study attempted to initiate the first steps in clarifying the relationships between antibiotic use and incidence of ESBL-producing pathogens in Bulgarian hospitals. Following investigations will take into account the use of other antimicrobials contributing to resistance rates with the construction of a multivariate analysis giving a more extensive view on these causal associations.

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LABORATORY-BASED DIAGNOSIS OF GONOCOCCAL AND GENITOURINARY CHLAMYDIAL INFECTIONS

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ABSTRACT

Gonococcal and genitourinary chlamydial infections remain a significant public health concern globally. The incresing burden of these infections, their severe complications that can compromise the general and reproductive health of infected individuals and frequent treatment failures due to increasing antimicrobial resistance require immediate attention. This review focuses on the main aspects and up-to-date recommenations of laboratory-based diagnosis of gonococcal and genitourinary chlamydial infections, on the definitions of a presumptive and definitive diagnosis, on collection and transport of clinical samples, current diagnostic methods (microscopy, culture, nucleic acid amplification tests [NAATs], etc.), antimicrobial resistance testing and molecular genotyping.

BACKGROUND (EPIDEMIOLOGY AND CLINICAL SIGNIFICANCE)

Sexually transmitted infections (STIs) are among the most common causes of illness in the world with severe consequences if not treated properly. There are more than 30 different sexually transmissible bacteria, viruses and parasites. The World Health Organization (WHO) estimated that there were 105.7 million new cases of *Chlamydia trachomatis* and 106.1 million gonococcal infections worldwide in 2008 [1], which make gonorrhoea and chlamydiasis the most common STIs. *Neisseria gonorrhoeae*, a Gram-negative diplococcus [2], is the etiological agent responsible for gonorrhoea. *C.trachomatis* are obligate intracellular bacteria multiplying in eukaryotic hosts and cause ocular infection (trachoma) and genitourinary tract infections [3]. Any sexually active person can be infected with gonorrhea or chlamydia through unprotected anal, vaginal, or oral sex, yet there are distinct risk groups such as commercial sex workers, men-who-have-sex-with-men (MSM) and young people with risk behavior. A relative high co-infection rate with both chlamydia and gonorrhoea is gaining importance in relation to proper treatment and avoiding treatment failures [4]. Alarming is the fact that recent reports on gonococcal treatment failures are becoming more frequent [5-7]. Failures in treatment of chlamydial infection also represent an interest for study [8]. If these infections become untreatable then complications, including pelvic inflammatory disease, ectopic pregnancy, infertility, neonatal eye infections, and consequences such as facilitation of HIV co-transmission, pose a threat to become common.

In Bulgaria, gonorrhoea and chlamydial genital infections are mandatory for reporting [9], however, the reported incidences are likely to be underestimated. This is due to suboptimal diagnostics (lack of appropriate methods or access to testing), syndromic management of the patients and incomplete case reporting. In the last 20 years there have been published only a few studies on the prevalence of *N.gonorrhoeae* and *C.trachomatis* infections [10-13].

Laboratory Identification of *N.gonorrhoeae* and *C.trachomatis*: Definitions

The diagnosis of gonorrhea and chlamydial genitourinary tract infection can be classified into two levels: presumptive and definitive [9]. A **presumptive** diagnosis of gonorrhea is defined by (1) the presence of clinical symptoms and (2) sexual exposure to a person infected with *N.gonorrhoeae* and/or *C.tra-*

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chomatis. Clincal symptoms of gonorrhea are one or more of the following: mucopurulent endocervical or urethral exudate on physical examination, epididymitis, proctitis, acute salpingitis, pelvic inflammatory disease, pharyngitis, arthritis. The clinical manifestations in chlamydial infection include urethritis, epididimitis, acute salpingitis, acute endometritis, endocervicitis and proctitis. Laboratory test results are generally unavailable when only a presumptive diagnosis is made. A **presumptive** diagnosis of gonorrhea should be considered in any newborn child with conjunctivitis and a vertically transmitted infection from the mother should be suspected.

A **definitive** diagnosis of gonorrhea requires (1) isolation of *N.gonorrhoeae* from sites of exposure (e.g., urethra, endocervix, throat, or rectum) by culture; (2) detection of *N.gonorrhoeae* in a non-culture laboratory test (e.g., nucleic acid amplification tests or non-amplified DNA/RNA-hybridization probe); and (3) typical gram-negative intracellular diplococci on a gram stain (for men only).

A **definitive** diagnosis of chlamydial genitourinary tract infection requires (1) isolation of *C.trachomatis* from a specimen from genotourinary tract, the anal area or from conjunctiva; (2) detection of *C.trachomatis* by direct fluorescent antibody assay (DFA) in clinical specimen or (3) detection of nucleic acid by nucleic-acid amplification methods (NAATs) from *C.trachomatis* in clinical specimen.

Guidelines for the Collection and Transport of clinical specimens

The specimen collection should fulfill the following basic requirements (1) Collecting the *most suitable material*, depending on the clinical manifestations and diagnostic method used; (2) Using sterile swabs, placing the specimens in a special transport medium and timely transportation to the microbiology laboratory at the appropriate temperature regime; (3) Specimens for microscopic examination are taken before all other samples [14]. The most informative material is taken prior to systemic and/or local antimicrobial treatment, at least two hours after the last urination and sexual intercourse and after menstruation in women [14]. Specimens collected for gonorrhea and *C.trachomatis* culture should be obtained by using swabs with plastic or wire shafts and Dacron or rayon tips. Other swab material such as wood shafts and cotton/calcium alginate tips might be inhibitory or toxic [15, 16] and should be avoided.

COLLECTION OF URETHRAL SPECIMENS FROM MALES

In the case of a typical gonococcal infection the abundant discharge could be easily collected. When the discharge is scant, collecting an early-morning specimen before urination may be helpful. Exudate may be expressed from the urethral orifice by gently „milking“ the penis or the tip of a narrow-diameter swab may be inserted 3 to 4 cm into the anterior urethra and left in place for a few seconds to allow the fibers to become saturated with the exudate. Specimen collection for *C.trachomatis* culture requires rotations of the swab to collect sufficient columnar or cuboidal epithelial cells [17]. If NAATs are to be used, urine is a satisfactory specimen simpler to obtain and more accetable by the patient than an urethral swab is. In contrast to the midstream urine appropriate for the diagnosis of bacterial cystitis, in this case, the first portion containing urethral epithelial cells and bacteria should be collected [17].

COLLECTION OF CERVICAL SPECIMENS FROM FEMALES

In females, the most appropriate specimens are obtained from the uterine cervix. Cervical specimens are collected with the aid of a speculum after clearing off the cervical mucus with a large swab. A smaller swab with plastic shaft and a Dacron or polyester tip is recommended for obtaining the specimen. The tip of the swab is inserted a few millimeters past the cervical os, rotated firmly to obtain both exudate and cervical cells, and carefully removed without touching the lateral walls of the vaginal canal [17]. Urine and vaginal swab specimen are excellent for diagnosis of chlamydial or gonococcal infection by NAATs [18, 19].

TRANSPORT

The collected specimens must be transported to the microbiology laboratory as quickly

as possible in order to maximize recovery of viable organisms. For successful culture of chlamydiae the specimens should be forwarded to the laboratory in a special chlamydial transport medium, such as 2-sucrose phosphate, while keeping them cold (4-8^{O}C) [20, 21]. Specimen for *N.gonorrhoeae* culturing can be transported by using Amies-based semi-solid media which should be kept at room temperature and not be refrigerated during transportation [14, 22].
NAATs testing does not require the organism to be viable and specimens are easier to transport and store [23]. Therefore, the transportation can be carried out at ambient temperature and there is no need for a special transport medium.

DESCRIPTION OF CURRENT DIAGNOSTIC METHODS FOR *C.TRACHOMATIS*

Serologic tests

Serological testing is not recommended for diagnosis of uncomplicated genitourinary chlamydial infections as well as for *C.trachomatis* screening because a present and/or previous chlamydial infection might or might not elicit a systemic antibody response [20, 24].

Isolation and identification of *C.trachomatis*

For isolation of chlamydiae from clinical specimens, properly collected and transported samples are inoculated onto preformed cell monolayers of McCoy, HeLa 229, or similar one that supports growth of *C.trachomatis*. Cultures are incubated for 48 to 72 h in the presence of host cell protein synthesis inhibitor cycloheximide [20]. Staining of inoculated cell monolayers with either genus-specific [25] or species-specific fluorescein-conjugated monoclonal antibodies allows specific visualization of the chlamydial inclusions with a fluorescence microscope. The less specific inclusion-detection methods using iodine or Giemsa stains are not recommended [24].

Nucleic Acid Amplification Tests (NAATs)

Problems associated with cell culture isolation of chlamydiae have driven the development of non-culture based methods. Of the nonculture tests available, only NAATs are recommended for routine use whereas other tests (e.g., enzyme immunoassays, nucleic acid probe tests, and genetic transformation tests) are not recommended [24]. The performance of NAATs with respect to overall sensitivity, specificity, and ease of specimen transport, is better than that of any other tests available for the diagnosis of chlamydial and gonococcal infections [19, 26]. The main targets for amplification are nucleotide sequences of *ompA* gene and of the 7.5 kbp cryptic plasmid of *C.trachomatis* [27]. The cryptic plasmid is present in an average copy number of about four plasmids per chromosome in Ebs and up to seven plasmids per chromosome in replicating RBs [28]. DNA targets for NAATs should be chosen cautiously as *ompA* is one of the most polymorphic single-copy genes known in bacteria [29]Amino Acid</keyword><keyword>Time Factors</keyword></keywords><dates><year>1993</year><pub-dates><date>Jul</date></pub-dates></dates><isbn>0737-4038 (Print and furthermore, *C.trachomatis* strains, that do not harbour the cryptic plasmid or have a 377-bp deletion in the cryptic plasmid target sequence, have also been isolated (new variant *C.trachomatis*) [30].

