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PROBLEMS

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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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DISSEMINATION OF CTX-M EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING ISOLATES OF *KLEBSIELLA PNEUMONIAE* IN FOUR HOSPITALS IN SOFIA AND PLEVEN

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7 - MICOER Institute Munich

SUMMARY

Objectives: To prove ESBL production in acollection of isolates *Klebsiella spp* with reduced susceptibility or resistant towards cephalosporins third generation, which were recovered during the period 2007-2009 in four hospitals in Sofia and Pleven. To characterize ESBL types, antibiotic susceptibility of the producers as well as to detect possibility for conjugative plasmid transfer and clonal relatedness of the strains.

Materials and methods: A total of 44 clinically significant isolates *Klebsiella pneumoniae* and 1 *Raoultella terrigena* with reduced susceptibility or resistant to III[rd] generation cephalosporins were studied. Antimicrobial susceptibility, ESBL-type and transferability of resistance determinants were analyzed. The epidemiology typing was performed by ERIC -1, 2A PCR.

Results: Production of ESBL was confirmed in 42 studied strains. The CTX-M group ESBL was the predominant one (98%). Only one strain, isolated in 2008, was SHV-12 producer. CTX-M-3 enzymes were found in 45% of the isolates. Among them we have detected two clones with one of them appearing in all centers in Sofia and the other in a hospital in Pleven, each representing 50% of isolates. CTX-M-15 positive isolates (53%) belonged to seven clones with 1 to 7 members. The high rate of conjugation transfer in the collection of CTX-M-3 producers (89%) was detected. In the case of CTX-M-15 producers the rate was 45%. The ESBLs producers demonstrated high resistant rates to tobramycin, gentamicin and amoxicillin/clavulanic acid.

Conclusions: On the base of these results it can be concluded that clonal dissemination most likely plays the important role for the disseminations of CTX-M-3 and 15 producers, while plasmid transfer is associated with distribution of CTX- M-3 enzymes among *Klebsiella* strains.

KEYWARDS: ESBL, CTX-M, epidemiology typing, Bulgaria

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INTRODUCTION

Beta-lactam resistance due to the production of Extended-Spectrum Beta-Lactamases (ESBL) was discovered in the eighties and since that time they have become epidemic and endemic in hospitals and community worldwide (1-6). The majority of ESBLs are class A enzymes and were classified into eight different groups, according comparison in deduced aminoacids substitutions (TEM, SHV, CTX-M, VEB, GES, TLA, BES и OXA)(1,2). The main groups were TEM and SHV families. Recently CTX-M ESBLs with enhanced activity against ceftazidime have become predominant in many countries (2,4,5). Among them, CTX-M-15, first described by Karim *et al*. (2-6), became the most frequent ESBL and has been found world-wide(2-6).

The global spread of some ESBL variants greatly depends on the dissemination of particular clones of the *Klebsiella pneumoniae* and *Escherichia coli* and/or on specific types plasmids carrying ESBL genes (1,3,5).

In Bulgaria, similarly to the world-wide trend, the prevalence of ESBL producing *E. coli* and *K. pneumoniae* has increased. The European Antimicrobial Resistance Surveillance System (EARSS) annual report 2008 showed dramatically increased rates of clinically significant Bulgarian invasive *E. coli* and *Klebsiella pneumoniae* isolates, mostly from bacteremia, resistant to oxyimino-cephalosporins(7). Other data also confirmed these results (8). This worrying finding prompted more detailed investigations.

THE AIMS OF THIS WORK were:

To prove ESBL production in collection of isolates *Klebsiella spp* with reduced susceptibility or resistant towards cephalosporins third generation, recovered during the period 2007-2009 in four hospitals in Sofia and Pleven, and to characterize ESBL types, antibiotic susceptibility of the producers as well as to detect possibility for conjugative plasmid transfer and clonal relatedness of the strains.

MATERIALS AND METHODS

Bacterial isolates

Forty-one clinically significant *K. pneumoniae and* 1 *Raoultella terrigena* with reduced susceptibility or resistant towards cephalosporins third generation, recovered during the period 2007-2009 in four hospitals in Sofia (Ministry of the Inferior (n=11), UMHAT Alexandrovska (n=12), MHATUM "H.I.Pirogov" (n=10) and Pleven - UMHAT Pleven (n=9) from: urine – 22, blood – 4, wound – 10, ear/eye swab – 4, upper respiratory tract secretions - 2. Isolates were from patients in neonatology unit (n=9), internal (n=7), urology (n=10), surgery and intensive care units (n=14) and from ambulatory patients (n=2).

Confirmation of ESBL production was by:

1. Double disk synergy (DDS) method by Jarlier et al (9)with disks of 3[rd] generation cephalosporins – ceftazidime, cefotaxime and amoxicillin/clavulanic acid

2. CLSI disk confirmatory method with disks ceftazidime, ceftazidime/clavulanic acid and cefotaxime, cefotaxime/clavulanic acid

Susceptibility testing was according to CLSI, 2010 guidelines with Mueller Hinton II agar (BD) (10).

Conjugative plasmid transfer was carried out on a solid medium with *E.coli* K_{12}: W_{3110} Rif lac (-) or J_{62} Nx lac (-). Selection: Mc Conkey agar + 2 mg/L Ceftazidime or Cefotaxime + 50 mg/L Rifampicin or Nalidixic acid 50 mg/L.

Preliminary characterization of ESBL groups:

1. **Isoelectric focusing (IEF):** Enzyme extracts of the strains were prepared with ultrasonic disintegration of bacterial cultures. IEF was performed by a procedure of Mathew et al as described previously (11, 12) with polyacrylamid gel 30% containing Ampholyte pH 3-10
2. **Bioassay** was performed by a procedure of Bauernfeind et al (13) to reveal the hydrolytic activity of the bands. The growth of the indicator strain identifies the position of which the chosen antibiotic has been inactivated.

Polymerase chain reaction and sequencing. PCR was performed to detect the presence of beta-lactamase genes of the SHV-, TEM-, and CTX-M-group as described previously (12). PCR amplification products obtained with oligonucleotides binding to the flanking region of the genes, as described previously (12), were subjected to automatic sequencing (ABI 3700, Applied Biosystems, Foster City, CA, USA).The nucleotide and deduced amino acid sequences were analysed and multiple alignments were performed using Chromas Lite 2.01 (Technelysium Pty Ltd, Brisbane, Australia) and DNAMAN 4.11 Software (Lynnon BioSoft, Vaudreuil-Dorion, Canada).

Epidemiology typing. Whole-cell DNA was prepared using the GFX Genomic DNA Purification Kit (Amersham Biosciences, Little Chalfont, UK) for RAPD analysis with ERIC 1 and ERIC 2A primers (14). Strains showing more than 85% to two band differences were interpreted as clonally related.

RESULTS

ESBL detection

Forty-two isolates gave positive results for ESBL production with DDS test and 32 were positive in CLSI confirmatory method except for 10 strains that haven't shown an increasing of the zone of inhibition around disk ceftazidime + clavulanic acid in comparison with ceftazidime alone. Thus we have confirmed 42 from 44 strains, which have been sent from the laboratories.

Antimicrobial susceptibility

The rates of resistance among ESBL producing *Klebsiella spp* (R+I) were shown in Fig 1. The resistance to ceftazidime was not 100%; however their therapeutic use should be avoided because of treatment failure. (6). In the case of cefotaxime and cefepime the strains were 100% resistant and these antimicrobial disks could be used for detection of ESBL production in *Klebsiella* isolates.

From a clinical point of view very important is the detection of higher resistance rates towards amoxicillin/clavulanic acid and tobramycin and gentamicin. The investigated strains also showed significant rates of resistance towards amikacin, ciprofloxacin and trimethoprim/sulfomethoxazole - 60-70% (Fig 1). All strains were 100% susceptible towards imipenem.

Phenotypic characterization of beta-lactamases

The results from the isoelectric focusing were shown on Table 1. All isolates demonstrated beta-lactamases with isoelectric point (pI) 5.4 *и* pI 7.6, without cefotaxime or ceftazidime hydrolyses – most probably broad spectrum TEM-1 and chromosomal SHV-1 (1) enzymes. ESBLs with pIs of 8.2, 8.4 and 8.8 were found (Table 1). The enzymes with pI 8.2, which were able to hydrolyze ceftazidime, probably belong to the SHV-group, while the cefotaxime-hydrolyzing beta-lactamases with pIs of 8.4 and 8.8 were suspected to be cefotaximases. All isolates produced a single ESBL.

Molecular identification of beta-lactamases

ESBL-group specific PCR carried out with all 42 isolates or their transconjugants confirmed the group of beta-lactamase expected from the phenotypic characteristics. Among them, 12 isolates, representing different centers, isolation periods, species and PCR groups, were selected to determine the distinct type of beta-lactamase by sequencing of their genes. Three different ESBLs were identified, namely SHV-12; CTX-M -3, and -15 (Table 1). Only one strain from a center in Sofia produced SHV-12. CTX-M-3 enzymes were found in 45% of the isolates in all four centers. The predominant ones were CTX-M-15 enzymes with 53% detection, they were found only in centers in Sofia (Table 1).

Molecular typing

Results of molecular typing are shown in Table 1. Among *K. pneumoniae* isolates, RAPD identified 10 types. Two of them, "d" and "e", were predominant, produced CTX-M-3 ESBLs and represent 45% of all isolates, other strains were grouped in 7 smaller clusters consisting of one to seven isolates and were associated with CTX-M-15 ESBL. One strain that expressed SHV-12 had an unique profile.

Conjugation experiments

The conjugation experiments were successfully performed on 28 *Klebsiella spp*. strains from all tested. The high rate of conjugation transfer in the collection of CTX-M-3 producers (89%) was detected. In the case of CTX-M-15 producers the rate was 45%. Table 1 showed resistotypes among transconjugant strains. Different resistance determinants for non-beta-lactam antibiotics were co-transferred with the ESBL-genes.

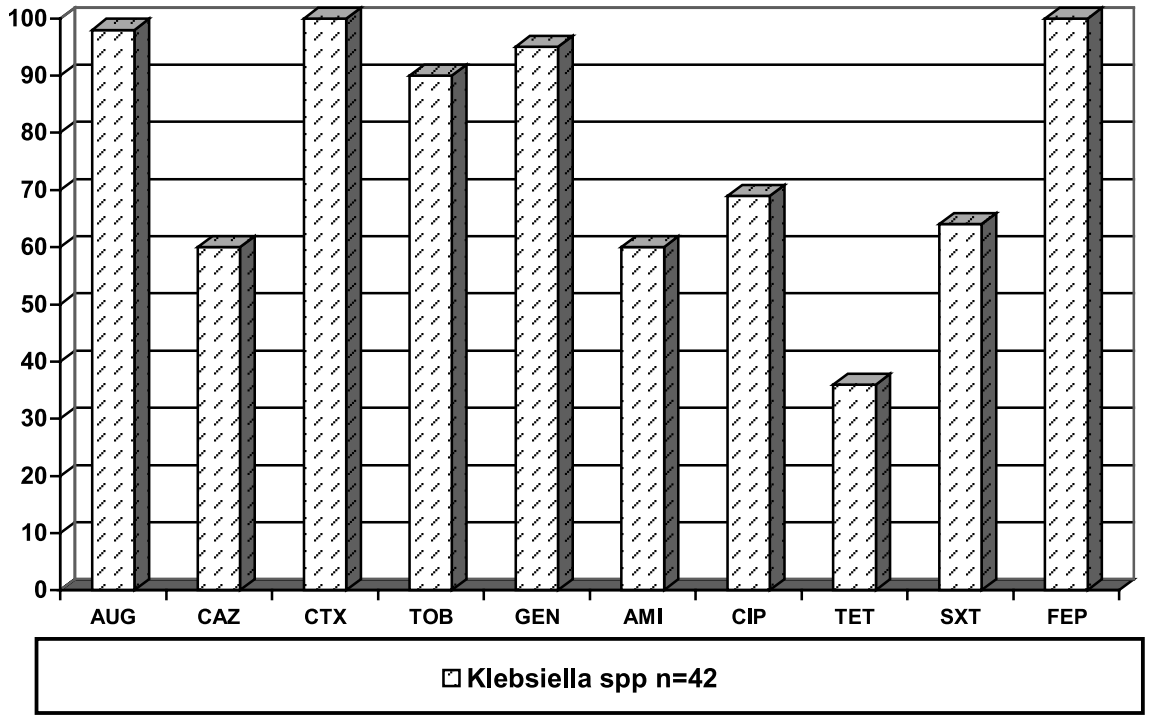
Table 1. Distribution of ESBL types in *Klebsiella spp* isolates according the center, year of isolation and RAPD type

Center	Number	IEF[1]	Sequencing	Year на isolation	RAPD тип number	Transconjugant phenotype number
UH-Pleven	9	5.47.6<u>8.4</u>	CTX-M-3	2007	v_9	TOB,GEN,AMI,SXT_9
MI-MI	1	5.4 7.6 <u>8.2</u>	SHV-12	2008	unique	TOB, AMI
	5	5.4 7.4/7.6 <u>8.8</u>	CTX-M-15	2008	e_5	TOB, GEN, TET_2
	5*	5.47.6<u>8.4</u>	CTX-M-3	2008	d_4 (K pn)	GEN_5
UH-Alexandrovska	11	5.4 7.4/7.6 <u>8.8</u>	CTX-M-15	2008, 2009	k_7;b_2;c_1; f_1	TOB,GEN_1 TOB, GEN, TET_1 $Susceptible_1$
	1	5.47.6<u>8.4</u>	CTX-M-3	2009	d	-
UH-Pirogov	6	5.4 7.4/7.6 <u>8.8</u>	CTX-M-15	2009	p_5;q_1	TOB,GEN_4 $Susceptible_1$
	4	5.47.6<u>8.4</u>	CTX-M-3	2009	d_4	TOB,GEN,AMI_3

Abbreviations:IEF – isoelectric focusing; [1] underlined pIs showed CTX hydrolytic activity in bioassay; * including one strain *Raoltiella terrigena*

Figure 1. Resistance (I+R) of ESBL producing *Klebsiella spp.* isolates towards some beta-lactam and non beta-lactam antibiotics

Abbreviations: amoxicillin+ clavulanic acid (AUG) ; ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP); tobramycin (TOB), gentamicin (GEN), amikacin (AMK), ciprofloxacin (CIP), tetracycline (TET), co-trimoxazole (SXT)R – resistant, I - intermediate



Most often it was gentamycin – in 89%; tobramycin – 75% and amikacin – 46%. The phenotype TOB, GEN, AMI, SXT was associated with carrying of *bla*CTX-M-3. 4 resistotypes were detected among transconjugants which produced CTX-M-15, and three among transconjugants which produced CTX-M-3.

DISCUSSION

Chronologically, SHV-type beta-lactamases appeared to be the first ESBLs emerging in Bulgaria. In the previous study among *Enterobacteriaceae* strains from Bulgarian hospitals (12,15) they persisted through the entire study period and were found in all studied centers.In this study we detected only a single SHV-12 producing *K. pneumoniae* in Sofia.

In the same study TEM-type ESBL has been detected for the first time in 1999 in Pleven. They were restricted there and were rarely detected in Sofia. Interestingly, in this study we did not detect TEM-139 enzymes among *Klebsiella* strains.

In Bulgaria CTX-M-type beta-lactamase producing isolates emerged in 2001 (CTX-M-15). Their percentage among all ESBL-producing strains rose rapidly from 16.7% in 2001 to 65.2% in 2003 (12). In the entire investigation period we also observed higher prevalence of CTX-M ESBL – 98% (41 from *Klebsiella* 42 strains) , divided into two groups CTX-M-15 in 53% and CTX-M-3 in 45%. The results showed that CTX-M ESBLs have completely replaced the other type enzymes. This data were similar to the other reports from all over the world – for increasing number of CTX-M producers, particularly CTX-M-15 (2, 4, 5). CTX-M-15 producers in our study belonged to 7 clones with an appearance of different clones in the separate centers as predominant. We observed 45% rate of conjugation transfer, thus showing that most likely their clonal distribution supports the dissemination of CTX-M-15 producers in Sofia. It is well known that these producers have great potential for mobilization and dissemination mainly due to different IS elements, transposons and integrons. IS*Ecp*1 transfer $bla_{\text{CTX-M-15}}$ with onestep transposition and play a role in gene expression (2,5). Recently it has been detected that a high percentage of *E. coli* strains (68%) in Bulgaria belonged to Pan European O25b-ST131 clone (17).