DESCRIPTION OF CURRENT DIAGNOSTIC METHODS FOR *N. GONORRHOEAE*

Microscopic examination

Gram stain of a male urethral specimen that demonstrates polymorphonuclear leukocytes with intracellular Gram-negative diplococci can be considered diagnostic for infection with *N.gonorrhoeae* in symptomatic men. In some settings, a method with methylene blue stain showing good correlation with the Gram stain, is also used [31]. However, because of the lower sensitivity, Gram stains of endocervical, pharyngeal, or rectal specimens are regarded as suboptimal and therefore are not recommended [32]. In addition, a negative Gram stain cannot rule out the infection [24].

Culture

Despite the introduction of new tests, identification of *N.gonorrhoeae* by culture remains the „gold standard“ owing to its exellent sensitivity and specificity and also because it

allows further characterization of the isolate with antimicrobial susceptibility testing and genetic analysis. However, factors limiting the sensitivity and specificity are improper transportation and collection of specimens from some anatomical sites such as the rectum and pharynx.

Culture media for *N.gonorrhoeae* isolation include a base medium supplemented with chocolatized (heated) equine or bovine blood to support the growth of the gonococcus. Commercially prepared chocolate agar containing synthetic hemin and growth factors for *N.gonorrhoeae* are available from various vendors. The use of selective media is highly recommended in order to avoid growth of other commensal bacteria. The prototype selective medium, developed by Thayer and Martin [33] has now been modified with additional antimicrobials and improves the control against commensal bacteria while allowing the recovery of rare vancomycin-sensitive *N.gonorrhoeae* isolates.

The inoculated plates should be incubated at 35 - 36.5^{O}C with 5% CO_2 in a moist atmosphere. Cultures should be examined daily for growth and held for a minimum of 72 hours. Supplemental CO_2 can be supplied in a CO_2 incubator or in a candle extinction jars [24]. After 48 hours of incubation colonies of *N.gonorrhoeae* are up to 1 mm in diameter, opaque, graysh white, glistening, convex and may vary in appearance due to different colony forms, designated T1, T2, T3, and T4.

Identification

Isolates recovered from a genital specimen on a selective medium that are Gram-negative diplococci- and oxidase-positive might be presumptively identified as *N.gonorrhoeae* [22]. A confirmed laboratory diagnosis of *N.gonorrhoeae* requires additional biochemical, enzymatic, serological, or nucleic acid tests. The most widely used are the multitest systems which combine carbohydrate utilization with enzyme substrate tests and the automated bacterial identification platforms with more than 30 biochemical tests.

Nucleic Acid Amplification Tests: PCR.

NAATs for *N.gonorrhoeae* diagnosis became available in the early 1990s [22] and are now the most common method used for gonorrhoea diagnosis in many countries [34]. Various NAATs are available for gonococcal DNA detection, including polymerase chain reaction (PCR), transcription mediated amplification (TMA) and strand displacement amplification (SDA) [35]. All these share the advantages of molecular diagnostics: (1) superior sensitivity and specificity; (2) NAATs can be automated and multiplexed detecting both *C. trachomatis* and *N.gonorrhoeae*; (3) specimens are easier to transport and store because there is no requirement for the organism to be viable for detection and (4) analysis can be done on non-invasive and/or selfcollected specimens like urine and vaginal swabs. The most frequently used molecular targets for amplification include nucleotid sequences from the *cppB,*16S rRNA, *pivNG, gyrA* and *opa* genes*, porA* pseudogene, etc*.* An enhanced awareness of the emergence of gonococcal mutants is essential when choosing the targets in molecular diagnostics to avoid false-negative results such as the case with *N.gonorrhoeae porA* mutant strains [36].

ANTIMICROBIAL RESISTANCE IN *C.TRACHOMATIS* AND *N.GONORRHOEAE*

Antimicrobial susceptibility testing of *C.trachomatis* hasn't been standardized, in-vitro resistance does not correlate with any clinical outcome [37] and is currently performed only in some research laboratories. Resistance to recommended antimicrobial agents such as tetracyclines, macrolides, fluoroquinolones, and rifampin apears to be rare. Nevertheless, there are studies on recurrent or persistent chlamydial infections in adequately treated individuals [8].

The emergence of antimicrobial resistance in *N.gonorhoeae* is an issue of a global concern. Resistance has been developed against a wide range of antimicrobials e.g. penicillins, tetracyclines, fluoroquinolones and in recent years broad-spectrum cephalosporins and azithromycin. Therefore gonorrhea may become a threat as an intractable and potentially incurable infection [23].

TYPING SYSTEMS

A number of methods have been developed and applied for genotyping of *C.trachomatis*

and *N.gonorrhoeae* isolates for epidemiological purposes, when medicolegal issues are involved, investigating the transmission patterns and surveillance.

Genotyping of *C.trachomatis* isolates usually involves either restriction fragment length polymorphism analysis (RFLP) of the MOMP-encoding *ompA* gene or sequence analysis of the four variable domains (VDs) in *ompA*. These variable regions encode peptides responsible for species, serovar, and serogroup specificities. Direct genotyping from *C.trachomatis*-positive specimens without isolation of the organisms is accomplished by PCR amplification and sequencing of *ompA* using extracted DNA from clinical specimens [20]. In addition, new high-resolution genotyping methods aplying a multilocus variable tandem repeat assay (MLVA) or multilocus sequence typing (MLST) have been introduced [38].

Precise methods for typing *N.gonorrhoeae*, together with epidemiological information, are crucial for an enhanced understanding of the issues involving epidemiology, test of cure and contact tracing, identifying core groups and risk behaviors, recommending effective antimicrobial treatment, control, and preventive measures. Genotypic methods based on DNA sequencing are preferred. Currently, for microepidemiological questions, the best methods for fast, objective, portable, highly discriminatory, reproducible, typeable, and high-throughput characterization are *N.gonorrhoeae* multiantigen sequence typing (NG-MAST) or full- or extended-length porB gene sequencing. However, pulsed-field gel electrophoresis (PFGE) and Opa typing, despite their limitations, can be valuable in specific situations, i.e., extreme microepidemiology. For macroepidemiological studies and phylogenetic studies, DNA sequencing of chromosomal housekeeping genes, such as multilocus sequence typing (MLST), provides a more nuanced understanding [39].

CONCLUSION

The performance of NAATs with respect to overall sensitivity, specificity, and ease of specimen transport is highly recommended for the diagnosis of chlamydial and gonococcal infections and these methods are increasingly represented in Bulgarian microbiological laboratories. However, maintaining the capability to culture for both *N.gonorrhoeae* and *C.trachomatis* in some laboratories is essential, when antimicrobial susceptibility testing is carried out or medico-legal issues are involved. As example, in the National reference laboratory Mycology And Sexually Transmitted Infections (STIs) along with NAATs is performed culturing for *N. gonorrhoeae* and antimicrobial susceptibility testing. Networking of laboratories and using up-to-date recommended methods for the diagnostics will benfit not only the managemnet of individual cases but also will improve underreporting and epidemiological surveillance in Bulgaria. The maintenance of the ability to culture for *N.gonorrhoeae* will implement the gonococcal antimicrobial resistance surveillance, and that could contribute to the treatment guidelines of gonorrhea and will ensure the appropriate therapy. Monitoring molecular types linked to antimicrobial resistance and epidemiological data can elucidate many public health issues like identification of base-line of gonococcal genotypes, genetic resistance determinants, emergence and dissemination of resistant gonococcal strains.

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VIROLOGICAL SURVEILLANCE OF ENTEROVIRAL INFECTIONS AMONG REFUGEES RESIDING WITHIN THE TERRITORY OF BULGARIA, 2013

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ABSTRACT

Owing to the raging conflict in Syria in 2013, the outbreak of polio and refugee wave towards Bulgaria posed a risk for importation of wild poliovirus in our country. Fecal samples were collected from refugee children up to 5 years of age, originating from Syria and other Arab countries and residing on the territory of Bulgaria, as urgent measures required for strengthening the monitoring of polioviruses.

Total of 155 fecal samples collected from eight refugee centers were examined at the National Reference Laboratory of Enteroviruses. The assay was performed by utilization of both classical methods and modern molecular techniques (RT-PCR).

Out of 155 samples tested, 43 (27,7%) were enterovirus positive by RT-PCR. Seventeen strains (11%) were isolated in cell cultures, including eleven ECHO 3 (7,1 %), one ECHO 29 (0,7%) and two ECHO 6 viruses (1,3%). Thirteen specimens showed positive result by both methods.

Three polioviruses were isolated for the first time since the introduction of inactivated polio vaccine in the country: two polioviruses type 3 (1,3%) and one poliovirus type 1 (0,7%). Isolated poliovirus strains were sent to the WHO Regional Reference Laboratory in Rome, Italy for intratypic differentiation. PCR and sequence analysis results showed that the isolates are Sabin-like polioviruses.

The largest number of enteroviruses were isolated from children in the age group 2-3 year-olds (41,2%), followed by the groups of 0-1 and 4-5 year-olds with an equal number of isolates (29,4%).

The results indicate the need of continued surveillance of enteroviruses in order to maintain the polio-free status of the country.