Dissemination of CTX-M-3 producing *K. pneumoniae* in Pleven is due to the persistence of one clone as well as the horizontal transfer of plasmids, carrying resistant determinants for TOB, GEN, AMI and SXT. It is well known that not only cephalosporins third generation but also other groups such as aminoglycosides and co-trimoxazole have selective pressure (1). Interestingly in Pleven all investigated *Klebsiella* strains produced only CTX-M-3. The rate of CTX-M-3 enzymes among *K. pneumoniae* is also higher in Varna – 89%, and transconjugants with phenotype TOB, GEN, AMI and SXT have been detected (18). Similar data were reported from Poland where investigators revealed identical *Pst*I conjugative plasmid carrying $bla_{\text{CTX-M-3}}$ (19). Only 31% of *K. pneumoniae* isolates in Sofia were associated with CTX-M-3 production. The epidemiology typing shows clonal relatedness among them. We may conclude that clonal dissemination and wide plasmid transfer equally contribute in the distribution of CTX-M-3 in Sofia.

Antimicrobial susceptibility showed that investigated *Klebsiella* strains were highly resistant towards tobramycin, gentamycin and amoxicillin/clavulanic acid, but resistance to ciprofloxacin and co-trimoxazole was not so high (Fig 1). For comparison, resistance towards ciprofloxacin in ESBL producing *E.coli* O25b-ST131 strains detected in Bulgaria was 96% (17). These antimicrobials can give an alternative for the treatment of multiple resistant ESBL producing *K. pneumoniae* in investigated hospitals.

All *Klebsiella* strains were susceptible to imipenem. This is the only opportunity for treatment of serious infections.

In conclusion, we have detected high prevalence of CTX-M producing *Klebsiella* strains, highly resistant towards aminoglycosides and amoxicillin/clavulanic acid. Clonal dissemination most probably plays an important role for the disseminations of CTX-M-3 and -15 producers, while plasmid transfer is associated with distribution of CTX- M-3 enzymes among *K. pneumoniae*.

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FIVE YEARS OF ANTIBIOTIC CONSUMPTION IN BULGARIA. SYNOPSIS OF THE MAJOR TRENDS IN 2007-2011

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ABSTRACT

Resistance to antibiotics is a major public-health problem and antibiotic use is being increasingly recognised as the main selective pressure driving this resistance. Our aim was to assess outpatient, hospital and total use of antibiotics in Bulgaria during the period 2007-2011.

Materials and Methods: Consumption data were obtained from the pharmaceutical marketing provider IMS Health as number of packages for each antimicrobial product for systemic use. We calculated antibiotic consumption in the three sectors as the number of defined daily doses (DDD) per 1000 inhabitants per day, according to WHO anatomic therapeutic chemical classification and DDD measurement methodology.

Results: Overview of total consumption shows slight decrease between 2008-2010 from 24.6 to 22.5 DDDs/1000 inhabitants per day, respectively. The most frequently used antimicrobials in primary care are arranged in a similar way as in the results for the total consumption. The penicillin group ranks first, followed by MLS (macrolides and lincosamides) and cephalosporin groups. Hospital consumption results have different pattern of the most widely used groups. Almost half of the total hospital consumption is in favor of the cephalosporins with measured values distributed between 0.8 and 1.1 DDDs/1000 inhabitants per day. Second are penicillins and after them – fluoroquinolones.

Conclusion: We measured beta-lactam antibiotics as the largest proportion of antibiotic consumption in the three sectors. An unfavourable trend during the five years of our monitoring is the increasing percentage of broad-spectrum cephalosporins used in Bulgarian hospitals.

INTRODUCTION

For the past 60 years, antimicrobial chemotherapy has been the mainstay of medical intervention against infectious diseases caused by bacterial pathogens. The continuous decline of therapeutic effectiveness as a result of extensive use of antimicrobial chemotherapy has been long predicted and seems inevitable. It is therefore critical to realize that antimicrobial drug effectiveness, widely accepted as a common good, cannot be taken for granted and that such substances are increasingly attaining the status of nonrenewable resources [1-3].

Developed originally to treat human infectious diseases, their properties in veterinary, animal and plant agriculture and aquaculture were applied soon thereafter. The emergence of resistance in nosocomial pathogens has been shown to be associated with antibiotic misuse (overuse plus inappropriate use) in therapy and prophylaxis. Similar associations can be found in the community. Broad use has created a strong selective pressure, which consistently has resulted in the survival and spread of resistant bacteria. Both the amount of antibiotics used and how they are used contribute to the development of resistance. The use of broad-spectrum antibiotics rather than narrow-spectrum drugs is known to favor the emergence of resistance by broadly eliminating competing susceptible flora. Antibiotics are frequently prescribed in the treatment of viral infections or at wrong doses for incorrect periods of time [4-6].

To better understand and analyse national, regional, and local trends in development of resistance, assessment of national data on antibiotic consumption is of great significance.

The National Reference Laboratory “Control and Monitoring of Antibiotic Resistance” in the Department of Microbiology at NCIPD is conducting the national surveillance system for antimicrobial resistance and antibiotic consumption in Bulgaria. Our data summarize national trends in the most frequently used antimicrobial agents at the substance level and as an antimicrobial class. Our surveillance covers the most important antiinfectives – antimicrobials, antivirals, and antimycotic agents. General results on overall (total) antibiotic consumption in Bulgaria assume the following structure: penicillins hold the first place of all antimicrobial agents with 42% in 2007 and 36% in 2011. Macrolides and lincosamides are second and cephalosporins are the third most frequently used antimicrobials. Hospital consumption was measured as 7-8% of the overall rates. In this sector the most used agents are cephalosporins and our data show that since 2008 the percentage of broad spectrum compounds within this group has increased.

MATERIALS AND METHODS

The primary aims of this study are to measure and summarize the national consumption of antiinfectives for systemic use in Bulgaria for the period of 5 years between 2007 and 2011. Surveillance of antimicrobial use is a key strategy to monitor prudence in antimicrobial therapy.

Information on expended antimicrobials for systemic use as number of packages is collected annually from the hospital and ambulatory care sector and these data are obtained from the pharmaceutical marketing provider IMS Health. In order to measure antibiotic

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consumption more accurately the Anatomical Therapeutic Chemical (ATC) classification and the unified Defined Daily Dose (DDD) measurement unit were assigned to the data (ATC/DDD version 2012) [7]. The amount in grams for an antimicrobial agent was converted to a number of defined daily doses (DDD). Consumption data are analyzed and expressed in numbers of DDDs per 1000 inhabitants per day for each antimicrobial class and for the overall annual rates. Trends in the most frequently used antiinfectives are summarized in the three sectors – hospital and outpatient use, and as total consumption.

Antimicrobial drug use in ambulatory and hospital care was summarized into the following major antimicrobial classes: penicillins and inhibitor combinations (J01C); other β-lactam antimicrobial agents (cephalosporins, monobactams and carbapenems, J01D); macrolides, lincosamines, and streptogramins (MLS-class, J01F), fluoroquinolones (J01M), tetracyclines (J01A), aminoglycosides (J01G), antitubercular drugs (J04), antimycotics (J02), and antivirals (J05).

RESULTS

The results showing national consumption measurements are outlined in Fig. 1, 2, 3, and 4, comprising total, hospital, and ambulatory care sectors. In order to be comparable all calculations and measurements of the antimicrobial consumption were determined as numbers of DDDs/1000 inhabitants per day. Only antimicrobials for systemic use are included in this survey. Overall results for the total consumption in the period of 2007-2011 are depicted in Fig. 1. Penicillin agents are the most widely used during all five years with values distributed between 8.1 and 10 DDDs/1000 inhabitants/day. The total use of penicillins in 2007 (alone and with inhibitor combinations) accounts for 42% of all other antimicrobial classes and in 2011 - for 37%. They are followed by the MLS group (macrolides and lincosamides) with 2.9 to 3.6 DDDs/1000 inhabitants/day. The group of cephalosporins and carbapenems ranks third (2.6 to 3.4 DDDs/1000 inhabitants/day) followed by fluoroquinolones and tetracyclines. The total consumption marks slight decrease between 2008-2010 from 24.6 to 22.5 DDDs/1000 inhabitants per day, respectively.

The antibiotic consumption in ambulatory care sector is presented in Figure 2. The most frequently used antimicrobials are arranged in a similar way as in the results for the total consumption. The penicillin group accounts for 44% (9.6 DDDs/1000 inhabitants/day in 2007) and 38% (8.4 DDDs/1000 inhabitants/day in 2011) of the measured usage. MLS and cephalosporin groups are in the second and third place, respectively. Once again during 2008 is reached the highest level of antibiotic consumption with 22.5 DDDs/1000 inhabitants per day, followed by a decrease which in 2010 reaches 20.8 DDDs/1000 inhabitants/day.

In Bulgarian hospitals (Fig.3) highlights of antibiotic consumption form a quite different structure. During the years 2007-2011 almost half of the total hospital consumption is in favor of the cephalosporins with measured values distributed between 0.8 and 1.1 DDDs/1000 inhabitants per day. Considering these values they occupy between 46-54% of the overall hospital consumption compared with other classes of antimicrobials. Penicillins are second, alone or in a combination with an inhibitor, with 0.2 to 0.4 DDDs/1000 inhabitants per day. They are followed by fluoroquinolones and the MLS group. Aminoglycosides remain in the fifth place. The highest value of measured consumption in this sector is in 2009 with 2.1 DDDs/1000 inhabitants per day. Overall, the proportion of ambulatory consumption (average 21

Figure 1. Total antibiotic consumption (DDDs/1000 inhabitants/day)

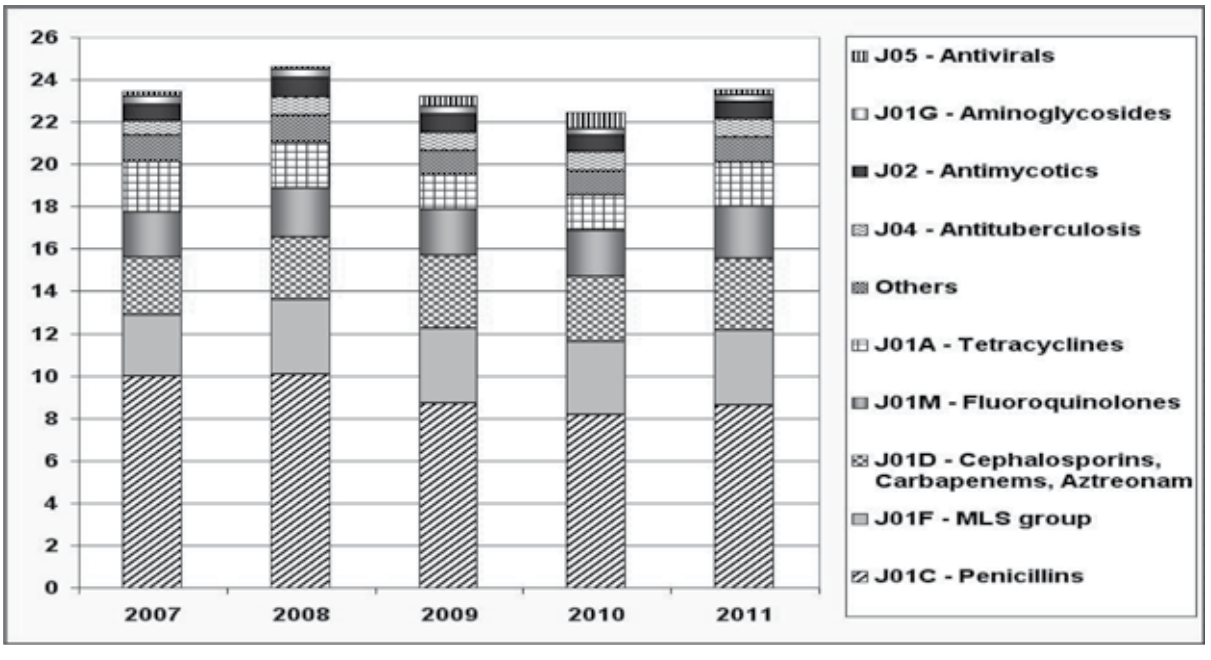


Figure 2. Outpatient antibiotic consumption (DDDs/1000 inhabitants/day)

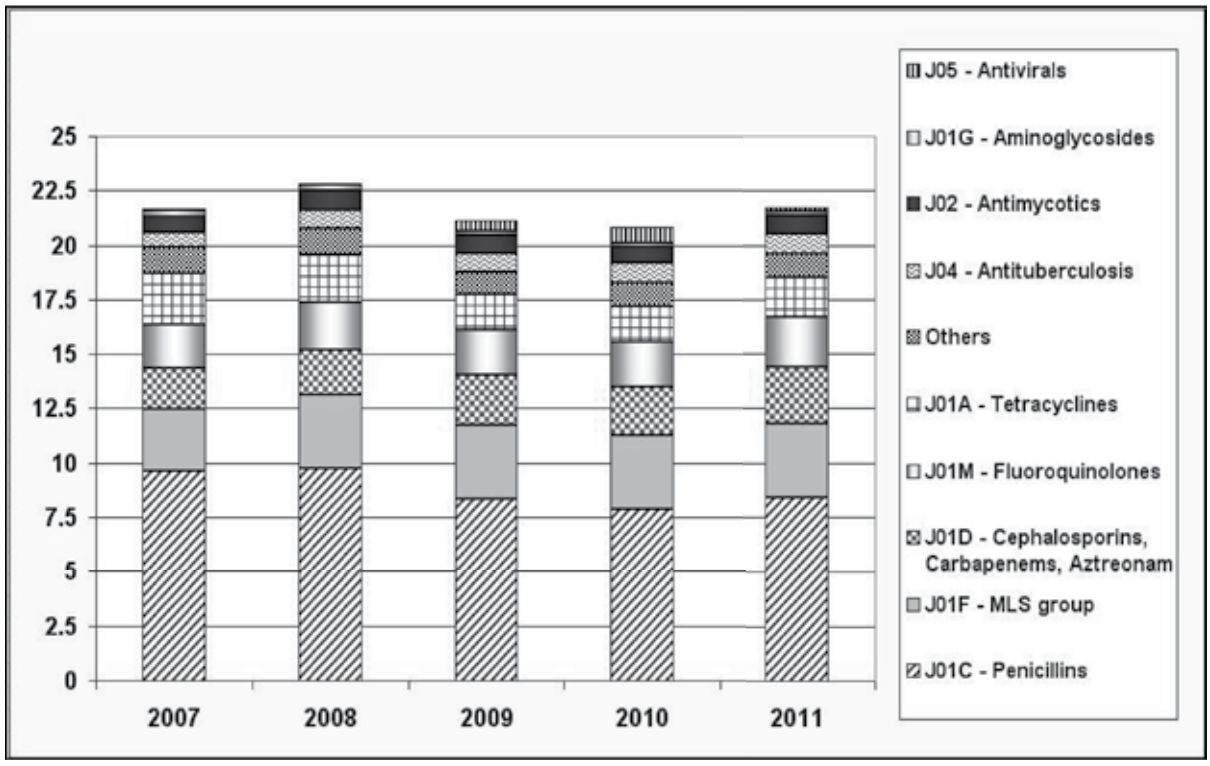
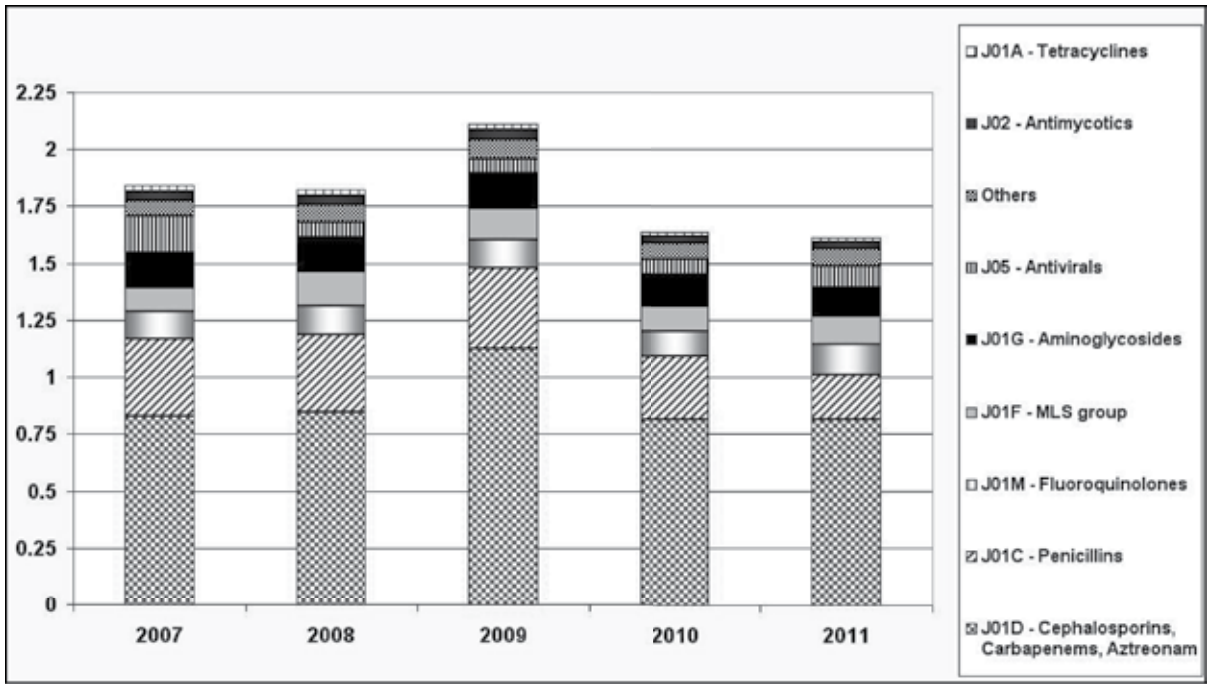