Keywords: enterovirus, echovirus, poliovirus, refugees

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INTRODUCTION

In 1988 the World Health Assembly passed a resolution to eradicate poliomyelitis by the year 2000. The Global Polio Eradication Initiative was created with the purpose of combating this serious illness. National and international efforts to eradicate polio led to a significant reduction of wild poliovirus circulation in the world. The estimated number of polio cases has decreased from 350 000 in 1988 to 416 in 2013 and the number of endemic countries has decreased from 125 to 3 (Afghanistan, Nigeria and Pakistan). Wild poliovirus type 2 has not been detected anywhere in the world since October 1999, and poliovirus type 3 – since November 2012 [1]. However, polioviruses continue to circulate in countries where immunization coverage is low and where many people live in close proximity and in poor hygienic conditions (especially during the wars and disasters).

The last reported cases of wild poliovirus type 1 infection in Bulgaria occurred in 2001, following a 9-year-period of zero-reported cases, as a result of importation from the Indian subcontinent [2]. In June 2002, the WHO European Region was certified as polio-free. Currently Bulgaria continues to maintain this status. Owing to the raging conflict in Syria in 2013, the outbreak of polio and refugee wave towards Bulgaria posed a risk for importation of wild poliovirus in the country. Fecal samples were collected from refugee children up to 5 years of age, orig-

inating from Syria and other Arab countries and residing on the territory of Bulgaria, as urgent measures required for strengthening the monitoring of polioviruses.

MATERIAL AND METHODS

Total of 155 fecal samples of children <5 years old collected from eight refugee centers were examined at the National Reference Laboratory of Enteroviruses. The samples are as follows: 43 numbers of RHI Haskovo - 20 of village Pastrogor and 23 from city Harmanli; 66 numbers of SRZI - 20 of Vrajdebna, 21 of Voenna rampa and 25 of Ovcha kupel; 20 numbers of Kovachevtsi (RHI Pernik); 20 numbers of Banya (RHI Sliven) and 6 of Elhovo (RHI Yambol).

All samples were tested by classical virological method - isolation of enteroviruses on WHO recommended cell lines - RD (human rhabdomyosarcoma) and L20B (gene-modified murine fibroblasts - selective cell line for polioviruses). The received samples were subjected to pre-processing in a 50 ml centrifuge tube at 5 ml PBS, with dissolved therein an antibiotic (penicillin) was added 2-3 glass beads (for mechanical reduction of the sample), 0,5 ml chloroform and 100-150 g fecal sample. Mix well and centrifuge at 2700 rpm for 20 minutes at 4 °C. After centrifugation, the supernatant was sucked off, which was used to inoculate into cell cultures (i.e. this is so called primary material). Virus isolation was used fresh cell monolayer, which is obtained after about 24 hours after pouring of cell suspension in tube (glass or polypropylene). In monolayer obtained was poured to 10% growth medium and replaced with 1 ml of 2% maintenance culture medium in which was inoculated 200 µL of primary material (Figure 1).

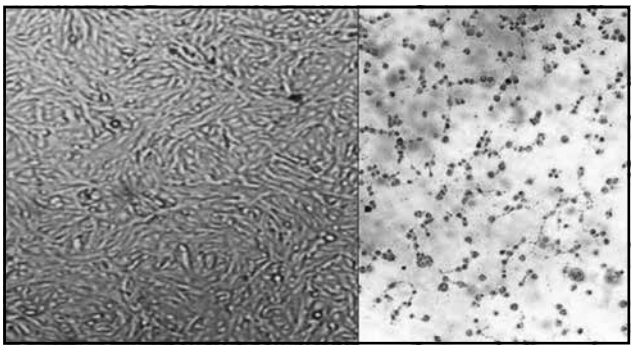

Fig. 1. Monolayer of RD cell line (in left) and specific virus-induced CPE (in right)

Identification of isolated strains was conducted by microneutralization test with pools of horse antisera, including antibodies to the most common enteroviruses (RIVM, Netherlands) [3]. Each box of RIVM enterovirus typing antisera contains enterovirus pools A, B, C, D, E, F, G, H; a Coxsackie B (1-6) virus pool and a poliovirus (1-3) pool. Briefly, equal volumes (50 µL) of each serum pool and virus were mixing in a microplate and then the mixtures were incubated at 36^{0}C allowing binding of the antibodies and virus. Subsequently, 100 µL suspensions of cells were added to the microtitre plate which were examined daily for the presence of CPE. The antiserum that prevents the development of CPE indicates the identity of the virus (Figure 2)

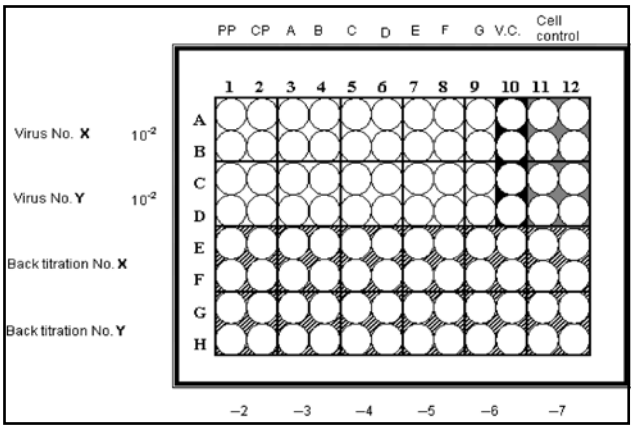

Fig. 2. The view of the micro-neutralization technique (microtitre plate) for identification

Molecular detection of enteroviruses were carried out by Real Time RT-PCR method with the use of kit *AccuPower® Dual-Hot-Start™ RT-qPCR PreMix*, group-specific primers targeting 5'UTR region of the enteroviral genome (EV-F and EV-R2) and probe marked with FAM and BHQ1. The RT-PCR was performed with a *Exicycler™96* (Bioneer, Korea) thermal cycler using the following conditions: reverse transcription at 50^{0}C for 15 min, RT inactivation and Taq polymerase activation at 95^{0}C for 10 min, followed by the 40 cycles of denaturation at 95^{0}C for 30 s and annealing at 60^{0}C for 1 min. A Ct value <38 was regarded as positive. Positive and negative controls were included in each run.

RESULTS AND DISCUSSION

Out of 155 samples tested, 43 (27,7%) were enterovirus positive by RT-PCR. Overall, 17 (11%) enteroviral strains were isolated on cell culture RD, including eleven ECHO 3 (7,1%), one ECHO 29 (0,7%) and two ECHO 6 viruses 1,3%). Thirteen specimens showed positive result by both methods. Isolation of enteroviruses from fecal samples of healthy children confirms the possibility that enteroviruses may replicate in the gut in the absence of clinically apparent disease [4].

Three polioviruses were isolated on cell cultures RD and L20B for the first time since the introduction of inactivated polio vaccine in the country: two (1,3%) polioviruses type 3 in children aged 1 m and 3 yrs 11 m and one (0,7%) poliovirus type 1 in child aged 3 yrs 9 m (Figure 3). Children who were poliovirus positive were probably vaccinated with the oral polio vaccine (OPV) or were in contact with the recipients of OPV.

Isolated poliovirus strains were sent to the Regional Reference Laboratory in Istituto Superiore di Sanità (ISS), Rome, Italy for intratypic differentiation. Real-time RT-PCR and sequence analysis results showed that the isolates are Sabin-like (vaccine) polioviruses. One mutation in both type 3 polioviruses (in different positions) and two mutations in type 1 poliovirus in genome region VP1 were identified. As is known vaccine polioviruses as other RNA viruses are highly variable. During the process of their replication in the gut, they acquire mutations, especially in the genomic region VP1, which is the most variable. Polioviruses containing less than 1% variation in this region compared to the corresponding vaccine virus are considered as Sabin-like.

The largest number of enteroviruses were isolated from children in the age group of 2-3 year old (41,2%), followed by the groups of 0-1 and 4-5 year with an equal number of isolates (29,4%) (Table 1).

Age groups (years)	% positive samples
0-1	29,4 (5/17)
2-3	41,2 (7/17)
4-5	29,4 (5/17)

Table 1. Age distribution of enterovirus positive children

CONCLUSION

The present study demonstrated a variety of identified enteroviruses. The sequencing of VP1 genome regions of isolated polioviruses showed a very high homology with reference Sabin strains. Sabin-like polioviruses were isolated for the first time since the introduction of IPV in Bulgaria. Our results confirm the need of a systematic surveillance of enteroviruses, as well as the need to maintain high vaccination coverage in order to protect people from eventual import of polioviruses [5, 6, 7].

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PARASITIC DISEASES IN BULGARIA IN 2012

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ABSTRACT

Background. Parasitic diseases are still widespread in Bulgaria and remain as factors adversely affecting human health. The aim of the performed analysis was to determine the status and trends of various parasitic diseases in the country and evaluate the surveillance and control activities conducted in 2012.

Materials. Official surveillance information data was used as well as the the annual reports of parasitological structures in the Regional Health Inspectorates (RHI), which summarize all parasitological situation and activities regarding parasitic diseases in the relevant region.

Results. The results showed that in 2012 a total of 738 155 persons were tested for parasitic diseases and positives accounted for 1.5% (1.63% in 2011). Of the indigenous parasitic diseases helminthozoonoses retain their medical and social importance.The tendency to renewed increase increase in human echinococcosis incidence since 2010, was observed in 2012 as well. In Bulgaria the soil-transmitted parasitoses ascariasis and trichuriasis are annually registered. The prevalence of ascariasis showed no changes in 2012 and there is considerable decrease of trichuriasis prevalence in comparison with the previous years.. The distribution of communicable diseases (enterobiasis, giardiasis, hymenolepiasis) remained at the same level as in 2011. Regarding the opportunistic parasitoses, a significant number of tests were carried out mainly for toxoplasmosis and blastocystosis. The registration of autochthonous cases of visceral leishmaniasis is probably due to the presence of a local source of the infection. Among imported from endemic countries parasitic diseases malaria is a leading disease.