Figure 3. Antibiotic consumption in hospital care (DDDs/1000 inhabitants/day)



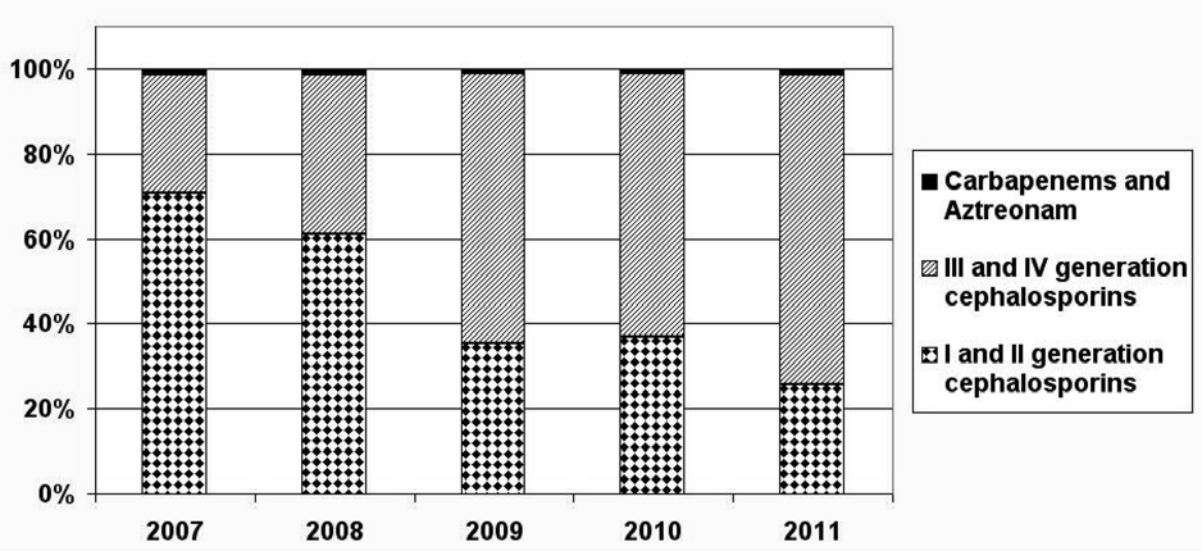
DDDs/1000 inhabitants per day) covers a much greater part than the hospital (average 1.8 DDDs/1000 inhabitants per day).

Because of the large use of cephalosporins in Bulgarian hospitals we extracted from the database the distribution of consumption values of the J01D group. Fig.4 shows the progressive increase in the percentage of use of broad-spectrum cephalosporins in hospital settings. In 2007 they account for 29% (0.23 DDDs/1000 inhabitants/day) of consumption of the entire group cephalosporins, carbapenems, and aztreonam. This value has increased to 71% (0.57 DDDs/1000 inhabitants/day) in 2011.

DISCUSSION

The publication by Cars et al. [8] in 2001, followed in the same year by the implementation of the European Surveillance of Antimicrobial Consumption (ESAC) project suddenly shed light on the large differences in the consumption of antibacterial agents among European countries [9]. In the present study we summarized major trends in the consumption of diverse

Figure 4. Percentage values in hospital consumption of the J01D group



antimicrobial classes for systemic use in Bulgaria for five consecutive years.

For hospital prescribing the standard unit of measurement is defined daily doses (DDDs) per 1000 (or per 100) bed days [10]. The present study measures hospital consumption in DDDs per 1000 inhabitants per day. DDDs provide a more reliable, portable numerator. DDDs per 1000 population served per day has been used to compare both out-of-hospital and hospital antibiotic use internationally, and such data have been useful both for benchmarking and for illuminating the relationship between antibiotic use and resistance at national level [11]. In Bulgarian hospitals the predominant pressure is highly in favor of the cephalosporin group. Other studies also describe the same trend [12, 13]. In 2007 and 2008 first and second generation cephalosporins form 73% and 63%, respectively, of total consumption within the group. Over the next three years these percentage values shifted to broad-spectrum compounds and in 2011 reached 71%. In the hospital sector penicillins (alone or with a β-lactamase inhibitor combination) and fluoroquinolones remain in the second and the third place with measured 0.2 to 0.3 DDDs/1000 inhabitants per day and 0.1 DDDs/1000 inhabitants per day, respectively. According to the latest ESAC-Net survey for the year 2010 [14] Bulgaria is in the 16th place for hospital antibiotic consumption among 19 participating European countries. Inappropriate or overuse of some antimicrobial classes in hospital settings is still an important issue and there are many surveys focusing on problematic nosocomial pathogens with the potential to become very difficult to treat [15, 16].

Overall analysis of our data concerning antibiotic consumption in society shows that for the period 2007 to 2011 it occupies between 92-93% of the total. In a recent European survey [14] Bulgaria stands at eight place in the ambulatory sector consumption among 23 participating countries. Over the five years covered in the present study the penicillin group ranks first in the primary care sector with values between 8-10 DDDs/1000 inhabitants per day. Following are macrolides and lincosamides, which together with penicillins account for 50-60% of ambulatory consumption throughout the period of monitoring. The cephalosporin group is in the third place.

When reviewing the total consumption in 2008 to 2010 a slight decrease from 25 DDDs/1000 inhabitants per day to 22 DDDs/1000 inhabitants per day can be noted. The total antibiotic consumption follows the same pattern as in the outpatient sector. Beta-lactam antibiotics constitute the largest proportion of antibiotic consumption in the three sectors.

The next step to a more comprehensive study of our national data is to assess the relationship between antimicrobial resistance and consumption, likewise many other authors suggest [17, 18], as part of a continuous research monitored by our national surveillance system.

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HUMAN LEPTOSPIROSIS IN BULGARIA - CLINICAL, EPIDEMIOLOGICAL AND SEROLOGICAL ASPECTS OF THE INFECTION, 2010-2011

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Abstract

In the last years, a decrease in the number of registered cases of leptospirosis in our country was observed. This study was focused on the analysis of the circulating serovars of the genus *Leptospira* and their distribution, and the epidemiological characteristics of the laboratory confirmed cases of human leptospirosis, 2010-2011. A total of 28 cases of leptospirosis were registered and laboratory confirmed by a microagglutination test in Bulgaria in this period. The highest incidence was observed in the following areas : Shumen (25%), followed by Sofia-city (14.29%) and Pleven (10.71%). Infected people were mainly elderly men. The age groups 40-49 years - 7/28 and 60-69 years - 7/28 were mainly affected, respectively (25%). The infection is acquired mainly through recreational activities, mainly fishing - (47.37%). The socio-economic structure and the seasonal distribution of the disease were also analyzed. The clinical spectrum of the symptoms was analyzed in 19 patients with leptospirosis (67.86%). Fever and jaundice were the most frequently reported symptoms in 18/19 patients (94.74%) and in 14 patients (73.37%) resp. The analysis of the serological data showed that the serovars causing leptospirosis belong to five serogroups. The leading serogroup was *Leptospira icterohaemorrhagiae* 14/28 (50%), followed by serogroups *Sejroe* (21.43%) and *Australis* (14.29%). Analysis of the results of this two-year period showed that leptospirosis in Bulgaria is an infection with limited distribution, but the risk of leptospirosis should not be underestimated.

Key words: leptospirosis, microagglutination test, epidemiological data, Weil's disease, *L. icterohaemorrhagiae, L. Pomona*

Leptospirosis is the most widespread zoonosis worldwide. The disease occurs in all continents except Antarctica as evidences for carriers of leptospires were found in almost all mammalian species (7). People most often become infected through occupational and recreational activities, or through contact with urine of infected animals or through contaminated water or soil.

In the recent years, the infection is indicated as emerging (14). People in developed countries are now more frequently exposed to infection as a result of more frequent international travel and greater participation in some outdoor recreational activities. Today, leptospirosis remains a diagnostic challenge in medicine. It is often presented as a nonspecific febrile illness and the laboratory diagnostics currently is still not adequate in the early stage of the disease sometimes.

In Bulgaria, cases of human leptospirosis are registered by 1952 (1,18). A trend to decrease in number of registered cases of leptospirosis in our country has been observed. The average morbidity decreased from 0.42 / 100 000 for the period 1989 - 2001 (1,9) and 0.36 / 100 000 for 2002-2005 (3) to 0.18 / 100 000 for the period 2006-2009 (4).

This study was focused on the analysis of circulating serovars of the genus *Leptospira* and their distribution, and epidemiological characteristics of laboratory confirmed cases of human leptospirosis for 2010-2011. It was interesting to reveal the relationship between clinical symptoms, mode of transmission and sources of infection and to assess the status of the problem in our country.

MATERIALS AND METHODS

In this study, all investigated patients were with clinical diagnosis of leptospirosis. Cases of leptospirosis were analyzed on the following main points: epidemiological data such as age and sex of patient, month of the onset of the disease, a potential source of infection and probable route of transmission (contact with source of water, contact with domestic animals), and clinical data (symptoms of the disease). All patients were diagnosed with the reference method for serological diagnosis, microagglutination test, following protocols (12).

Suspensions of live serovars from 9 different serogroups known to circulate in Bulgaria, were used as antigens. Human serum samples were tested initially with routine micro-agglutination. Titer of the specific antibodies was processed in the positive sera subsequently (1).

RESULTS AND DISCUSSION

EPIDEMIOLOGICAL DATA

A total of 28 cases of leptospirosis were registered and laboratory confirmed in Bulgaria in the period 2010-2011. The cases were respectively: 17 in 2010 and 11 for 2011. The average morbidity was 0.15 / 100 000 (0, 15/100 000 in 2010 and 0.16 / 100 000 in 2011). The variation of morbidity showed decrease over the period, compared with previous studies in the country (2006-2009) - 0.18 / 100 000 (4). The average annual incidence during this period was respectively in 2010 - 17/116 (14.65%) and in 2011 - 11/98 (11.22%). A total of 28 patients from 214 tested had antibodies against *Leptospira interrogans* (13,08%) (Fig. 1). This percentage is significantly lower than in previous studies (4).

The average mortality was 0.04%. This is twice higher than in the previous period 2006-2009 (0.02%) (4). There was one fatal case, 76-year old man from Montana, recorded for the period. Weil's disease with acute renal failure (ARF) and multiple haemorrhages were identified as cause of death. A similar case was described in Turkey in 2007 with severe leptospirosis and death (8).

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Serologically confirmed cases of leptospirosis were reported in 10 of 28 Regional Inspectorates for Protection and Control of Public Health (RIPCPH). Distribution of Leptospira serogroups in these cases is shown in table 1.

The highest incidence was observed in the district of Shumen, where 7 clinical cases from 28 in the country (25%) were registered, followed by Sofia-city - 5 cases (14.29%), and district of Pleven - 3 cases (10.71%). Significant increase of leptospirosis cases was observed in these regions, compared with the previous studies. The number of patients in Shumen region was 5.36% in 2005-2009 (4). Decrease in mor-

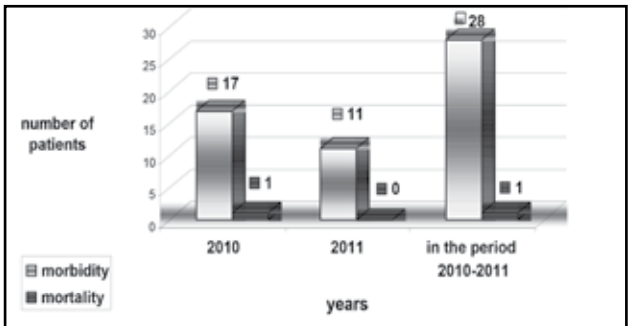

Fig.1 Average morbidity and mortality of leptospirosis patients in the period 2010-2011.

Table 1 Distribution of serologically proven cases of leptospirosis during the period 2010-2011 г. by RIPCPH and serogroups

Serogroups / RIPCPH	*L.icterohaemorragiae*	*L. Pomona*	*L. sejroe*	*L. Australis*	*L.Bataviae*	Total
Blagoevgrad						
Bourgas	2					2
Varna						
Veliko Tarnovo				1		1
Vidin						
Vratsa			1	1		2
Gabrovo						
Gotse Delchev	1					1
Dobrich						
Kardjali						
Kyustendil						
Lovech						
Montana			1			1
Pazardjik						
Pernik						
Pleven	3		1			4
Plovdiv					1	1
Razgrad						
Silistra						
Sliven						
Sofia-city	4		1			5
Sofia-region	1					1
Stara Zagora						
Targovishte						
Haskovo						
Shoumen	2	3	1	1		7
Yambol	2		1			3
Total	15	3	6	3	1	28

bidity was observed in Bourgas region, endemic for leptospirosis, compared with the period 2005-2009 (8.93%). One case (3.57%) per year was reported in the areas of Veliko Tarnovo, Gotse Delchev, Montana, Plovdiv and Sofia. A trend to a significant reduction in the percentage of patients was observed in the areas of Sofia region, Montana and Gotse Delchev, where percentage of patients in 2005-2009 was respectively 10.71%, 8.93% and 6.36% (4). The percentage of infected people was higher in urban (82.14%) than in rural areas (17.86%), which is associated with urbanization and confirmed findings in our previous studies. In Croatia, just the opposite was seen - the majority of infected people are concentrated in rural areas (11).

Data were analyzed according to age and sex of the patients in this period. Infected people were mainly elderly men. A total of 26 from 28 patients were men (92.86%) (Fig. 2). Distribution by sex of patients with leptospirosis in the country could be compared with similar studies from Korea, France, Croatia, Taiwan, Romania (5,11,13,16,17). Age distribution of patients is presented in Figure 3. Age groups of 40-49 years - 7/28 and 60-69 years - 7/28 were mainly affected (25%). This is probably related to increased outdoor activities outside professional activities. In Korea, a similar study was conducted and found that the most affected age group was those of adult men aged 60-69 years (59.6%) (13). There is a tendency to displacement of the age limit of the patients toward more people active in adulthood (4). Such data could be cited also from France, Romania and Taiwan, where affected people aged average 45 year (5,16,17).

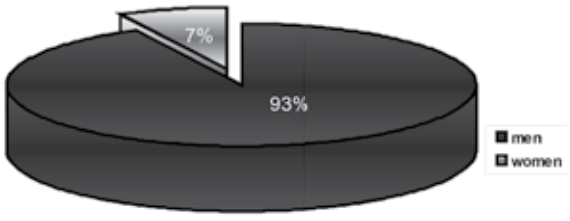

Fig. 2 Distribution by sex of patients with leptospirosis, 2010-2011.

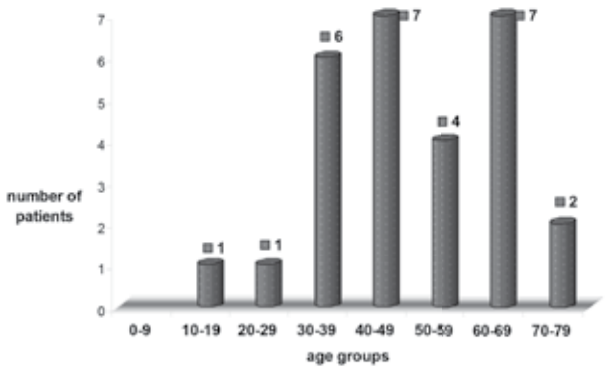

Fig.3 Distribution by age of leptospirosis patients, 2010-2011.

Seasonal distribution of leptospirosis was similar to the previous studies (3,4). Cases of leptospirosis were registered mainly in early summer and early autumn with two peaks in June and September (Fig. 4) - 5/28 (17.86%) of all serologically proven leptospirosis. A total of 21 cases of leptospirosis were registered between June and October, representing 75% of all reported cases. The lowest incidence was observed in December, January and May. Trend shift of the peak of infected people from August to September was observed in this two-year period. This is most likely due to the higher temperatures in the summer of 2011. These observations are consistent with data from Croatia and Taiwan (11,16) and previous studies in Bulgaria (4). In Korea, outbreaks of infection with leptospires have been proven more frequently in autumn (peak in September) (13).

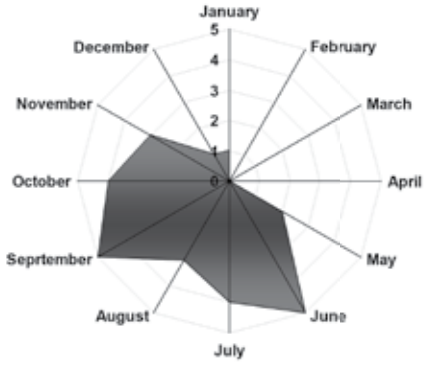

Fig. 4 Seasonal distribution of leptospirosis cases, 2010-2011.

All patients were infected in the territory of Bulgaria. The exception was a man working periodically in Greece under extremely unhygienic conditions in the presence of rodents.

We had information on risk factors in 19 of the cases with leptospirosis in this two-year period. Analysis of epidemiological data showed that infection was acquired mainly: 1) through recreational activities, mainly fishing - 9/19 (47.37%), 2) by activities associated with profession - 7/19 (36.84%) and 3) by other factors - 3/19 (15.79%). Two types of activity favoured effectiveness of leptospira transmission and they were associated with livestock and fishing, responsible for 57.89% of all cases of leptospirosis in this study period (Table 2). Epidemiological study done in 2011 in Korea found increased incidence of leptospirosis in people infected outside professional activities (outdoor activities) (13).

Only three patients (15.79%) were exposed to more than one risk factor, namely livestock, farming, presence of rodents in workplaces and homes, eating food and drinking water contaminated with excreta from rodents. The rodents in the houses were the major risk factor for infection with leptospirosis 15/28 - (10.71%) at home.

Possible sources of infection are shown in table 2. In 4 out of 19 patients, the source of infection was direct contact with domestic animals, mainly pigs (21.05%) and with sheep and goats (5.26%). Three patients (15.79%) were infected while fishing in the river Iskar.

Bathing in the river Danube was mentioned in one case (5.26%) as a risk factor and in three of them (15.79%) infection was associated with wading in sewage. Retrospective study was conducted in Serbia, in which stagnant waters, wetlands and lakes in areas with fish were indicated as a source of infection. The percentage of infected with letpospirosis was very high in these regions (21). Another risk factor was improper food storage and possibility for contamination with excreta of rodents (10.53%). Factors related to profession - working in slaughterhouses (5.26%) also had regard to transmission of infection.

Table 2. Possible sources of infection and mode of transmission of cases of leptospirosis in Bulgaria for 2010-2011г.

Mode of transmission	Frequency	%	Sources of infection	Frequency	%
Activity			Contact with:		
I. Related profession			1. Rats	7	36,84%
1. Livestock production	4	21,05%	2. Swine	4	21,05%
2. Work in carpentry workshop	1	5,26%	3.Sheep and goats		
3. Securing the farm	1	5,26%		1	5,26%
4. Working in a slaughterhous	1	5,26%	4. Feed from stores	2	10,53%
Total	7		5. Sewage	3	15,79%
II. Activities outside the profession					
1. Swimming in lakes and open reservoirs	2	10,53%	6. Unknown source	2	10,53%
2. Fishing	7	36,84%	Total	**19**	
Total	**9**				
III. Additional factors					
1. Contaminated food	2	10,53%			
2. Contaminated water	1	5,26%			
Total	**3**				

Data analysis showed downward trend of leptospirosis associated with occupation and rise of those associated with recreational activities in the two-year research period. These data differ from the previous studies done in our country (4).

Socio-economic structure of the patients was also analysed in the present study (Fig. 5). Workers were mainly infected - 8/19 (42.10%), including workers in the carpentry workshop and slaughterhouses, guard of farm. Pensioners were less affected 4/19 (21.05%), mainly those dealing with livestock production. Percentage of unemployed was slightly higher - 5/19 (26.32%) compared with the period 2005-2009 (12%) (4), while that of infected workers had decreased (48%). This is most probably due to the economic crisis in our country and reduced employment of people. In the similar survey conducted in Korea in 2011, significantly higher percentage of disease in farmers - 52.2% and lower in the unemployed - 14% and in the serviceman - 0.7% were found (13) .

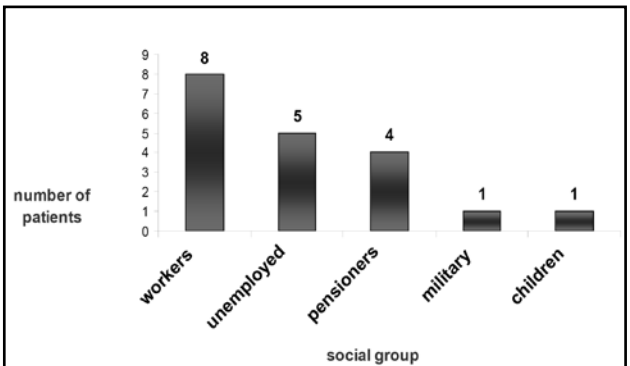

Fig. 5 Socio-economic structure of leptospirosis cases, 2010-2011.

Clinical data

The clinical spectrum of symptoms was analyzed in 19 patients with leptospirosis (67.86%), respectively 11 patients in 2010 (39.29%) and 8 patients in 2011 (28.57%). A lot of combinations of symptoms were observed. Data analysis showed that 48.21% of these patients suffer from severe disease (Weil's disease), probably because the majority of benign forms remain undiagnosed. Fever and jaundice were the most frequently reported symptoms in 18 (94.74%) and in 14 (73.37%) patients, respectively. Myalgia and splenomegaly were observed in 13 patients (68.42%). Myalgia was reported as the leading symptom of the disease in patients in our previous studies. The percentage of patients with jaundice was significantly lower - 39.47% (4). The disease was often accompanied by nausea and oliguria (57.89%), vomiting and abdominal pain in 9/19 (47.37%). The percentage of patients with headache was increased - 7/19 (36.84%) and in those with conjunctivitis also - 8/19 (42.11%) compared with previous studies (3,4,9) (фиг.6). The leading symptoms in the clinical spectrum of leptospirosis in many countries of Europe have been cited as myalgia, headache, gastrointestinal complaints (5,23).

Acute renal failure, diagnosed in 1/19 (5.26%) patients, was the main cause for fatality, which was noted also in the previous studies (4,9). In contrast with findings from many European countries, in Malaysia, the cause of death more frequently is severe pulmonary hemorrhagic form of the infection (15). In Romania, for example, six fatal cases have been recorded and the cause of death was liver and renal failure (17).

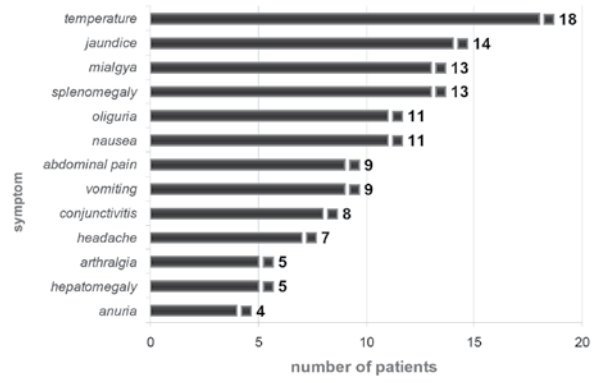

Fig.6 Clinical spectrum of the symptoms in the infected with leptospirosis for the period 2010-2011.

Cases of leptospirosis were classified into seven main clinical forms (Table 3). Hepatorenal syndrome and toxic infectious syndrome, alone or in combination with other symptoms, accounted for 57.90% of all cases, which coincides with studies done previously in our country (4).

Table 3. Clinical forms identified in 19 patients with leptospirosis from 28 serologically confirmed cases in the period 2010-2011.

Clinical forms	Number of patients	%
1. Hepatorenal syndrome	6	31,58%
2. Toxico infectious syndrome	5	26,32%
3. Flu-like form	3	15,79%
4. Renal form	2	10,53%
5. Conjunctivitis	1	5,26%
6. Hepatitis	1	5,26%
7. Hemorrhagic syndrome	1	5,26%

In 15.79% of cases leptospirosis appeared as flu-like infection, not associated with jaundice. Renal form was registered only in 10.53% of patients. It is necessary to test for leptospirosis patients with hemorrhagic fever with renal syndrome, especially if there are epidemiological data on the mode of infection (10,19).

Serological data

Serologically confirmed cases of leptospirosis by microagglutination test in the period 2010-2011 are shown in Figure 7.

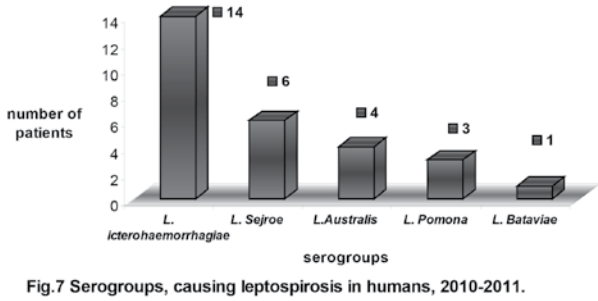

Fig.7 Serogroups, causing leptospirosis in humans, 2010-2011.

Antibodies against more than one serovar (coagglutination) were detected in the serum samples of 10 patients (35.71%). Coagglutination with one serovar was detected in 8 (28.57%) and with two serovars in two of the patients (7.14%). Coagglutination was apparent early in the disease, which is explained by the fact that Leptospira serovars are vague to distinguish and could not be identified precisely (23). The analysis of serological data showed that serovars causing leptospirosis belonged to five serogroups. Leading serogroup was *Leptospira icterohaemorrhagiae* 14/28 (50%) and titre of antibodies against this serogroup ranged from 1:400 to 1: 12 800 (Table 4). Infection was confirmed in early disease in 14.29% (4/28) of the patients with antibody titre 1:400, and in later stage - only in 1 patient with antibody titre 1:12 800. The percentage of infections caused by this serogroup was reduced in the present study, whereas in previous studies - in the period 2005-2009 it was 66.07% (4). Application of antibiotic therapy early in the course of infection resulted in low titer of the specific antibodies. Antibiotic treatment in the course of infection is crucial for the outcome of the disease, given that Weil's disease is severe and affects many organs (8). Serogroup *L.icterohaemorrhagiae* was reported as leading serogroup in the etiological structure of leptospirosis in studies from other European countries (Serbia, Turkey, Romania) (8,17,21). Significant change in the etiological structure of leptospirosis was established in this two-year period. Serogroup *Sejroe* was the second most frequently detected serigroup, found in 21.43% of our patients (6/28). Role of this serogroup was significantly increased compared with the previous studies (8.93%) (1,9,3,4) (Fig. 8). The dynamics of the antibodies against serogroup *Sejroe* ranged from 1:3200 to 1:12800. This is most likely due to a slight development of leptospirosis and misdiagnosis of the infection in the early stage by physicians (Table 4).

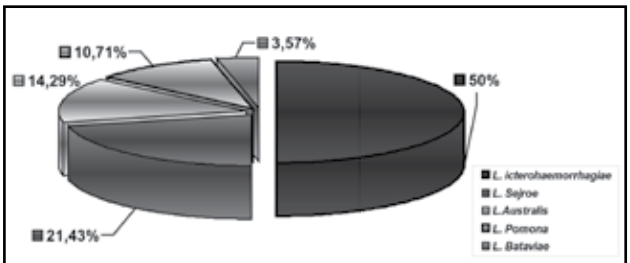

Fig 8. Serogroups causing leptospircsis during the period 2010-2011.

Serogroup *Australis* (serovar *Bratislava*) was found only in 1.79% of the patients in the previous seroepidemiological studies (3,4,9), while in the present study, this serogroup was demonstrated in 14.29%, thus occupying the third place of the most frequently detected leptospira serogroups (Fig. 8). Antibodies against serogroup *Australis* in low titers 1:400 were detected in three patients (Table 4). The significant role of *L. Australis* has been demonstrated in other studies, such in Serbia, where together with *L. ictero-*

Table 4. Dynamics of antibody titres in different serogroups causing leptospirosis during the period 2010 - 2011.

Serogroup	*L. icterohaemorrhagiae*/ Number of patients	*L. Pomona*/ Number of patients	*L. sejroe*/ Number of patients	*L. Australis*/ Number of patients	*L. Bataviae*/ Number of patients
Titre of the antibodies					
1: 400	4	1		3	1
1: 800		1			
1: 1600	2			1	
1: 3200	4		1		
1: 6400	3		3		
1: 12 800	1		2		
1: 25 600		1			
Total	14	3	6	4	1

haemorrhagiae caused 72.72% of leptospirosis cases (21). In Croatia, this serogroup has demonstrated its leadership with serogroups *Saxkoebing* and *Grippotyphosa* (22).

In Bulgaria in the period 1986-1989, the high prevalence of serogroup *Pomona* was registered. This serogroup was reported in all leptospirosis cases followed by serogroup *Icterohaemorrhagiae* (1). *L. Pomona* has held first place in the etiological structure of leptospirosis (4.22% of cases) in 1999 in the region of Plovdiv (20). Significant change was observed in etiological structure of leptospirosis during the period 1989-2001 (9) when the leading serogroup was *L icterohaemorrhagiae*. Progressive reduction of the proportion of serogroup *Pomona* was observed in this study and this serogroup was ranked fourth (10.71%) (Fig. 8). Antibodies against *L. pomona* were found in lower number of patients, which could be explained by vaccination of pigs. High antibody titre 1: 25 600 was proven only in one patient (Table 4).

Proportion of serogroup *Bataviae* was very low - 3.37%, indicating that the circulation of this serovar was low and the data correlate with the previous studies (1,3,9).

Data analysis showed that jaundice was observed in 5 (35.71%) of 14 infections caused by serogroup Icterohaemorrhagiae. In other two (14.29%) disturbances in kidney and liver, abdominal pain, myalgia, and only in one patient (7.14%) - arthralgia were reported. In the rest of the patients these symptoms were not registered. In six clinical cases, caused by serogroup *Sejroe,* jaundice was observed. Correlation between clinical forms and other infecting serogroups was not statistically significant because the incidence of infections caused by them was very low.