Surveillance and control of parasitic diseases are complex and involve case detection and etiological treatment as well as conduction of prophylactic and anti-epidemic measures against the main groups of indigenous and imported parasitic diseases.

Conclusions

Parasitic diseases continue to be an important health problem in Bulgaria. Some of parasitozoonoses are widely distributed in the country; high incidence rates of human echinococcosis are maintained over years and trichinellosis outbreaks are annually registered.

Imported from endemic countries parasitic diseases, and especially malaria, pose a risk of clinical and epidemiological consequences unless strong epidemiological surveillance is in place.

To limit the spread of indigenous parasitic diseases and prevention of the local transmission of imported parasitoses a high level of vigilance and epidemiological surveillance are required

Key words: *parasitoses, annual analysis, zoonoses, soil-transmitted parasitoses, communicable parasitoses, opportunistic parasitoses*

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INTRODUCTION

Bulgaria is a country with a number of endemic parasitic diseases and some of them pose important medical and health issues. The migration of Bulgarian and foreign citizens to and from tropical

countries has increased in recent years, which entails a higher risk of importation of parasitic diseases that have no local distribution. This raises a number of issues facing public health in order to prevent local spread of a number of imported parasitic diseases for which there are favorable climatic and faunistical factors. In Bulgaria more than 20 indigenous parasitic diseases are registered and 11 of them are subject to obligatory notification and registration. The incidence of echinococcosis and trichinellosis remain higher than in the other European countries.

The surveillance of parasitic diseases in the country is performed by the Regional Health Inspectorates (RHI) and the National Centre of Infectious and Parasitic Diseases (NCIPD) under the leadership of the Ministry of Health (MoH). **The aim of the study was to evaluate the status of various parasitoses in the country and the surveillance and control activities conducted in 2012.**

MATERIALS

For the analysis were used the annual accounts of the RHI of registered cases of parasitic diseases - local and imported. Under the existing regulations for each reported case subject to mandatory registration a standard form is completed with epidemiological and clinical data. Based on annual reports from individual RHI in the country collaborators from the Department of Parasitology and Tropical Medicine at NCIPD prepared a general report for the status of parasitic diseases with some **epidemiological parameters like prevalence and incidence.**

RESULTS

The report represents the status of parasitic diseases in Bulgaria in 2012. A total number of 738 155 (782 336 in 2011) patients were examined for parasitoses and 11 026 (1.5%) (1.63% in 2011) of them showed positive results (Figure 1).

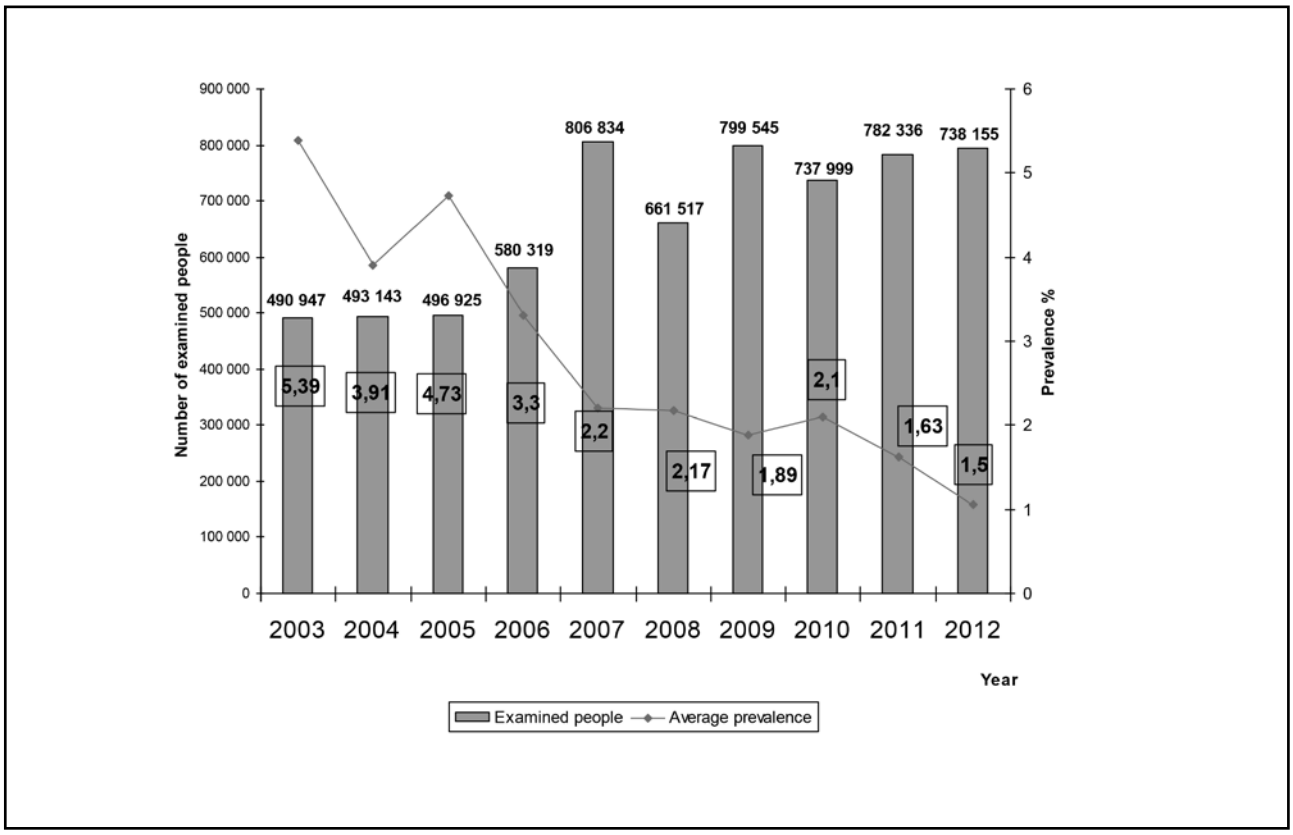

Fig.1. People examined for parasitic diseases and average prevalence (2003-2012)

INDIGENOUS PARASITIC DISEASES

Zoonotic parasitic diseases continue to have major medical and social impact because of their high incidence, which exceeds significantly the parameters of the other Member States in the European Union.

Echinococcosis. The number of the officially registered cases was 346, 320 of them were newly diagnosed (annual incidence 4.37 per 100 000 population). The fact that 16.18% (56) of the cases are children and adolescents (0 - 19 years) is very disturbing (Figure 2, 3, 4). This fact indicates the presence of very active transmission of the parasite from main reservoir hosts to humans. The highest annual incidence is registered in the following regions: Sliven – 15.76 per 100 000 population; Yambol – 11.53 per 100 000 population; Razgrad – 8.09 per 100 000 population, Targovishte – 7.51 per 100 000 population and Dobritch - 7.44 per 100 000 population.

Trichinellosis. Meat products, infected with larvae of *Trichinella* were consumed by 79 people and 30 (43%) developed clinical symptoms of the disease (Figure 5). On the territory of Bulgaria four outbreaks were registered in 2012 – in the regions of Blagoevgrad, Razgrad, Plovdiv and Sofia with thirteen, twelve, three and two diseased persons respectively. One outbreak was caused by consumption of meet of a domestic pig and three - by consumption of meet of wild boars. Isolated sporadic cases were not registered.

Taeniasis. Throughout the year 28 cases of taeniarrhynchosis (*Taenia saginata*) were reported and the incidence of the disease was 0.38 per 100 000 population. This rate was above the average in the following regions: Shumen – 3.33 per 100 000 population; Pazardjik – 2.19 per 100 000 population and Targovishte – 1.02 per 100 000 population.

Soil-transmitted helminthoses.

The prevalence of **ascariasis** and **trichuriasis** was 0.1% and 0.01%, respectively