Conclusion

Analyzing the results from this two-year period, we can conclude that leptospirosis in Bulgaria is an infection with limited distribution, but the risk of leptospirosis should not be underestimated. Fatal cases continue to appear, although there is a tendency to reduce the incidence of leptospirosis. Variety of clinical manifestations was observed with prevalence of clinical icteric form of the disease. Serological findings revealed predominance of serogroup *Icterohaemorrhagiae* over serogroups *Pomona* and *Sejroe*. Neurological, eye, respiratory and cardiac symptoms occur less frequently, but should be kept in mind in the differential diagnosis of the disease. Leptospirosis is still difficult for recognition by clinicians. Diagnosis is difficult especially in cases with flu-like symptoms, accompanied by fever, headache and intense myalgia associated with elevated liver enzymes, proteinuria and thrombocytopenia (6). The prevalence of leptospirosis is largely dependent on the general hygiene measures and control of rodents.

Furthermore, some climatic and socio-economic phenomena observed in recent decades can increase the a range of favourable conditions for transmission of infection from animals to humans. This requires surveillance of the status of this infection, better communication with clinicians and timely laboratory diagnosis. This study provides useful information as a starting point for further research on leptospirosis in Bulgaria and the problems associated with this infection in the context of public health protection.

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EVALUATION OF A NEW MOLECULAR TEST FOR THE IDENTIFICATION OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATES

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ABSTRACT

***Mycobacterium tuberculosis* is a clonal organism, once a single nucleotide polymorphism (SNP) is introduced into the genome it will be passed on to the next generation. Drug resistance in *M. tuberculosis* strains is to a great extent caused by the presence of SNPs in genes conferring resistance to tuberculostatics. SNPs can be used as discriminative markers for drug resistance as well as to generate robust phylogenetic trees. SNP based species identification is also possible. A new molecular method was recently developed for the analysis of drug resistance and strain typing of *M. tuberculosis* strains, based on the detection of informative genetic markers such as deletions or single nucleotide polymorphisms (SNPs). The method evaluated in this study, multiplex ligation-dependent probe amplification (MLPA) [Schouten, 2002] contains three essential steps, a hybridization step followed by a ligation step and an amplification step. The final read-out is performed on the MAGPIX device using the Luminex xTAG bead assay. The multiplexing capacity of the assay allows the simultaneous analysis of up to 47 different molecular markers within one sample and therefore provides complex information from a single assay. 13 markers provided information about resistance to first- and second line drugs based on the detection of specific mutations in the bacterial genome. We have analysed DNA isolated from bacterial culture by MLPA and compared the results to the current molecular method for the detection of drug resistance in TB strains, the MTBDR*plus* and MTBDR*sl* assays (HAIN Lifesciences).**

Key words: MLVA, tuberculosis, drug resistance, MDR, Luminex

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INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is one of the most devastating bacterial diseases. Current identification of drug resistance in MTB and proper strain identification is time consuming as it requires combinations of several techniques such as microscopy, bacterial culture and molecular methods. This delays the change from empirical to accurate personalized treatment and limits prevention of transmission.

A new molecular method was recently developed for the analysis of drug resistance and strain typing of MTB strains [Bergval, 2008], based on the detection of informative genetic markers such as deletions or single nucleotide polymorphisms (SNPs). MTB is a clonal organism, once a SNP is introduced into the genome it will be passed on to the next generation. Drug resistance in TB strains is to a great extent caused by the presence of SNPs in genes conferring resistance to specific antibiotics. Therefore SNPs can be used as discriminative markers for drug resistance as well as to generate robust phylogenetic trees. SNP based species identification is also possible.

The method evaluated in this study, multiplex ligation-dependent probe amplification (MLPA) [Schouten, 2002] (Fig. 1) contains three essential steps, a hybridization step followed by a ligation step and an amplification step. The final read-out is performed on the MAGPIX device using the Luminex xTAG bead assay. The multiplexing capacity of the assay allows the simultaneous analysis of up to 47 different molecular markers within one sample and therefore provides complex information from a single assay after only a short period of time and with easy hands-on performance.

In this study we have established the MLPA in the laboratory. We have analysed DNA isolated from bacterial culture by MLPA and compared the results to the current molecular method for the detection of drug resistance in TB strains, the MTBDR*plus* and MTBDR*sl* assays (HAIN Lifesciences), routinely performed in the TB reference laboratory in Sofia [Bachiyska 2010].

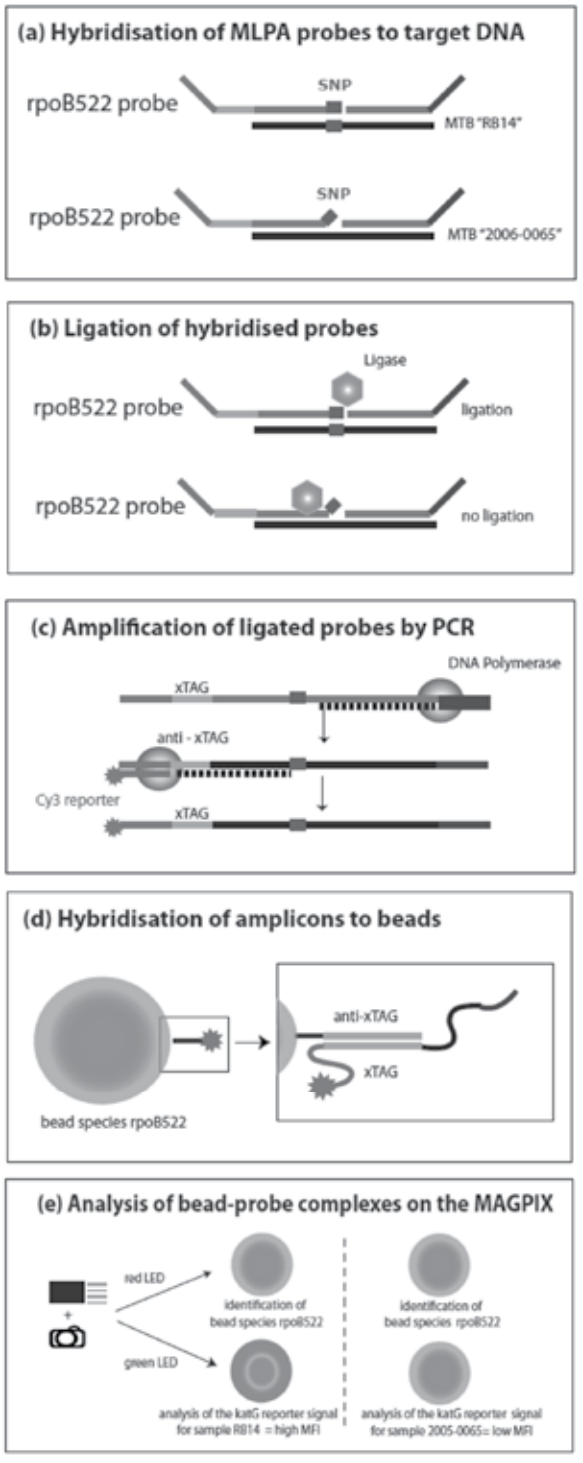

Fig. 1. Overview of the Multiplex Ligation-dependent Probe Amplification (MLPA) assay.
(a) Sequence-specific probes hybridise to the target DNA over night. Each probe consists of a target-specific sequence, xTAG, forward and reverse primer sequences. The rpoB522 probes perfectly match the sequence of the strain RB14 but not strain 2005-0065, indicating that the SNP is only present in strain RB14. **(b)** Only probes that are 100% complementary to their target sequence hybridise and are ligated by a highly specific ligase. **(c)** Ligated probes are amplified in a PCR reaction. The reverse primer binds first and amplifies the probes before the labelled forward primer can bind to its complementary sequence generating a probe labelled with a reporter dye. **(d)** Amplified probes hybridise to their specific bead. Each bead species has a unique xTAG sequence that is complementary to the probe xTAG and is present in multiple copies on the bead surface. **(e)** Analysis of the bead-probe complexes takes place on the MAGPIX device. A red light emitting diode (LED) and a CCD camera identify first the individual bead species before green LEDs excite the reporter molecules on the probes. The signal is translated into Median Fluorescence Intensity (MFI). The MFI is proportional to the amount of probes bound to the bead.

OBJECTIVE

The purpose of this study was to assess the feasibility of the recently developed MLPA assay in a setting which is endemic for multidrug-resistant TB (MDR–TB) and where the information gained by the assay is thought to be of added value. Therefore we analysed a selection of MDR-strains with the recently developed MLPA assay at the National Institute of Infectious and Parasitic Diseases in Sofia.
The demonstration study performed at the National Institute of Infectious and Parasitic Diseases in Sofia is part of a joint project between The Netherlands, France, Georgia and Bulgaria. The leading partner is the Royal Tropical Institute in Amsterdam, The Netherlands.

METHODS

MLPA was performed on nine selected culture isolates from patients with MDR-TB collected between 2009 and 2011 in Bulgaria. The clinical isolates have been defined as MDR-TB based on phenotypic drug resistance testing (DST) using the nitrate-reductase assay [Panaiotov and Kantardjiev, 2002] and Bactec 460TB or Bactec MIGIT 960 system for first line and second line drugs. DNA was extracted from MGIT cultures using buffers containing cetyltrimethylammonium bromide (CTAB). PCR positivity and genotype of the *M. tuberculosis* strains was evaluated as previously described [Panaiotov, 2010 and Panaiotov, 2004]. For comparison between the MLPA and MTBDR*sl*/MTBDR*plus*, the isolates were analysed by the two reverse hybridisation assays MTBDR*plus* detecting resistance to first line drugs and MTBDR*sl* detecting resistance to second line drugs [Hillemann, 2007; Kiet, 2010]. The principle of the MLPA assay was previously described [Bergval, 2008] and is briefly outlined in Figure 1. The MLPA assay consists of a hybridisation, ligation and amplification step followed by analysis of the amplicons using a bead-based assay on the MAGPIX device (Luminex Corporation). In addition to the nine samples, a negative control, a contamination control and an assay control are analysed as extra samples. No DNA template or probe mix is added to the negative control thereby sensing for contamination with PCR products. The contamination control contains DNA from a species unrelated to mycobacteria e.g. *Staphylococcus aureus* (*S. aureus*). Contamination with MTB DNA will be detected with this control. The assay control contains DNA from a MTB strain that has been previously analysed by MLPA and quality controls the assay.
For this study, the results of 13 informative markers for drug resistance were analysed. These markers represent the most prevalent mutations revealed from literature conferring resistance to rifampicin, isoniazid, ethambutol, streptomycin, amikacin, kanamycin, capreomycin and fluoroquinolones. An additional marker detects an MTB-specific sequence within the 16S rRNA locus. Only strains that are positive for this marker are further analysed. Markers for drug resistance included in the assay are listed in Table 1.

Table 1. Drug resistance markers included in the MLPA assay

FLD	**Ethambutol**	embB-306 (MLPA probes detect wild type MLPA sequence)
	Isoniazid	katG-S315T (high level INH) , inhA-C(-)15T (low level INH)
	Rifampicin	rpoB-V176F, rpoB-S522L, rpoB-H526D, rpoB-H526Y, rpoB-S531L
	Streptomycin	rpsl-43 (MLPA marker detects wt sequence)
SLD	**Aminoglycosides Macrocyclic peptides**	rrs-1401 (AMK/KAN/CAP) (MLPA probes detect wild type sequence) rrs-1402 (nucleotide change C to G) (CAP)
	Fluoroquinolones	gyrA-A90V, gyrA-D94G

Results

The MLPA technique contains a hybridisation, ligation and amplification step. The successful generation of amplicons can be checked by agarose gel electrophoresis prior to analysis. Products of about 150 bp were amplified in all nine analysed strains (Fig. 2, lane 4,6-13). No products are present in the negative control and products of smaller size are present in the contamination control, which is possibly indicative of unspecific amplification products (Fig. 2, lane 1-3). Following the amplification step, the amplicons are analysed using the bead-based assay. All nine strains analysed were positive for the 16S rRNA marker indicating a genetic background of MTB (data not shown).

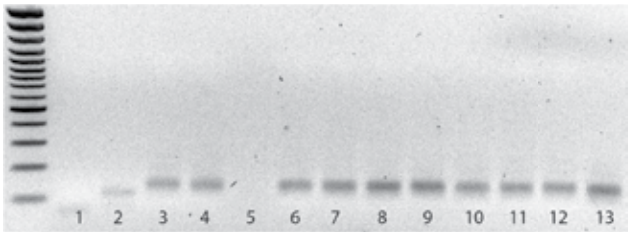

Fig. 2. PCR products obtained after the PCR step of the MLPA assay.
Probes that could bind to their target sequence were amplified and are visible as a PCR product of about 150 nt. Lane 1, negative control. Lane 2, control with *S .aureus* DNA. Lane 3, assay control with DNA from a well characterised MTB strain. Lanes 4,6-13, samples. Lane 5, empty. 100bp ladder.

The MLPA was positive for markers indicating resistance to rifampicin and isoniazid for all nine strains analysed (Table 2). This finding is consistent with the results from the MTBDR*plus* assay and the DST.
DST for second line drugs and MLPA was performed for all nine strains but only three strains (32-10, 91-10, 96-10) were investigated for second line drugs using MTBDR*sl*.
The five strains that were streptomycin resistant on the basis of DST (52-20, 72-10, 90-10, 91-10, 101-10) were determined as streptomycin sensitive by MLPA. A single marker is included in the MLPA assay for the detection of streptomycin resistance. It is possible that the mutation conferring resistance to streptomycin in the five strains is different to the one that can be detected with the marker included in the MLPA assay. Resistance to streptomycin cannot be detected by the MTBDR*sl* since no specific markers are included.
Phenotypic resistance to ethambutol was detected in five strains (32-10, 9-10, 91-10, 96-10, 101-10). Mutations in the conferring resistance to ethambutol were detected in all three strains analysed by MTBDR*sl* and in five strains analysed by MLPA (32-10, 52-10, 72-10, 91-10, 101-10). The results of two strains (32-10, 96-10) were consistent between MLPA, MTBDR*sl* and DST. Mutations conferring resistance to ethambutol were also detected by MLPA in two strains that were phenotypically ethambutol susceptible (52-10, 72-10). Performance of DST for ethambutol is difficult and might in some cases lead to false negative results. Further, mutations indicating resistance to ethambutol could not be detected by MLPA in one phenotypically ethambutol resistant strain that was also positive for the marker indicating ethambutol resistance by MTBDR*sl*. It is likely that the mutation conferring resistance to ethambutol in the clinical isolates differs from the one which can be detected by the marker included in the MLPA assay.

Table 2. Comparison of identified drug resistance of phenotypically MDR TB revealed by different molecular methods.

	Molecular test			Phenotypic test
Sample	**MLPA[a]**	**MTBDR*plus*[b]**	**MTBDR*sl*[b]**	**DST[c]**
30-10	INH, RIF	INH, RIF	ND	INH, RIF
32-10	INH, RIF, EMB, FLQ	INH, RIF	EMB, FLQ	INH, RIF, EMB, FLQ
52-10	INH, RIF, EMB, FLQ	INH, RIF	ND	INH, RIF, STR, FLQ
72-10	INH, RIF, EMB	ND	ND	INH, RIF, STR
90-10	INH, RIF	ND	ND	INH, RIF, STR, EMB
91-10	INH, RIF, EMB, AMK/KM	INH, RIF	EMB, FLQ, AMK/KAN, CAP	INH,RIF, STR, EMB, FLQ,AMK, KAN, CAP
96-10	INH, RIF, FLQ	INH, RIF	EMB, FLQ	INH, RIF, EMB, FLQ
97-10	INH, RIF	ND	ND	INH, RIF
101-10	INH, RIF, EMB	ND	ND	INH, RIF, STR, EMB

[a] MLPA can detect resistance to INH, RIF, STR, EMB, FLQ, AMK, KM, CAP
[b] MTBDR*plus* and MTBDR*sl* detect resistance to INH, RIF, EMB, FLQ, AMK, KM, CAP
[c] Drug Susceptibility Test (DST) was performed for INH, RIF, STR, EMB, FLQ, AMK, KAN and CAP
INH, isoniazid; FLQ, fluoroquinolones; EMB, ethambutol; RIF, rifampicin; AMK, amikacin; KM, kanamycin; CAP, capreomycin.