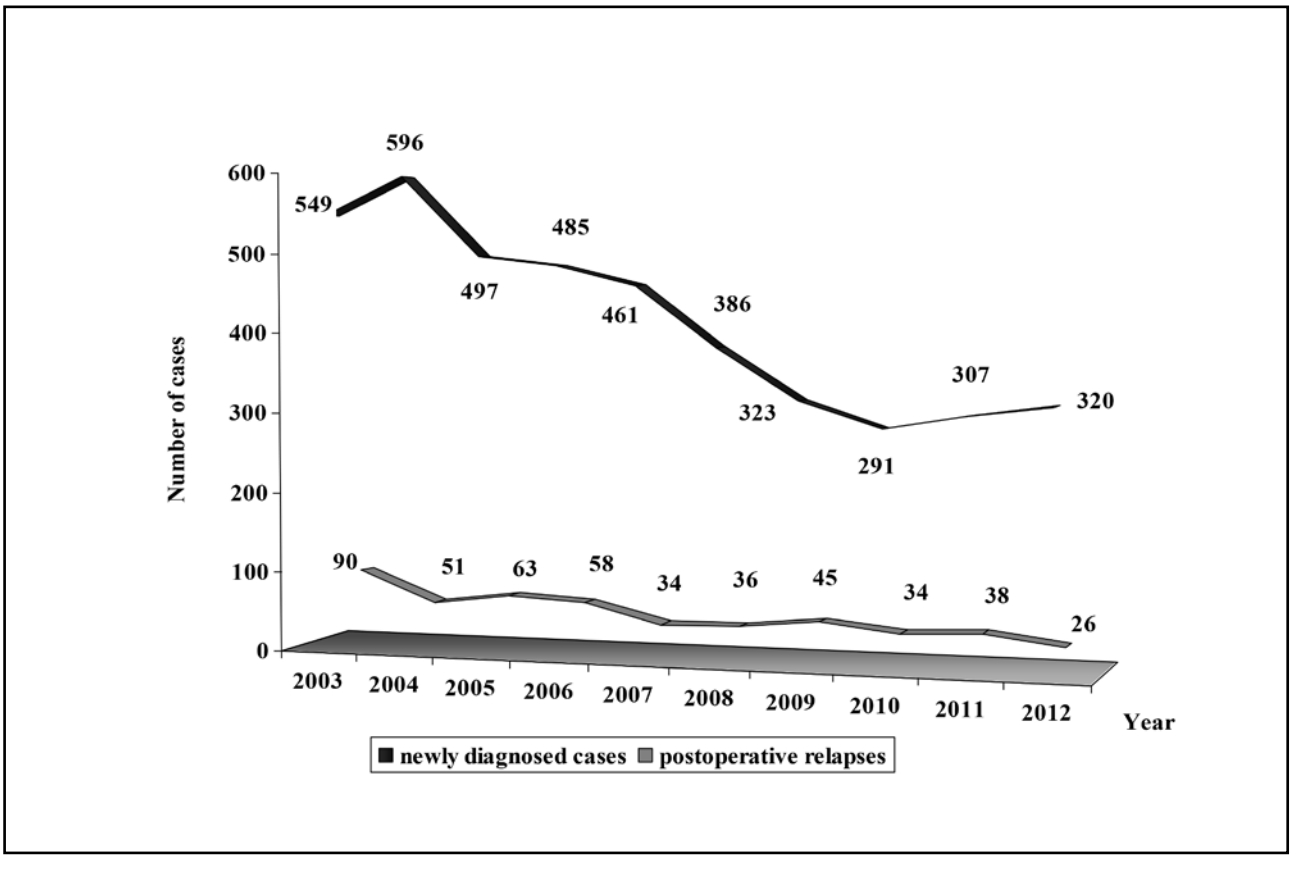

Fig. 2. Newly diagnosed cases of cystic echiococcosis and relapses (2003-2012)

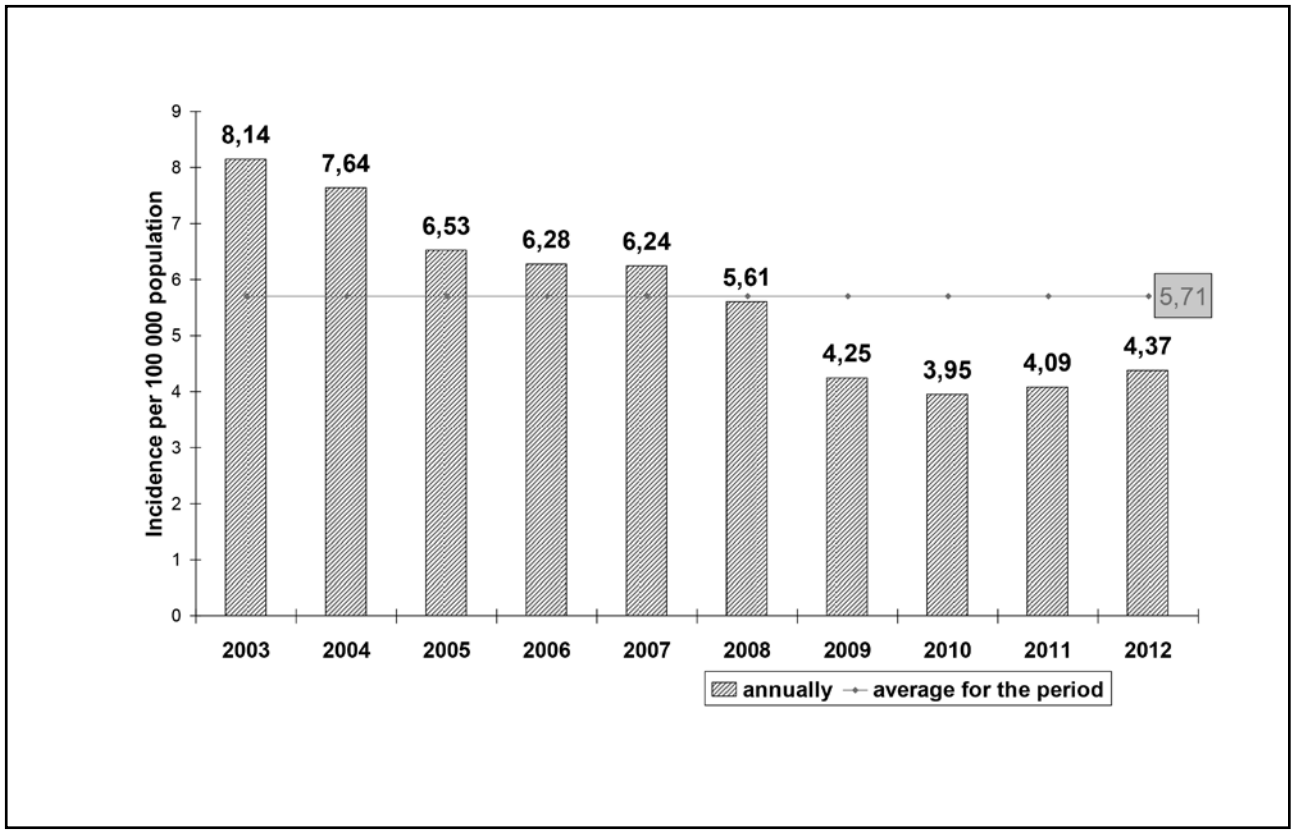

Fig. 3. Incidence of cystic echinococcosis in Bulgaria (2003-2012)

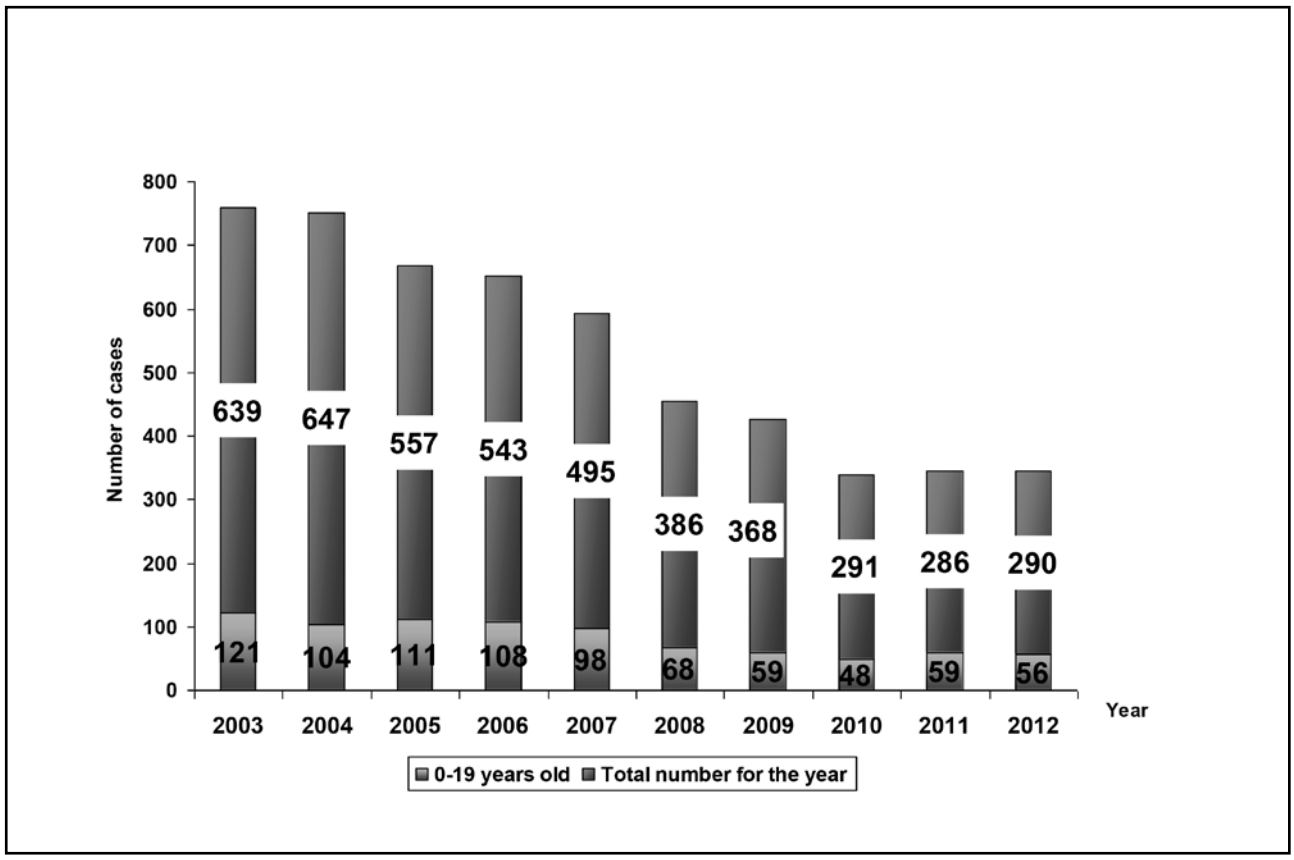

Fig. 4. Cases of cystic echinococcosis among adults and children (0-19 years old) for the period 2003-2012

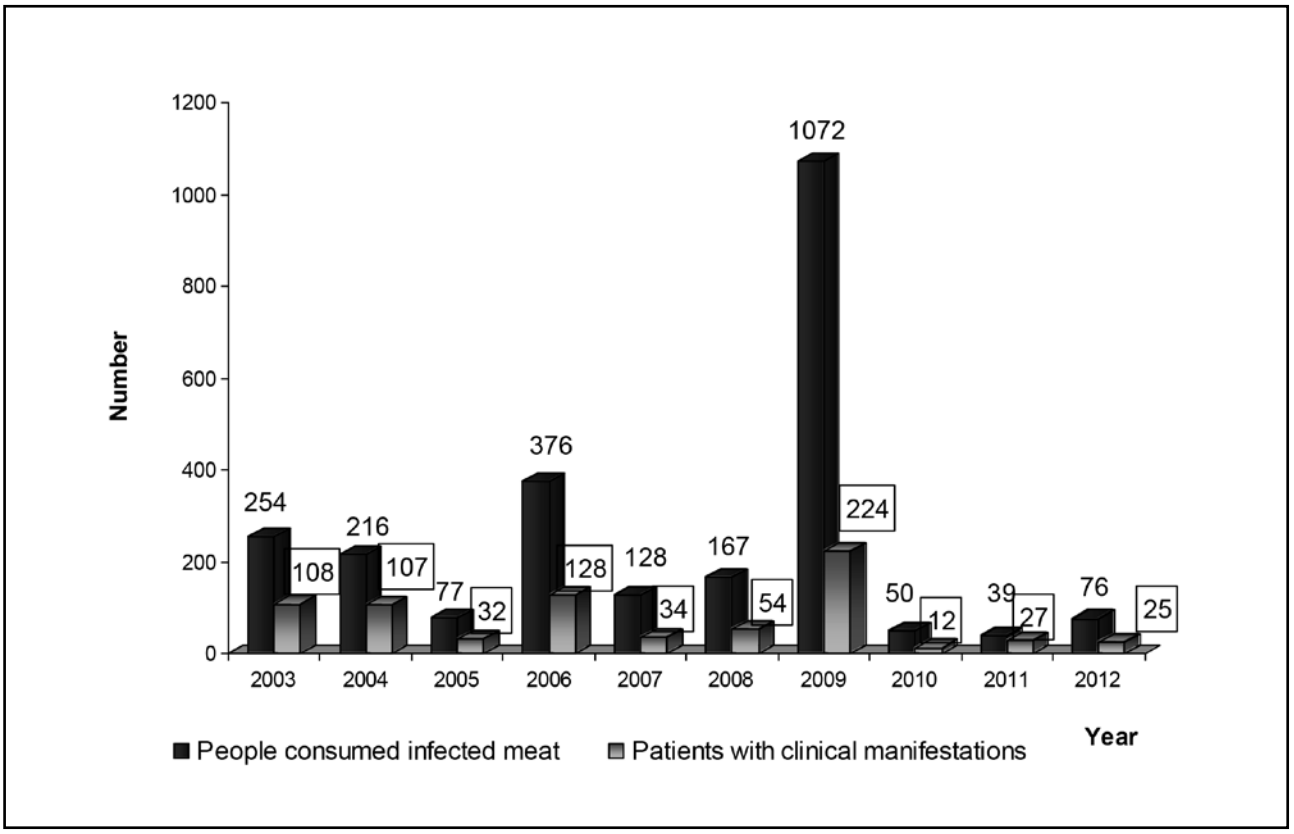

Fig.5. Cases of trichinellosis (2003–2012)

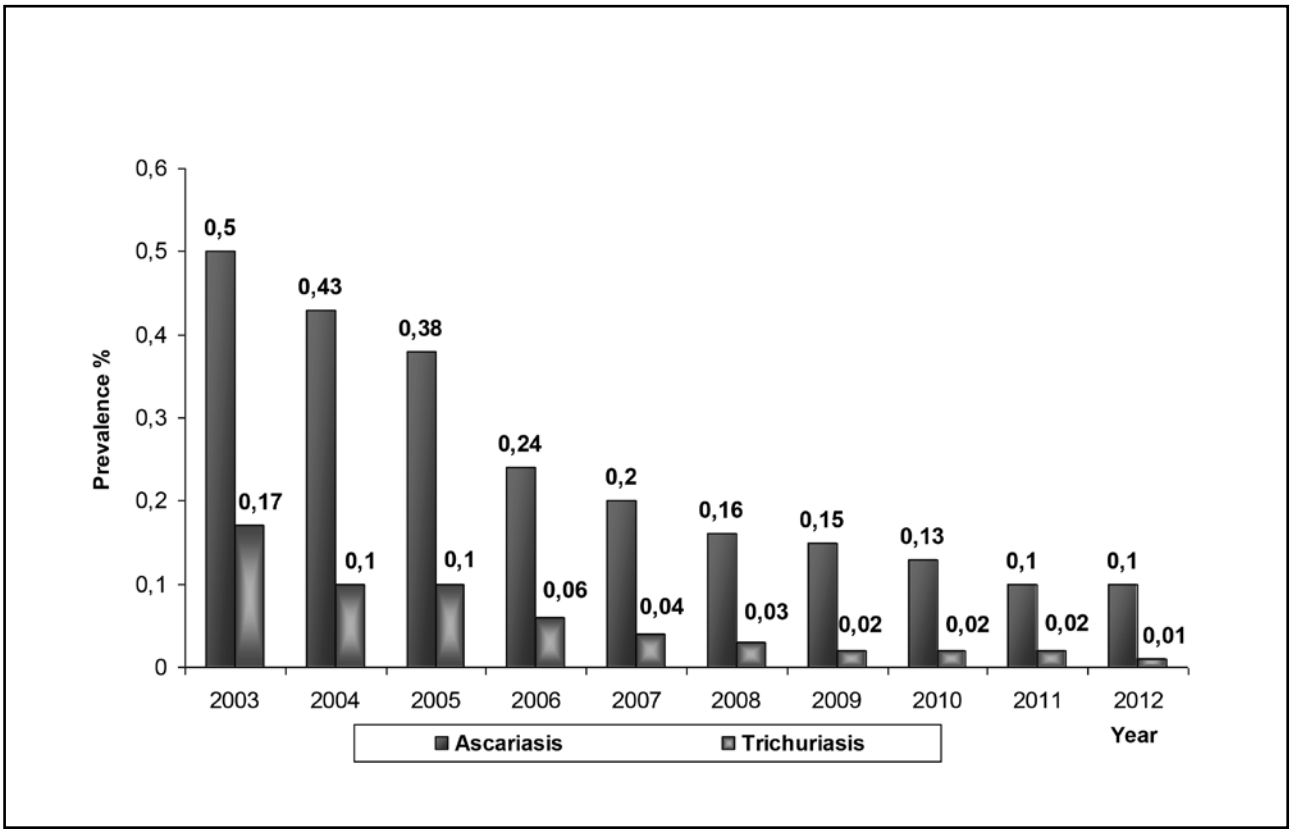

Fig. 6. Prevalence of soil-transmitted parasitoses (2003-2012)

(Figure 6). Regions with the highest prevalence of ascariasis were: Kardjali (4.43%), Blagoevgrad (1.27%), Sofia (1.26%) and Veliko Tarnovo (0.21%). 760 852 villages and towns endemic for ascariasis were monitored during the year. Control measures were conducted in 88 (28.94%) among them, which is a good indication of the work and continuous activities of parasitologists at RHI in these endemic regions.

Community acquired parasitic diseases.

Enterobiasis. There were 568 502 patients examined for the disease with 3 366 registered positive cases (prevalence 0.6%,) (0.7% in 2011) (Figure 7). Tests for enterobiasis were conducted on 182 559 children (95% from different childcare facilities) and the registered prevalence was 0.9%.

Giardiasis – the prevalence was 0.44% among 457 273 examined patients. Prevalence rates above the country average were registered in the regions of Yambol and Sliven.

Hymenolepiasis – 0.04% prevalence among 333 654 examined patients. Most of the cases were diagnosed in the regions of Yambol (46), Sliven (34) and Varna (22).

Urogenital trichomoniasis. The total number of examined people in RHI, NCIPD and some private laboratories was 1 457 with 252 positive cases (17.3%).

Opportunistic parasitic diseases.

Toxoplasmosis. During the year 11 826 patients were examined for toxoplasmosis and positive results were obtained from 2 363 persons. The average annual seropositivity was 20%. The highest seropositivity was registered in the following regions: Vratsa – 52.94%, Gabrovo – 69.77% and Silistra – 46.43%. Acute toxoplasmosis was reported in 11 cases and the annual incidence was 0.15 per 100 000 population.