Four strains (32-10, 52-10, 91-10, 96-10) showed resistance to fluoroquinolones on the basis of DST. Resistance to fluoroquinolones was detected by the MTBDR*sl* assay in all three analysed strains (32-10, 91-10, 96-10) whereas the MLPA detected resistance to fluoroquinolones in three strains (32-10, 52-10, 96-10), two of them consistent with the strains analysed with the MTBDR*sl* assay. Although the two most prevalent markers for the detection of fluoroquinolone resistance are included in the MLPA assay it is possible that other mutations conferring resistance to fluoroquinolones are present which could not be detected by MLPA but with the MTBDR*sl* assay.
Resistance to amikacin/kanamycin/capreomycin was identified in one strain, 91-10, by all three methods.

DISCUSSION AND CONCLUSIONS

We set out to asses the performance of a new molecular method, MLPA, by comparing the results obtained by MLPA in the national TB reference lab in Sofia, Bulgaria to previously performed DST and MTBDR*sl*/*plus*. MLPA allows the detection of molecular markers that confer resistance to antimycobacterial drugs rifampicin, isoniazid, ethambutol, streptomycin, amikacin, kanamycin, capreomycin and fluoroquinolones. Results were obtained after a turn around time of 1 ½ days using DNA isolated from liquid cultures.
In the present study, 13 markers provided information about resistance to first- and second line drugs based on the detection of specific mutations in the bacterial genome. The MLPA assay identified mutations conferring resistance to rifampicin and isoniazid for all isolates and thereby confirmed the MDR-TB status of the clinical isolates.
For the second line drugs discrepancies were identified between MLPA and the MTBDR*sl* assay. The current MLPA assay is lacking some probes that are present in the MTBDR*sl* assay. Therefore mutations present in the bacterial genomes could only be detected by MTBDR*sl*. In case of the non-detected mutation in the embB306 locus, it was previously reported that not all possible codon changes can be detected by the MLPA embB probe [Bergval, 2008]. The strains that were sensitive for specific antibiotics but by interpretation of the MLPA results were resistant, it is most likely that DST led to false negatives due to the difficult performance of DST for drugs such as ethambutol [Da Silva, 2011].
Compared to the MTBDRsl/plus that requires two individual tests to obtain the same results, MLPA revealed the drug resistance in a single assay.
In addition to a shorter turn-around time that currently available methods can offer to obtain the same information, MLPA enables simultaneous detection of 13 markers per sample in a single assay which can be extended to 50 markers per sample. Therefore, with a single assay more information, useful for appropriate treatment or control measures, can be obtained than is currently feasible. Another benefit of MLPA over for instance reverse hybridisation assays such as the MTBDR*plus* and MTBDR*sl* is that the choice of genetic markers to be included is completely flexible and can be made dependent on the information required. Inclusion of additional drug resistance markers is of interest for diagnostic purposes whereas addition of genotypic markers would provide information on the species level. Country-specific marker panels could contain markers for the most prevalent genotypes circulating in e.g. Bulgaria, Eastern Europe or Asia presuming that indicative markers are available from the literature or can be identified by genome sequencing of representative strains. Another variation of the MLPA could be the addition of markers that identify the most clinically relevant non-tuberculous mycobacteria (NTM) [Ngan, 2011]. In summary, the MLPA is the only molecular test currently available that reveals complex information from a clinical isolate in only a single assay within 1 ½ days turn around time.
The MAGPIX device used for the bead-based read out of the MLPA is a robust and open platform that can be used for DNA assays as well as multiplexed immunological assays. These can be purchased as a kit or may also be partially developed by the researchers themselves.
We feel that the combination of MLPA with Luminex technology is promising and can be of great added value in the fight against (MDR-)TB. Initial results obtained in the reference laboratory in Sofia are promising and lessons learned from this feasibility study are a good starting point to further optimise the method.

ACKNOWLEDGMENTS

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VACCINATION AGAINST HPV IN THE BULGARIAN ARMY MILITARY FEMALE PERSONNEL

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ABSTRACT

On the initiative of representatives of the Ministry of Defence and under the guidance of experts from the Military Medical Academy in May 2009 a program to prevent the spread of human papillomavirus (HPV) among military female personnel has been started. The program covers voluntary female military personnel and includes clinical examination by a gynecologist with sampling for direct microscopy and some of them for Real-Time Polymerase Chain Reaction for HPV serotypes 16 and 18.

Women with negative clinical and laboratory findings were vaccinated immediately. Those with abnormal examinations underwent treatment and after re-examination their participation in the program was refined in each case. The vaccines provided were Cervarix, production of GlaxoSmithKline and Silgard, production of Merck Sharp&Dohme. The program includes five stages: 1) Preparation, 2) Information lectures, 3) Clinical examination, 4) Vaccination and 5) Clinical and laboratory monitoring of vaccinated individuals. For a period of two and a half years - from 2009 until the May 2012 SCMEH - MMA and Preventive Medicine Teams in Pleven, Varna, Sliven and Plovdiv have vaccinated with full course of three doses 480 women (of 631 examinated) - 396 with Cervarix and 84 with Silgard. The age of the women was 18 to 30 (on the average 27.4 years) with 472 (of 607 tested) and 30 to 40 (on the average 36, 8 years) in 8 of them (of 24 tested). The immunization course was interrupted in 25-female militaries due to pregnancy in 21 women, 1 because of breast cystosis, 1 because a discharge from the armed forces and 2 had not appeared for personal reasons.

Key words: *program, vaccine, female military personnel, human papiloma virus*

INTRODUCTION:

The vaccination against the cervical cancer (CC) is a long term investment in the population health. Its success is not achievable without a broad vaccination covering. The vaccine protects girls from contracting oncogenic human papilloma viruses (HPV) types 16 and 18 (causing about 70% of all CC) and overall efficacy against development of severe precancerous condition (CIN+3) exceeds 90%, when the vaccine is applied before the start of sexual activity. The goal of these vaccines is to protect young girls and women from persisting infection (above 6-12 months) with oncogenic HPV, which is obligatory etiological factor for CC development. The risk of infection's advance to precancerous lesions and CC depends on the HPV type as 15 types are highly risky with prevalence of HPV types 16 и 18 (74-77%), followed by HPV 45, 31 and 33. These types HPV are responsible for 80% of all CC cases [5, 6, 7]. They are responsible for 97% of precancerous and cancerous cervical lesions among women in Bulgaria – result with profound importance while choosing vaccine as well as giving ground to predict significant effect of the future CC vaccine prophylaxis in Bulgaria. A morbidity reduction tendency is shown in all countries with programs for primary and secondary CC prophylaxis. The disease frequency in Bulgaria goes up steadily – the number of new cases during the last few years is 1100-1200 cases annually with 300-400 deaths. The vaccination is the only method for effective and long lasting CC primary prophylaxis [1]. The prevention through vaccination would impact on CC morbidity and mortality nationwide only if the immunization would be done on population principle i.e. application of vaccines against CC causing viruses becomes a part of the state health policy and the state covers the expenses. Regarding the included in the vaccines two main oncogenic HPV types – HPV 16 and 18 – both vaccines provide high level of protection as Cervarix contains two types (HPV16, HPV 18), and Silgard contains four types (HPV 16, HPV 18 plus low risk types HPV 6 and HPV 11, associated with acute condylomas, a.k.a. genital verucas)[2,3]. Both vaccines are constructed by virus-like particles using DNA technology (they are not infectious), possess very good safety profile and can be applied simultaneously with other vaccines (for example dTpa, recombinant hepatitis B) on different application sites. Application schemes consists of three doses set in six months (0, 1-th and 6-th month for Cervarix and 0, 2-nd and 6-th month for Silgard) [2,3]. Both licensed vaccines show cross efficacy against the rest oncogenic non-vaccinal HPV types. Silgard (MSD) provides cross protection to moderate and high-level displasia (CIN 2/3) associated with 10 basic non-vaccinal types in 23.00%, while with Cervarix the protection to the rest of 12 oncogenic non-vaccinal types is respectively 56.2% regarding CIN2+ and 91.4% regarding CIN3+ [1,2,8]. Silgard (MSD) provides protection against low-risk types HPV 6 and HPV 11, causing cervical epithetial benign or low level lesions and ano-genital acute condylomas (verucas) [5]. Considering specificity and the lower age of the Bulgarian girls' sexual activity the experts recommend mass immunization at age of twelve. A mass, not a formal cervical screening under assumed rules and standards should be organized for all women (vaccinated and unvaccinated). There is no screening and vaccination program in place in Bulgaria at the moment. A program for HPV infection prevention amongst female personnel within Bulgarian Army has started since the beginning of March 2009 under the initiative of the Ministry of Defense officials and under the Military Medical Academy representative supervision. It covers on voluntarily principle female soldiers at age between 18 and 30 years of age. The age group definition was made in accordance with

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the available epidemiological data for HPV infection distribution [4]. This is the first program in the country as well as amongst other armies.

GOAL:
The aim is to study prospectively the HPV distribution among sexually active female soldiers at the age between 18 and 39 years, who were willing to be vaccinated; to vaccinate the eligible ones with the market available HPV vaccines (bivalent, GSK or tetravalent, MSD).

MATERIALS AND METHODS:
The program includes five main steps:
1. Preparation;
2. Information interchange;
3. Screening of the personnel, willing to be vaccinated;
4. Immunization, and
5. Control of vaccinated women.

We present our experience in phases 1,2,3 and 4. We expect in 2013 part of vaccinated women to be tested for anti HPV 16 and 18 antibodies production (ELISA method with HPV G (HPV IgG), produced by DIA PRO, Italy on the 12th , 36th and 60th month as well as groups vaccinated with different vaccine to be compared and efficacy result to be evaluated. The vaccination immune response data will be presented after the program conclusion.

Women went through a voluntarily gynecological exam, cervical smear, RT-PCR for HPV 16 и 18. Women with normal cervical smear data and negative for HPV-DNA result were vaccinated either with bivalent or with tetravalent vaccine. Women with positive cervical smear result underwent the corresponding treatment. Those with normal cervical smear data and positive for HPV-DNA result were advised to take another HPV test six months later; they were vaccinated then, if the infection proved to be self-eradicated. All women consecutive testing (cervical smear and HPV) are scheduled to be done on 12 months period for five years. The vaccines will be evaluated in both groups (vaccinated with GlaxoSmithKline's bivalent HPV vaccine and Merck's tetravalent HPV vaccine. Breaches in vaccination and cases of CIN (I, II, III) up to five years after vaccination will be subject of evaluation. Cases with failed immunity development will be reported to the vaccine manufacturer as well as to the regulatory agency. The data will be analyzed in several time points: HPV infection epidemiological data during the pre-vaccination screening; age groups HPV clearance after all HPV positive women would completed step 2 (six months after the screening); and vaccine comparative efficacy on the 3rd and the 5th year.

RESULTS:
During the period between May and June 2009 physicians from the General and Oncological gynecology ward and Scientific-Applied Center for military epidemiology and hygiene gave seven lectures about CC prevention to military female personnel in Sofia, Plovdiv, Karlovo, Varna, Veliko Tarnovo, Pleven and Sliven. Approximately 1500 women were made familiar with the problem. 480 women received complete course of vaccination (three doses) – 396 were immunized with Cervarix and 84 with Silgard in a two and a half years period – from 2009 till May 2012. The age of 472 women were in the range 18-30 years (median 27,4 years) and the age of 8 was in the range 30-40 years (median – 36.8 years). Twenty five women interrupted the vaccination course – 21 women because of pregnancy, 1 woman because of breast fibrocystic alterations, 1 woman because of duty discharge and 2 women because of personal reasons.
472 out of 631 women (74.35%) were suitable for vaccination after the preliminary screening. In the age group 18-30 years of age from 607 tested women 472 (77.35%) were suitable for vaccination, while in the age group 30-40 years of age from 24 tested women their number was only 8 (33.33%). Minimal side effects after vaccination were reported: swelling (2.6%), itching (4.2%), and application site redness (7.9%).

DISCUSSION:
The proposed program casts light upon the adult sexually active women vaccination algorythm applicability and the vaccination possible effect upon the screening results. We have chosen the age group to cover sexually active women at age 18-30 years in order to evaluate their vaccination advantages. The age group 30-40 years covers the HPV infection second peak and helps their vaccination benefits' evaluation as well as the HPV positive test in persisting HPV infection significance comparison [8] between age groups 18-30 and 30-40 years. Our studies show positive HPV results apparent difference for both age groups which defines their inclusion in the program. Suitable for vaccination in the age group 18-30 years were 77.35% while in the group 30-40 years they were 33.33%. Our observations are confirmed by other authors [4].

Table 1. Immunized women number – complete course and unterrupted vaccination:

	Vaccine	Sofia	Varna	Plovdiv	Sliven	Pleven	Total
Vaccinated women	**Cervarix**	67	89	148	88	4	396
	Sigard	19	0	4	25	36	84
Interrupted vaccinations:							
Pregnancy after 2 doses		1	5	0	1	5	12
Pregnancy after 1 dose		0	0	1	1	7	9
Illness				1			1
Discharge		1					1
Personal reasons after 1 dose			2				2

We give exceptional importance to the information campaign amongst female army personnel. An acknowledgment with the CC prevention latest achievements, differences between the vaccines available, screening methods, the other European countries approaches and experiences form the basis for the good immunization covering. Women choose the vaccine because of informational lectures about the vaccines' immunogenicity and efficacy to prevent disease.

Regarding the post-vaccination immune response the Silgard vaccine includes aluminum-containing adjuvant while in the case of Cervarix an innovation adjuvant system AS04 with proven immune response invigorating effect has been used [2,3,7}. During a both vaccines immune response direct comparative study in women of age between 18-45 years Cervarix (GSK) showed several times higher anti-HPV16 antibody (3,7 times) titters and anti-HPV18 antibody (7,3 times) titters compared to Silgard/Gardasil (MSD) in age subgroup 18-26 years, as the difference remains in other age subgroups [6]. The both vaccines studies data showed that in girls before entering sexual activity Silgard gives 42.7% protection against CIN2/3 regardless of the causative agent, while Cervarix provides 93.2% efficacy in CIN3+ and 64.9% against CIN2+. The vaccines' protective efficacy could be measured by their ability to lower local therapeutic procedures in the future. Cervarix ensures 70.2% reduction of all local therapeutic procedures while Silgard gives 41.99% reduction [2,3,5].

The higher number vaccinated by Cervarix women probably is due to the Cervarix lower price. Our study data show that both HPV vaccines has good tolerance and safety as the most common side effects are predominantly local (on the application site) and transient.

Regarding the will to vaccinate women in age group 30-40 wears are at least twice more motivated and convinced in the benefits than the women in the age group 18-30 years.

CONCLUSION:

The agencies, patients, medical specialists and mass media joint efforts will lead to positive changes in the disease negative statistic. Military HPV prevention program not only has made female army personnel to feel special and taken care of, but also made popular the latest medical achievements. As a model and experience the program certainly has its place for the vaccines 2012 reimbursement implementation in Bulgaria.

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CLINICAL, EPIDEMIOLOGICAL AND LABORATORY CHARACTERISTICS OF HEPATITIS A OUTBREAK IN THE VILLAGE OF ISKRA (PLOVDIV REGION), JANUARY - MARCH 2012

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Abstract:
Goal: To analyze the clinical, epidemiological and laboratory characteristics of the patients from Iskra village with VHA treated in the Clinic for Infectious Diseases during the period January-March 2012.
Materials/methods: For the period considered a total of 225 VHA patients were hospitalized. Of these, 125 were associated with the epidemic outbreak in the village of Iskra. The diagnosis was verified by anti HAV IgM. Routine laboratory methods, clinical observations and epidemiological analysis were applied.
Results: Males were 70 (62.9%) and women - 55 (37.1%). Thirty-three of the study subjests are not permanent residents in the locality, but have an epidemiological link. The index of affected contacts in the existing outbreaks of infection and the risk of occurrence of the disease during outbreaks have been assessed. The average hospital stay was 10 days. The disease was manifested in mild and moderate form. Cholestasis component was registered in 3 of the cases. So far no relapses have been registered.
Conclusion: The studied epidemic outbreak is another proof of the endemic-epidemic spread of VHA in Bulgaria. Therefore preventive measures and disease control need to be improved.
Key words: *viral hepatitis A (VHA) epidemic outbreak, index of affected contacts*

Introduction:

Hepatitis A is an acute infectious disease disseminated worldwide. It occurs either as sporadic cases or as epidemic outbreaks which sometimes involve many people (1, 2). The disease is observed with different frequency in the separate countries. A conclusion for the endemicity of hepatitis A is drawn based on the incidence rate and seroprevalence in any country (3, 4). Introduction of hepatitis A vaccine in some countries as routine practice has reduced considerably the morbidity and the control of the disease. So far the vaccine is only recommended in Bulgaria (5). The **aim** of the study is to analyze the clinical, epidemiological and laboratory features of the patients from Iskra village with hepatitis A who were treated in the Clinic for Infectious Diseases during the period January-March 2012.