Leishmaniasis. Indigenous cases of visceral leishmaniasis have been registered

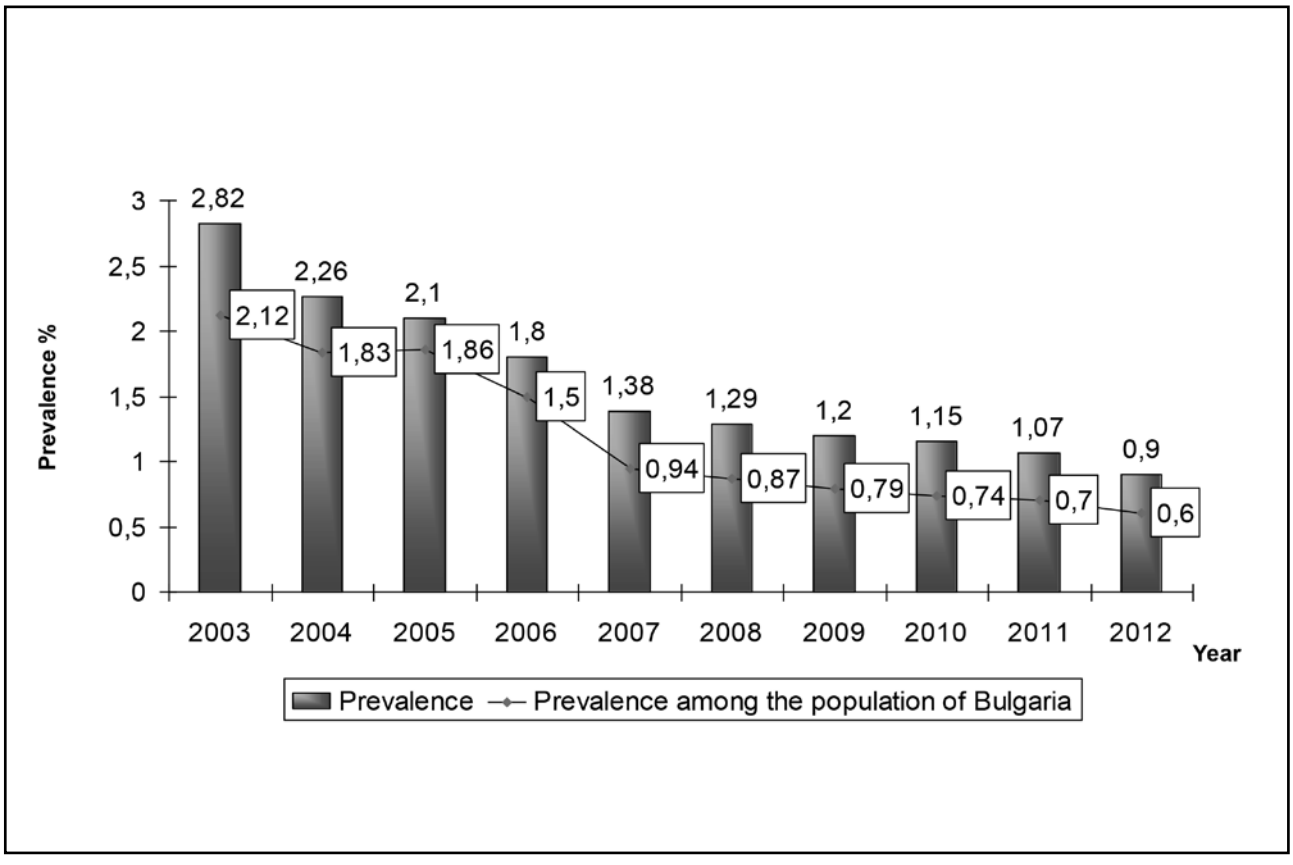

Fig. 7. Prevalence of enterobiasis (2003-2012)

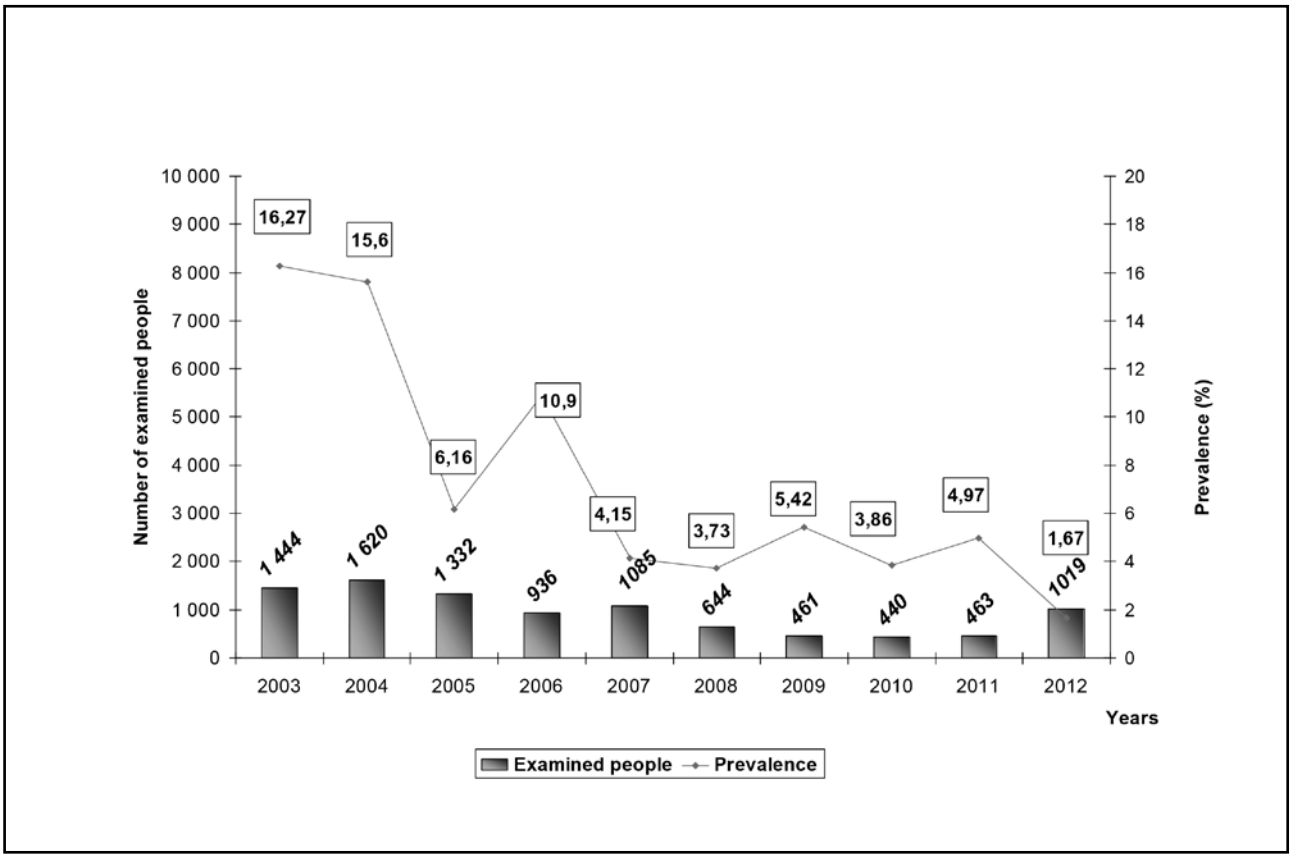

Fig. 8. Parasitological examinations for imported parasitic diseases (2003-2012)

annually in Bulgaria since 1988 (7). In 2012 the number of patients with leishmaniasis was 2 and both cases were from the region of Petrich.

***Cryptosporidiosis*.** During the year 248 patients were examined for the disease and 4 positive cases were reported from Burgas region. Examinations were conducted in 4 regions of Bulgaria – Burgas, Pernik, Plovdiv and Sofia-capital.

***Pneumocystosis*.** Only 27 patients were examined for this parasitosis and no positive results were registered. Tests were conducted in two regions – Plovdiv and Sofia-capital.

Blastocystosis. The prevalence was 0.26% among 478 189 examined patients in 2012. Prevalence above the country average was registered in the following regions: Pernik – 2.72 %, Pleven – 0.90 %, Burgas – 0.70 %, Sliven – 1.01 %, and Lovech – 0.93%.

Imported parasitic diseases

In 2012, 1 019 patients were examined for imported parasitoses and positive results were registered in 17 (1.7%) of them (Figure 8).

***Malaria*.** During the year 1006 patients were examined in 16 regions of Bulgaria and NCIPD, with positive results diagnosed in 16 cases – 15 with *P.falciparum* and 1 with *P.vivax*. Nine were Bulgarian citizens and 7 were foreigners. No lethal cases were registered in 2012.

Other imported parasitic diseases. The examination of 134 people (57 Bulgarian citizens and 77 foreigners) detected a positive result for *B.hominis* in only one person.

Activities regarding the surveillance and control of the parasitic diseases

***Laboratory diagnosis*.** A total of 738 155 patients were examined for parasitic diseases during the year 2012. The number of patients with positive result was 11 026 (1.5%) where 7 311 (66.3%) cases were diagnosed with morphological methods and