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MATERIALS AND METHODS:

For the period considered (01.01. – 31.03.2012) a total of 225 VHA patients were hospitalized. Of these, 125 were associated with the epidemic outbreak in the village of Iskra. The diagnosis was verified by anti HAV IgM. Routine laboratory methods, clinical observations and epidemiological analysis were applied. The complex method of epidemiological research has been used, including epidemiological history, timely isolation of the patients, observation and laboratory testing of the contacts in the source of infection, disinfection of the contaminated surfaces, dispensary observation of the discharched.

RESULTS AND DISCUSSION:

During the study period a total of 225 patients with hepatitis A were hospitalized (Table 1). Most of the cases were from Parvomay municipality (125 – 55.5%). In one of the municipality villages (Iskra) an epidemic outbreak burst out affecting 92 inhabitants of the village and 33 people who were temporarily residing there during the outbreak.

Table 1: Distribution of the patients with hepatitis A by communities

Communities	Plovdiv	Maritza	Rodopi	Stambolijski	Brezovo	Kaloianovo	Saedinenie	Sadovo	Rakovski	Karlovo	Hissar	Parvomai	Asenobgrad	TOTAL
N of cases with cumulation	48	16	8	21	1	5	1	4	3	13	1	100	4	225

Analysis of the dynamics (Chart 1) shows that the peak of the outbreak was from 03.02.2012 until 09.02.2012. During this period, 65 (52%) of all patients, registered during the epidemic outbreak, became infected.
Most affected among patients with hepatitis A in Iskra were in the age groups 5-9, 10-14, 15-19, 35-39 and 40-44 years. (Table 2). Analysis of age distribution found an increase in the relative share of patients with VHA from the older-age groups (notably from 35 to 44 years) compared with previous research.
The epidemiological survey carried out in Iskra during the epidemic outbreak revealed frequent failures in the plumbing system and in the water supply regime. This makes it difficult to observe good personal hygiene and instigates villagers to use water with unproven qualities from their own wells or from local water sources.

Diagram 1:
Dynamic of the registered cases with hepatitis A in v. Iskr

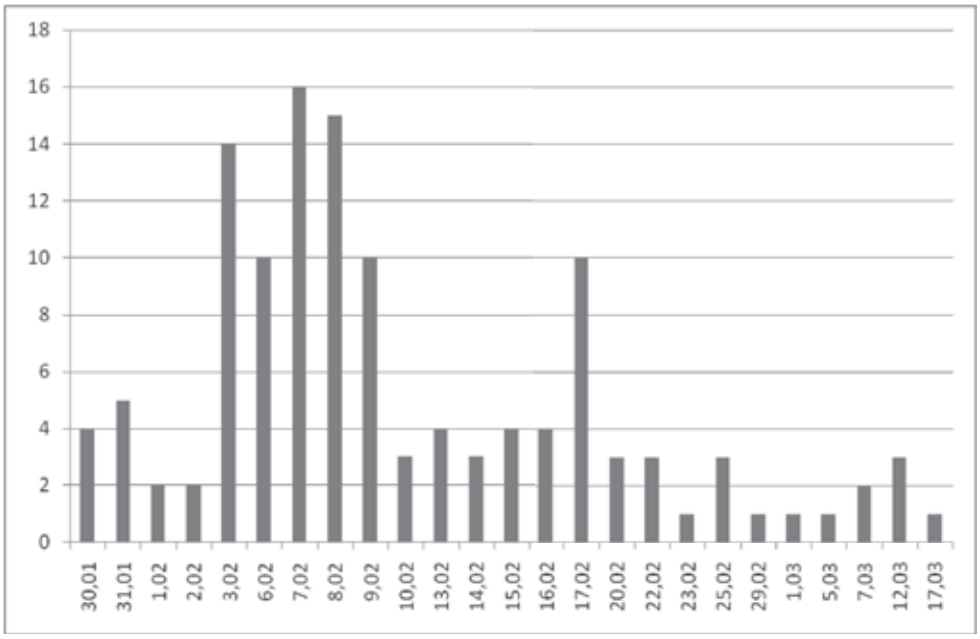


Table 2: Age distribution of the patients from v. Iskra

Age group	0-1	1-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65+	All
N of patients	0	7	15	13	23	12	6	5	14	13	7	6	1	3	0	125

Additional checks after the outbreak in different facilities, including those for production and marketing of food, found out illegal plumbing connections enabling the internal plumbing of the facilities to be supplied entirely with water from local wells or water from wells to be mixed with water from the central water supply system of the village.

Related to this, joint actions were taken by Plovdiv Regional Health Inspectorate, Plovdiv Regional Directorate for Food Safety and Plovdiv Water Supply and Sewerage Ltd. to spot and eliminate these illegal plumbing connections (orders were issued to stop operation of facilities with proven disorders). After the first VHA cases adequate anti-epidemic measure were taken by the Regional Health Inspectorate and Parvomay municipality Water Supply and Sewerage, by general practitioners and by village residents aimed at preventing the epidemic spread of viral hepatitis A, improving the sanitary living conditions, ensuring constant water supply of quality drinking water, and enhancing personal and health education of the population.

Clinical forms of hepatitis A in patients from Iskra village: the most frequently observed clinical form was moderately severe - in 88 (70.4%) of the patients (Table 3). Unlike our previous studies of outbreaks of hepatitis A, where the most common form was light, it was now observed in only 30 (24%) patients. This can be partly explained by the older age of patients.

Table 3: Distribution of the patients by clinical form of hepatitis A

Gender/number	Light form	Moderately severe	Severe
Male 70 /56.0%/	**19**	**47**	**4**
Female 55 /44.0%/	**11**	**41**	**3**
Total 125 /100%/	**30 /24%/**	**88 /70.4%/**	**7 /5.6%/**

Similar clinical forms have been observed from other authors (6, 7, 8).
Blood bilirubin and ALT dynamics are presented respectively in diagram 2 and diagram 3. Aminotransferase levels as an expression of cytolytic activity are in direct correlation with disease severity. Similar changes in paraclinical parameters are also reported by other authors (9).

CONCLUSIONS:
1. This outbreak is another proof of the endemic-epidemic spread of HAV in Bulgaria. It demands for better prevention and control of the disease.
2. A large number of the diseased (60%) were of Bulgarian descent unlike previously reported outbreaks in the region in which patients were predominantly of Roma origin.
3. The majority of cases (70.4%) are considered to be of moderately severe clinical form. In most of the reported outbreaks from previous years mild forms were prevalent. This is explained by the greater proportion of patients of older age.
4. The high VHA incidence in Plovdiv region (as in Bulgaria) - over 30-40/100 000 and much higher in specific years, determines the appropriateness of including VHA vaccine in the immunization calendar of Bulgaria.

Diagram 2:
Dynamic of the blood bilirubin

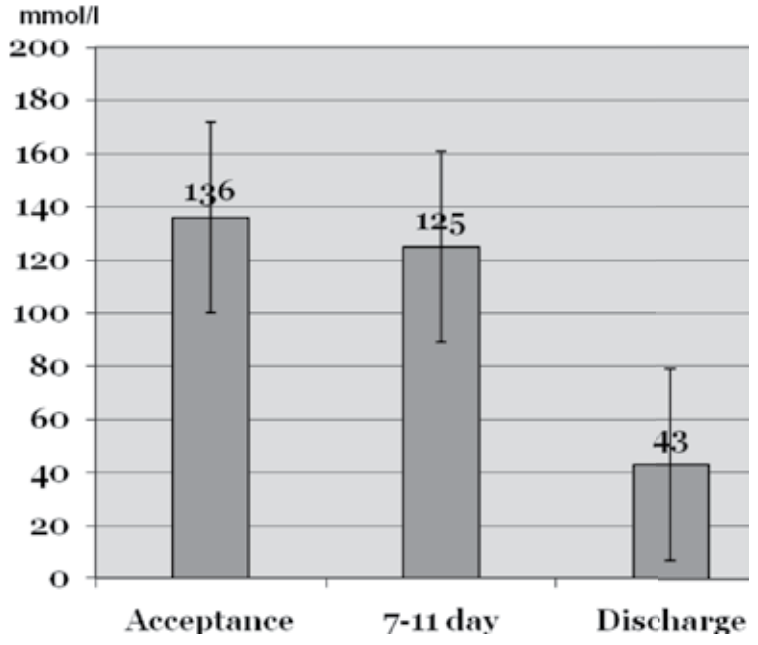
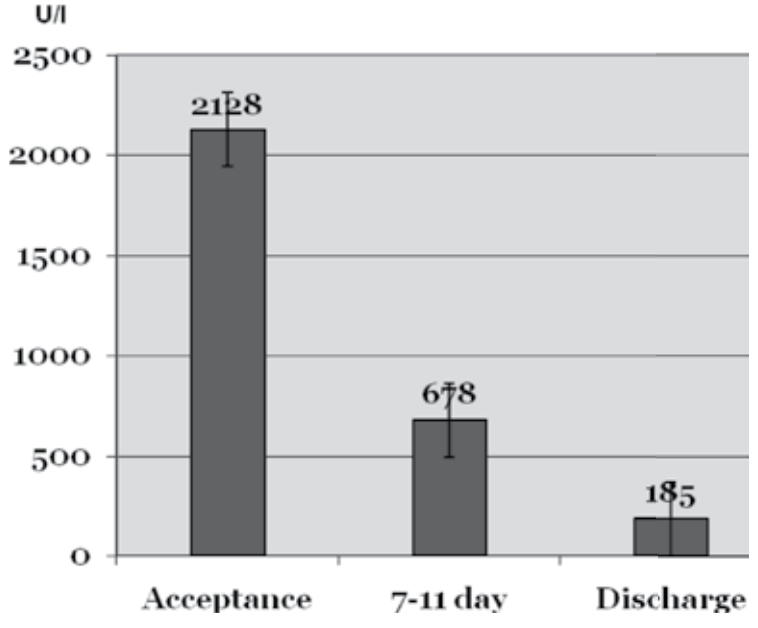


Diagram 3:
Dynamic of alanine amino-transferase (ALT)



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NOSOCOMIAL INFECTIONS, CAUSED BY CANDIDA SPP. -FREQUENCY AND SPREAD DYNAMICS DURING THE PERIOD 2000-2011

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ABSTRACT

During the period 2000 - 2011 genus Candida organisms had a significant share of the etiological structure of nosocomial infections in Bulgaria - an average of 4.14%. Spread dynamics of these pathogens tended to increase from 2.95% in 2000 to 5.13% in 2011. Over 40% of the infections caused by Candida spp. were due to C.albicans, with an increase of its relative share to 52.17% in 2011.

Anesthesiology and Intensive Care Units took leading positions in the spread frequency of Candida nosocomial infection with 51.09% of all registered Candida Nosocomial Infections (NI). Microorganisms of genus Candida were the most common agents of urinary (34.43%) and respiratory (33.29%) system infections.

Key words: *nosocomial infections, Candida spp. distribution, Wards at high risk of NI*

Etiological characteristics of the present-day nosocomial infections (NI) define the dominant position of conditionally pathogenic microorganisms.

NI agents' structure is too erratic. It varies not only in terms of the profile of the hospital ward, but in terms of time, being influenced by various factors – such as selection of "hospital strains", epidemic bursts and others.(1,2) Significant changes in microbial population occur under the influence of the large scale and not always scientifically sound use of antibiotics in medical practice. As a result the so-called "hospital strains" microorganisms, characterized by high virulence, invasiveness and poli-resistibility, circulate in the "risky" wards of hospitals (3,4).

In recent years, the spread of Gram-negative microorganisms such as E. coli, Proteus spp., Pseudomonas spp., Klebsiella spp. etc. has been dominant. Data, proving an increase in frequency of isolation of genus Candida organisms in NI in various profile wards both at home and globally, are of particular interest (1,2,7).

This fact determined the objective of this study - to track the frequency and dynamics of infections caused by microorganisms of the genus Candida in different hospital wards in Bulgaria and their role in the development of different clinical forms of the infection process in hospitalized patients.

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MATERIALS AND METHODS

This epidemiological study was conducted during the period 2000 -2011. It traced the emergence frequency and spread dynamics of infections caused by genus Candida microorganisms by years, in hospital wards at risk in terms of NI- Gynecological, Maternity, Neonatal, Child, Surgical, Urological, Therapeutic, Intensive and Resuscitation.

The clinical structure of reported NI caused by Candida spp. was determined after the indicator clinical forms had been tracked in Anesthesia and Intensive Care Units (AICU) - the wards at the highest risk with regard to this pathology.

The materials used were from our own epidemiological studies and the
official statistics presented in the information section of the National Centre for Public Health and Analysis.

In the processing and analysis of the collected materials, the complex epidemiological method was applied, together with some methods of the alternative analysis – assessment and comparison of relative shares.

RESULTS AND DISCUSSION

Candida spp. belongs to the group of conditionally pathogenic organisms with a high level of human organism colonization and continuous infestation with an upward trend in recent years (5,6).

Global data show frequency of Candida spp. isolation from nasopharynx-up to 52%, from vaginal discharge – up to 12.7%; in pregnant women – up to 86 % and from faeces – up to 9.5%. High level of Candida spp. is formed in carriers from 16-18 years of age and it remains at the same level in the following years without any significant changes (3,7).

Candida spp. can be found on objects in the environment, on the surface of medical instruments, toys, dishes and cutlery, in cosmetics (creams), etc (6).

Candida spp. is one of the etiological agents of nosocomial infections (NI). The infection is transmitted to susceptible persons in direct contact with the Candida carriers, by the hands of the medical staff in hospitals, by a kiss (2 ml saliva contains up to 500 cells). These pathogens are also isolated from raw and cooked food products, contaminated by hands, saliva, etc (3,4,5).

Tracing the frequency and dynamics of emergence and spread of NI caused by Candida spp. in Bulgarian hospitals in the period 2000-2011, (regardless of

incomplete registration of this group of infectious diseases) the following has been established (Figure 1).
- The average share of infections caused by Candida spp. in etiologically proven NI is 4.14%;
- Analysis of the data shows a gradual increase of this pathology in recent years – from 2.95% in 2000 to 5.13% in 2011;
- The infections which occurred after 2006 are above the average benchmark – 4.77% in 2007, 5.57% in 2008; 5.55% in 2009; 4.64% in 2010 and 5.13% in 2011.

The analysis of the spread of NI caused by Candid spp. in Bulgaria shows definitely an increasing trend of this indicator and is to confirm the global data.

Genus Candida contains over 100 different species, but only some of them are pathogenic to humans. Pathogenic Candida species differ in their spread frequency, in their virulence and clinical forms.

According to different authors, the most common representative of this genus is Candida albicans. It also appears to be the most virulent of all species, causing severe infections with the highest degree of mortality. An interesting fact is that latest global research shows reduction in the incidence of C.ablicans species and increase in the relative share of C.glabrata, C. tropicalis, C. parapsilosis. It is believed that Candida spp. is one of the five most frequent blood infections of hospitalized patients.

Genus Candida species structure of the isolated hospital strains in our country, which is presented in Figure 2, determines the leading position of Candida albicans - 40,57% and the highest value of this indicator in recent years - 53.41% in 2009; 59.84% in 2010 and 52.17% in 2011. The second position with a far smaller share belongs to C.krusei - an average of 2.63%. C.glabrata is with close values - 2.15%. C. tropicalis comes after with 1.67 % C.pseudotropicalis and C.stellatoidea follow with only 0.24%. More than half of the Candida strains (52.74%) are unidentified and few are referred to other species.

The dominance of C. ablicans species is associated with the marked adhesive activity of this species to epithelial cells, which provides the active colonization of mucous membranes where this pathogen is reproduced. Moderate adhesive activity is characteristic of C. tropicalis, weak of C. parapsilosis, but virtually devoid of such are C. guilliermondii, C, krusei and C. pseudotropicalis.