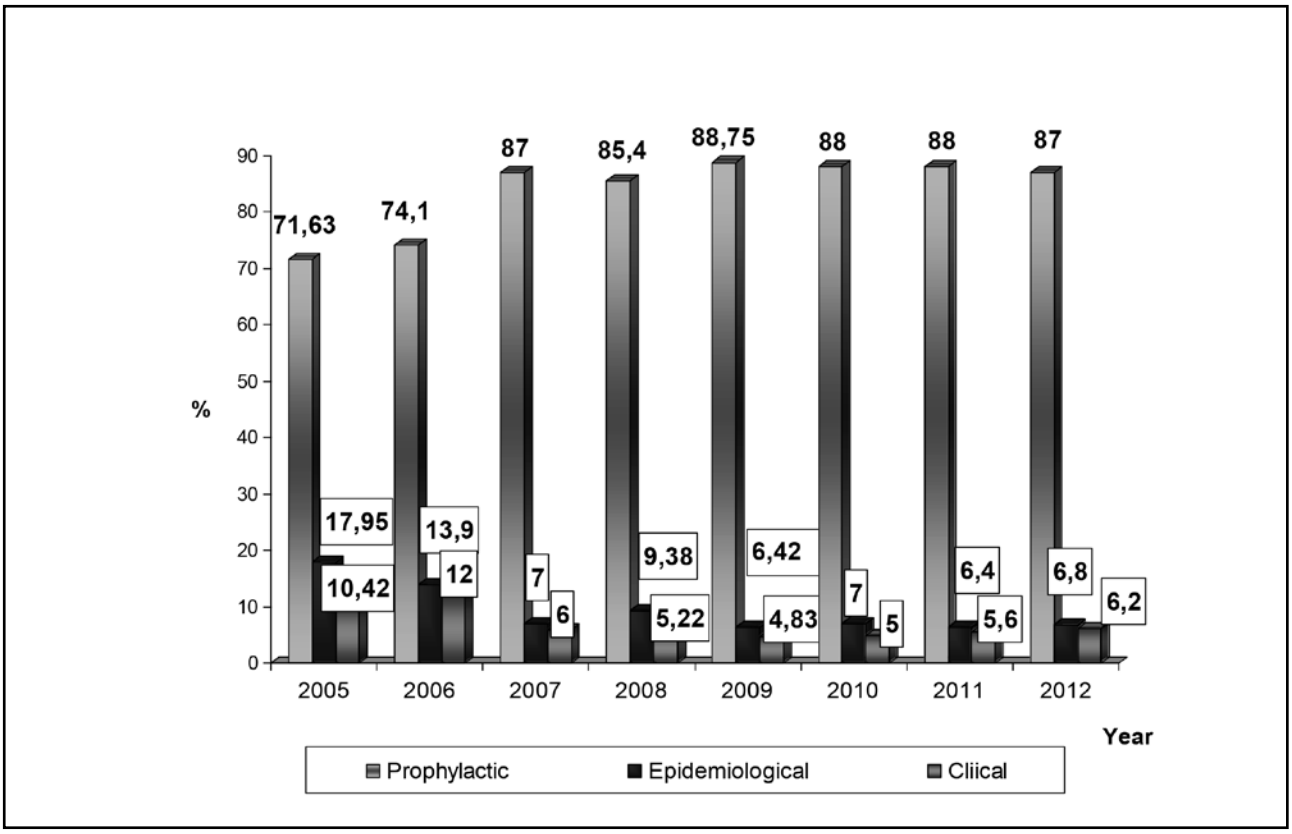

Fig. 9. Comparison of examined people in groups

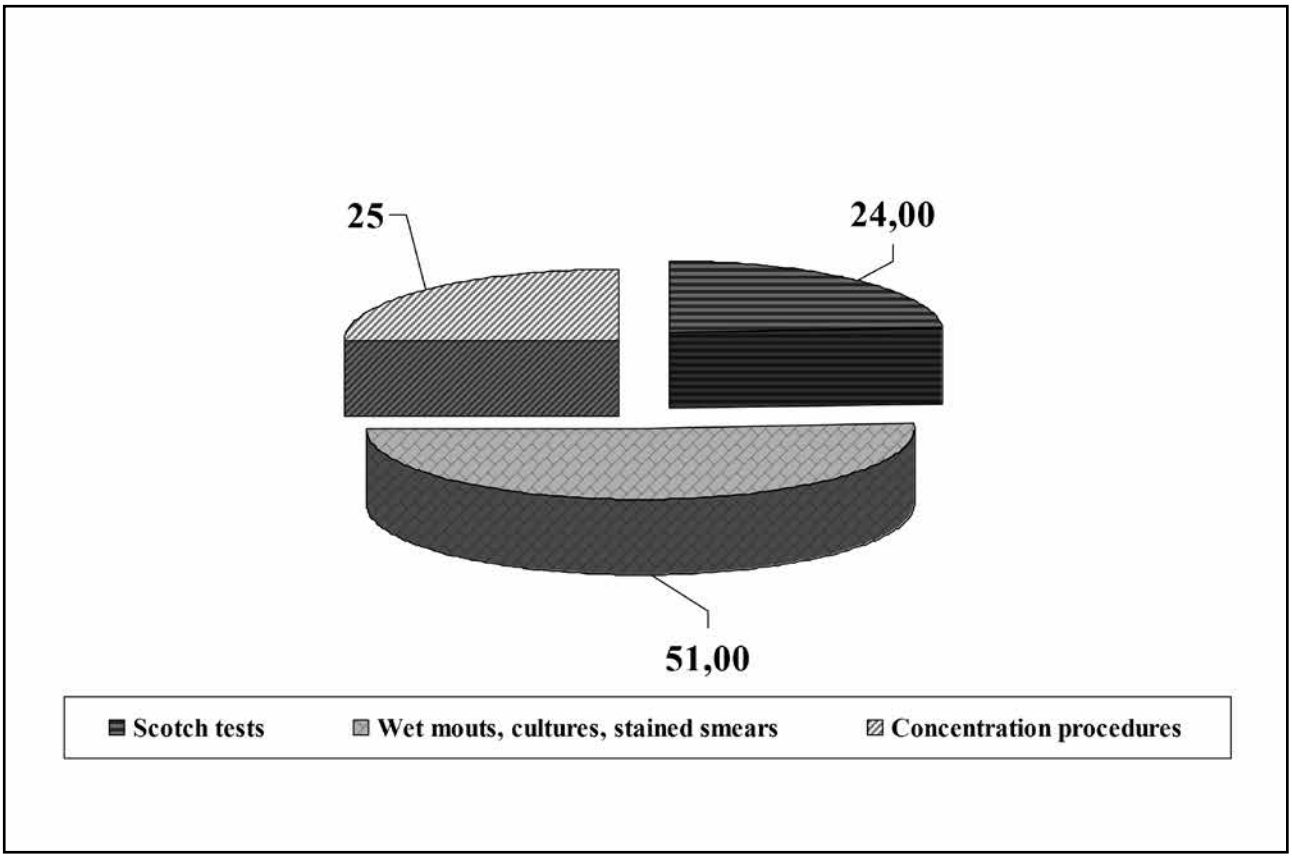

Fig. 10. Distribution of the morphological analyses according to the applied tests (2012) (n = 1 697 983)

3 715 (33.7%) – with immunological tests. The majority of patients - 641 739 (87%), were examined as part of prophylactic measures, 50 324 (6.8%) were examined due to epidemiological requirements and 46 092 (6.2%) on clinical indications (Figure 9).

In the country 1 719 247 analyses (1 401 793 in 2011) were conducted with 1 697 983 (98.5%) morphologial (microscopic) and 21 264 (1.5%) serological methods (3).

The majority of morphological tests were wet mounts, cultures and stained smears– 851 932 (51%), followed by the concentration procedures – 433 449 (25%) and scotch tests – 412 602 (24%) (Fig. 10).

Surveillance, control, organizational and technical assistance. Instead of planned 5 567 sites for inspection, 12 345 sites were investigated and 2 082 of them were examined with laboratory methods. During the year 3 458 inspections were conducted at childcare facilities. Inspections were made as follows: 142 in social facilities for children and adolescents, 58 in facilities for adults and all checks were accompanied by laboratory tests. In treatment facilities for hospital and outpatient care 5 258 inspections were conducted with technical assistance regarding parasitic diseases.

In 2012, 466 epidemiological investigations were carried out - 346 for cystic echinococcosis, 28 for taeniasis, 2 for leishmaniasis, 16 for malaria and 74 for trichinellosis.

A total of 18 221 samples were collected from the environment with 49 936 performed analyses. Among them 17 290 were samples from living environment, 452 – from soil with 8 478 analyses, drinking and wastewater – 171 samples with 312 analyses, as well as food (meat and meat-products, fruit and vegetables), related to *Trichinella* **outbreaks and geo helminthes foci in the country –310 samples with 716 analyses. The number of positive samples was 29 as follows: eggs of** *E.vermicularis – 12, Strongyloides* **and other nematode larvae - 10 and 7 samples with other parasites.**

***Health promotion*.** During the year 57 discussions regarding parasitic diseases such as echinococcosis, soil-transmitted helminthoses, malaria and comminicable parasitic diseases were broadcasted on regional and national TV and radio programmes. In addition, 1384 discussions were held among children, parents and minority groups and 1200 information sheets on echinococcosis and tropical parasitic diseases were distributed.

CONCLUSIONS

The prevalence of parasitic diseases in Bulgaria has remained at a steady level over the last years and varied between 1.5 and 2%. The number of examined patients was significant (between 700 000 and 800 000) (1, 2, 3, 4).

Regarding helminthozoonoses it should be noted that the tendency to maintain the incidence of echinococcosis and trichinellosis continues (1, 2, 3, 4, 5). The downward trend in prevalence of soil-transmitted parasitic diseases and reducing the number of endemic villages will continue over next year, probably due to the depopulation of rural areas (6). The prevalence of enterobiasis, giardiasis and hymenolepiasis holds the same values with a large number of examined children - approximately 95% of childcare institutions. For the detection of the opportunistic parasitic diseases a significant number of tests was performed for toxoplasmosis and blastocystosis. The number of examinations for cryptosporidiosis and pneumocystosis remains very limited and available only in individual regions. In recent years there are annually registered autochthonous cases of visceral leishmaniasis and this trend will continue due to the presence of a source of infection in the south part of the country (7).

Imported from endemic countries parasitic diseases, and especially malaria, pose

a risk of clinical and epidemiological consequences unless strong epidemiological surveillance is maintained.

The specific features of parasitic diseases require complex measures for diagnosis, treatment and prophylaxis in order to reduce the number of sources, as well as of strengthening the surveillance and control of parasitoses aiming at limitation of their spread.

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STATEMENT ABOUT PROTECTION OF HUMAN SUBJECTS
AND ANIMALS IN RESEARCH

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

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

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