There are various clinical symptoms of the candidiasis. They include affecting the mucosa with oropharyngeal and genital syndrome, infection of the skin and nails with paronychia development, lack of hairy coated armpits, groin and perirectal infections. Candidas which have entered the bloodstream (candidemia) can cause damage to several organs and systems - heart, eyes, kidneys, liver, spleen, bones, and lungs. Abscesses are likely to form in the intestinal tract or elsewhere.

The results of our study on the structure of clinical candidiasis as NI are presented in Figure 3:
- More than one third of the reported cases were uri-

Fig. 1

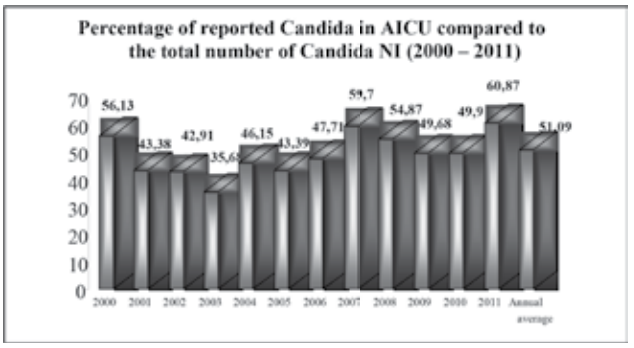


Fig. 2

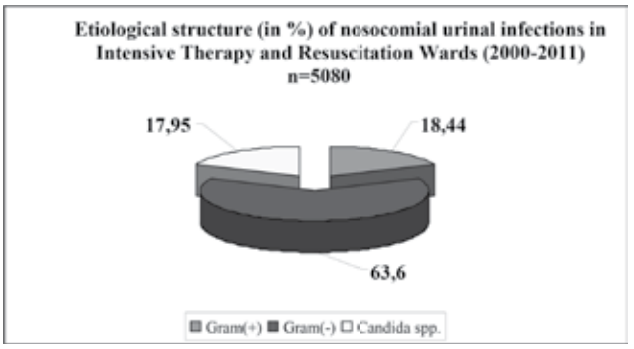


Fig. 3

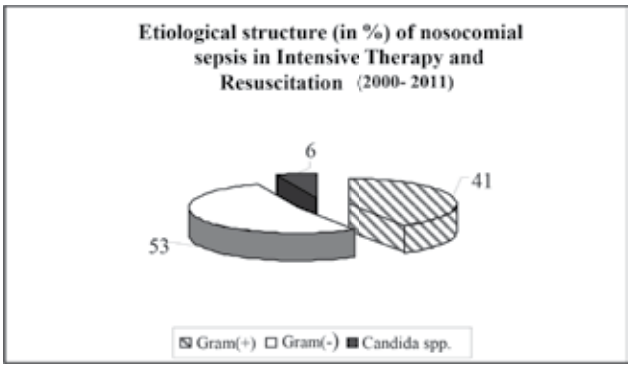


Fig. 4

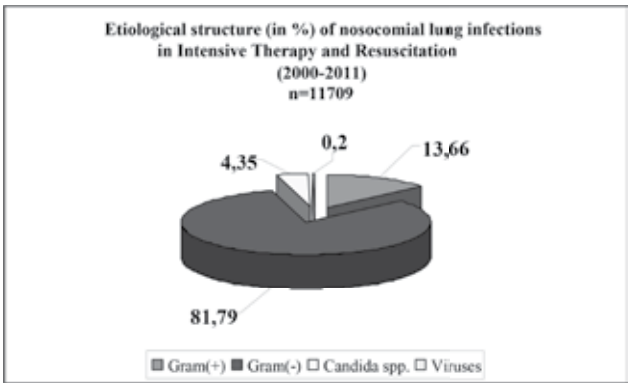
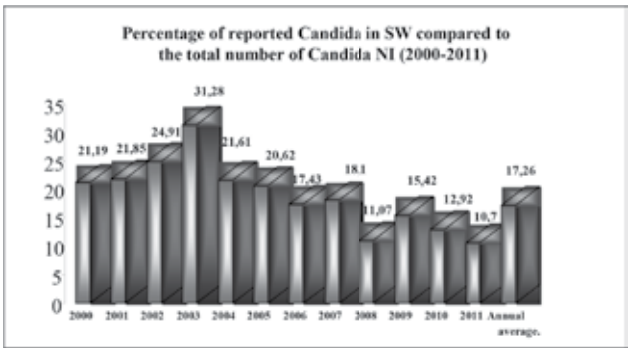


Fig. 5



nary (34.43%) and respiratory (33.29%) systems infections;

- Microorganisms of the genus Candida are the agents of other clinical forms:

* Surgical wound infection - 7.12%;
* IAI - 5.77%;
* Nosocomial sepsis - 4.71%;
*Infections of the intestinal tract (intestinal infection) - 2.74%;
* Infections of the sense organs - 2.23%;
* Infections of the skin and subcutaneous connective tissue - 1.75%;
* Gynecological infections - 1.55%.

The diverse clinical manifestations of candidiasis can be divided into four main groups:

- Surface candida infection of the mucous membranes, skin and nails.
- Visceral candidiasis.
- Allergic forms of Candida infection.
- System (disseminated) candidiasis.

Thrush of the urinary tract is characterized by candiduria, proteinuria, large amounts of erythrocytes and cylinders are found in sediment, which indicates the presence of disseminated candida infection.

Candidiasis of the respiratory system is the most common complication after an antibacterial treatment. Criteria for candida pneumonia are: absence of effect of standard (antibiotic) therapy, a history of prior stomatitis, laryngitis, tonsillitis,.cheilitis etc. and visible improvement in the patient's condition after the inclusion of antifungals.

Epidemiological study presents the incidence and spread dynamics of candida infection in the period 2000-2011, in various hospital wards in Bulgaria at risk in terms of NI (Figure 4).

Average data for the period under consideration show that more than half of NI caused by Candida spp. are registered in the Anesthesiology and Intensive Care (AICU) Units - 51.09%. A significant incidence of this pathology is found in surgical wards (SW) - 17.26%, therapeutic wards (TW) - 14.16%, urological wards (UW) - 5.67% and gynecological wards (GW) – 3.56%. Less than 2% are the reported cases in child wards (CW) - 1.71%, in neonatal wards (NW) - 1.59% and maternity wards (MW) - 1.15%.

Tracing the dynamics of the origin of candidiasis infection in AICU - wards with the highest incidence of this pathology - an uneven upward trend from 35.68% in 2003 to 60.87% in 2011 is noticed (Fig. 1).

During the observed period, our studies indicate that 17.95% of nosocomial urinary tract infections in AICU are caused by Candida spp., and in 2011 this indicator reached the value of 21.64% (Fig. 2).

In the etiological structure of nosocomial sepsis in these wards during the same period Candida spp. takes a share of 6.0% (Fig. 3).

Lower relative share of the agents of the genus Candida are registered with other socially significant clinical forms of NI in AICU - lung infection - 4.35% (Fig. 4) and surgical site infections - 3%.

Meanwhile, in surgical wards (occupying the second position in the frequency of infections caused by Candida spp. after AICU) the relative share of this pathogen gradually reduces - from 21.19% in 2000 to 10.70% in 2011 (Figure 5).

Table 1

Nozocomial infectons with the etiologic agent Candida spp.

Year	Etiologically proven NI, nr	Candida spp.causeed NI	
		nr	%
2000	9 111	269	2,95
2001	8 533	302	3,54
2002	9 694	289	2,98
2003	8 566	227	2,65
2004	8 561	273	3,19
2005	10 197	388	3,81
2006	11 207	436	3,89
2007	11 239	536	4,77
2008	10 704	596	5,57
2009	11 109	616	5,55
2010	10 844	503	4,64
2011	11 646	598	5,13
Annual Average	10 118	419	4,14

Table 2

Candida spp.caused NI-Species composition

Year	Candida spp. caused NI	of them:															
		C.albicans		C.krusei		C.tropicalis		C.pseudotropicalis		C.stellatoidea		C.guilliermondii		C.glabrata		Other and not identified	
		nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%
2000	269	51	18,96	3	1,12	0	0,00	0	0,00	1	0,37	0	0,00	0	0,00	214	79,55
2001	302	95	31,46	0	0,00	0	0,00	0	0,00	0	0,00	0	0,00	1	0,33	206	68,21
2002	289	75	25,95	2	0,69	4	1,38	0	0,00	0	0,00	0	0,00	1	0,35	207	71,63
2003	227	60	26,43	2	0,88	9	3,96	0	0,00	0	0,00	0	0,00	1	0,44	155	68,28
2004	273	80	29,30	7	2,56	3	1,10	0	0,00	0	0,00	0	0,00	5	1,83	178	65,20
2005	388	166	42,78	3	0,77	3	0,77	0	0,00	0	0,00	0	0,00	4	1,03	212	54,64
2006	436	163	37,39	13	2,98	5	1,15	0	0,00	2	0,46	0	0,00	3	0,69	250	57,34
2007	536	181	33,77	12	2,24	2	0,37	0	0,00	1	0,19	0	0,00	7	1,31	333	62,13
2008	596	228	38,26	19	3,19	20	3,36	2	0,34	2	0,34	0	0,00	42	7,05	283	47,48
2009	616	329	53,41	24	3,90	19	3,08	4	0,65	0	0,00	0	0,00	24	3,90	215	34,90
2010	503	301	59,84	15	2,98	14	2,78	1	0,20	2	0,40	1	0,20	24	4,77	145	28,83
2011	598	312	52,17	31	5,18	0	0,00	0	0,00	0	0,00	1	0,17	0	0,00	255	42,64
Annual Average	419	170	40,57	11	2,63	7	1,67	1	0,24	1	0,24	0	0,00	9	2,15	221	52,74

Table 3

Candida spp.caused NI- clinical structure

Year	Candida spp. caused NI	Clinical forms																					
		Respiratory systems infections		Intestinal		IAI		Urinary tract infections		Gynecological		Cardiovascular system		Sepsis		Surgical wound		Skin		Sense organs		Other	
		nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%
2000	269	68	25,28	4	1,49	5	1,86	130	48,33	2	0,74	2	0,74	6	2,23	15	5,58	22	8,18	3	1,12	12	4,46
2001	302	96	31,79	3	0,99	9	2,98	132	43,71	3	0,99	2	0,66	16	5,30	16	5,30	4	1,32	6	1,99	15	4,97
2002	289	101	34,95	7	2,42	3	1,04	102	35,29	2	0,69	0	0,00	15	5,19	34	11,76	3	1,04	2	0,69	20	6,92
2003	227	74	32,60	6	2,64	12	5,29	80	35,24	4	1,76	0	0,00	6	2,64	13	5,73	2	0,88	4	1,76	26	11,45
2004	273	61	22,34	5	1,83	13	4,76	99	36,26	1	0,37	1	0,37	22	8,06	27	9,89	3	1,10	13	4,76	28	10,26
2005	388	117	30,15	3	0,77	17	4,38	134	34,54	8	2,06	0	0,00	19	4,90	39	10,05	2	0,52	29	7,47	20	5,15
2006	436	119	27,29	18	4,13	56	12,84	126	28,90	8	1,83	0	0,00	15	3,44	45	10,32	10	2,29	16	3,67	23	5,28
2007	536	191	35,63	15	2,80	44	8,21	150	27,99	9	1,68	1	0,19	35	6,53	53	9,89	7	1,31	4	0,75	27	5,04
2008	596	269	45,13	30	5,03	33	5,54	178	29,87	5	0,84	4	0,67	14	2,35	26	4,36	8	1,34	11	1,85	18	3,02
2009	616	229	37,18	15	2,44	61	9,90	193	31,33	21	3,41	4	0,65	25	4,06	30	4,87	10	1,62	8	1,30	20	3,25
2010	503	198	39,36	18	3,58	24	4,77	184	36,58	6	1,19	0	0,00	18	3,58	24	4,77	7	1,39	5	0,99	19	3,78
2011	598	151	25,25	14	2,34	13	2,17	223	37,29	9	1,51	5	0,84	46	7,69	36	6,02	10	1,67	11	1,84	80	13,38
Annual Average	419	140	33,29	12	2,74	24	5,77	144	34,43	7	1,55	2	0,38	20	4,71	30	7,12	7	1,75	9	2,23	26	6,13

Table 4
Percentage of reported Candida spp caused infections by hospital wards compared to the total number of Candida NI

Year	Number of Candida spp. caused NI	At hospital wards at risk																	
		GW		MW		NW		CW		SW		UW		AICU		TW		Other	
		nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%
2000	269	0	0,00	2	0,74	3	1,12	11	4,09	57	21,19	19	7,06	151	56,13	23	8,55	3	1,12
2001	302	2	0,66	3	0,99	5	1,66	10	3,31	66	21,85	29	9,60	131	43,38	54	17,88	2	0,66
2002	289	2	0,69	0	0,00	3	1,04	6	2,08	72	24,91	8	2,77	124	42,91	72	24,91	2	0,69
2003	227	3	1,32	1	0,44	2	0,88	8	3,52	71	31,28	12	5,29	81	35,68	44	19,38	5	2,20
2004	273	16	5,86	5	1,83	6	2,20	8	2,93	59	21,61	25	9,16	126	46,15	24	8,79	4	1,47
2005	388	44	11,34	9	2,32	4	1,03	8	2,06	80	20,62	25	6,44	180	46,39	26	6,70	12	3,09
2006	436	24	5,50	10	2,29	9	2,06	2	0,46	76	17,43	21	4,82	208	47,71	69	15,83	17	3,90
2007	536	18	3,36	4	0,75	9	1,68	1	0,19	97	18,10	12	2,24	320	59,70	51	9,51	24	4,48
2008	596	14	2,35	5	0,84	15	2,52	5	0,84	66	11,07	40	6,71	327	54,87	98	16,44	26	4,36
2009	616	29	4,71	13	2,11	12	1,95	8	1,30	95	15,42	35	5,68	306	49,68	95	15,42	23	3,73
2010	503	18	3,58	1	0,20	5	0,99	15	2,98	65	12,92	30	5,96	251	49,90	90	17,89	28	5,57
2011	598	9	1,51	5	0,84	7	1,17	4	0,67	64	10,70	29	4,85	364	60,87	66	11,04	50	8,36
Annual Average	419	15	3,56	5	1,15	7	1,59	7	1,71	72	17,26	24	5,67	214	51,09	59	14,16	16	3,90

Conclusions:

1. During the period 2000-2011 genus Candida microorganisms presented a significant percentage of the etiological structure of NI in Bulgaria - an average of 4.14%.
2. The dynamics of Candida spp. spread in the hospitals in Bulgaria was a growing trend - from 2.95% in 2000 to 5.13% in 2011.
3. Species characteristic of genus Candida microorganisms showed indisputable dominance of C. albicans species - an average of 40.57% and the highest value of this indicator in recent years - 53.41% in 2009, 59.84% in 2010, 52.17% in 2011.
4. Microorganisms of genus Candida are the most common cause of urinary (34.43%) and respiratory (33.29%) systems infections.
5. Wards at high risk of NI caused by Candida spp. are: the Anesthesiology and Intensive Care Units - with 51.09% of all reported Candida NI; Surgical Wards - with 17.26%, Therapeutic Wards - with 14.16%, Urological Wards - with 5.67 percent. Candida spp. accounts for NI in Gynecology, Child, Neonatal and Maternity Wards.
6. The dynamics of the reported NI with the etiologic agent Candida spp. in the Anesthesiology and Intensive Care Units showed a tendency to increase in recent years - from 35.68% in 2003 to 60.87% in 2011.
7. In AICU 17.95% of nosocomial urinary tract infections were caused by Candida spp. In 2011 this indicator reached 21.64%, and Candida sepsis represented 6.0% of the general structure of this pathology.
8. In Surgical Wards the dynamics of the reported NI with Candida spp. agent is declining - from 21.19% in 2000 to 10.70% in 2011.

E. coli - with 14.87%, other viruses - with 11,52%, Klebsiella spp. - 7.56%, Rseudomonas spp. - 3,73%, Streptococcus spp. - 3.08%, Rgoteus spp. 2.84% and so on (Fig. 5) Candida spp.

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