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NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 37, NUMBER 1/2009

ISSN 0204-9155

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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 37, NUMBER 1/2009

Send to the printers on Published sheets
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STUDY OF SERUM LEVELS OF IL4 AND IL10 IN PATIENTS WITH DIFFERENT TYPES OF ECHINOCOCCOSIS (HYDATID DISEASE)?

P. Tonchev

Medical University, Pleven

SUMMARY

Echinococcosis is a severe parasitic disease, caused by larvae of cestodes Echinococcus granulosus. The immunological interaction between infected subject and E. granulosus is complex and depends on host's immune response against parasitic invasion and suppression and modification of this response by the parasite. The aim of this study was to measure the levels of IL4 and IL10 using ELISA in serum of patients with different forms of Echinococcosis, and to find the relationship (if exist) between serum levels of IL4 and IL10 and the number, size and locations of E. granulosus-cysts. Sera were obtained preoperatively from 25 patients(15-f 10-m) age 11-76 (median 45,4 ±18) and from comparably designed control group of healthy subjects. There was a significant(p<00.5) difference in IL4 levels in patients with liver hydatid disease compared with controls. Our results show that the serum levels of IL4 are a useful marker in the follow-up of patients with cystic disease.

Key words: Echinicoccosis, immunity, IL-4, IL-10

INTRODUCTION

Echinococcosis is a severe parasitic disease caused by the cestode Echinococcus granulosus. Larvae of the parasite develop in humans, transforming into cysts with different sizes .Usually cysts are located in liver and lungs although all other organs can be affected. Bulgaria is among most affected countries with morbidity of 6,28‰ for 2006. Although there is a small number of clinical studies for cytokine profile in human Echinococcosis, new studies suggest a possible relationship between cytokines and outcome of the disease.

Echinococcosis is a disease which unlocks cellular and humoral immune response in the host characterized by enhanced production of specific serum antibodies and simultaneous activation of both Th1 and Th2 cells helpers (12, 10). Th2 cells express the production of IL-4, IL - 5, IL-6 and IL-10 and according to some authors (Rigano, Profumo, Zhang) that is associated with predisposition to a more severe course of disease, while Th1 cells express production of IL-2 and IFN-γ, which is connected with host protective immunity (13.19). The different severity of clinical manifestation of disease may be predetermined by prevailing host immune response (14). On the other hand, clinical symptoms of the disease are varying and depend on many factors: the affected organ, size and number of ehinococcus cysts, possible complications (abscess formation and / or rupture of the cyst) and of the subsequent protective, host immunological reactions (3). According by Touil-Boukuffa et al. studying levels of serum cytokines in patients after surgical removal of cysts would allow early discovery of relapse.

ABBREVIATIONS USED IN THIS PAPER:

ACCEPTED FOR PUBLICATION: 10. 06. 2009

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Since the effects of IL-4 and IL-10 are different in their action, the immunological link between the host and the parasite is expressed as the complex interaction between host immune response against parasitic invasion and oppression and modification of that response by parasite (17).

The aim of our study was to determine the levels of IL4 and IL10 in the serum of patients with various forms of hydatid and to find (if exists) relationship between serum levels of these cytokines and the number of organ locations of the cysts.

MATERIALS AND METHODS

Human sera tested

Tested sera were obtained before operation from 25 patients with hydatid disease (15 women and 10 men) aged 11-76 years (mean 45.4 ±18), The diagnosis in all patients was confirmed serologically, during the operation, or histomorphologycaly. Studied patients are from departments of abdominal or visceral or pediatric surgery consulted in parasitological office in University Hospital, Pleven (Table 1).

As controls, sera obtained from 10 healthy subjects without changes in routine biochemical laboratory parameters, without a history of autoimmune disease, with normal results from last X-ray study of the lungs and ultrasound examination of the liver, grouped respectively by age and gender. To exclude other parasitic invasion all individuals were tested serologically for toxoplasmosis and trichinellosis, and coprologically studied for intestinal parasites.

All procedures of the study were authorized by the ethical committee of the University-Pleven, Pleven University Hospital and all patients gave informed consent for this study.

Sera were stored at temperature of 20°C before testing.

Serum levels of IL-4 and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, the test included the following steps:

Polisterenovite plates (Dynatech) were coated with sera from patients with hydatid disease in 1:5 dilution with PBS, incubated at room temperature 18 hours and flushed three times with phosphate-buffered saline (PBS, pH 7,2) + Tween 20. Then unrelated active centers in the holes were blocked by incubation of the plaques for 24 hours with 1% solution of bovine serum albumin (SIGMA) at room temperature.

Incubation with anti-human interleukin 4/anti-human interleukin-10 (SIGMA) diluted in 0.05% RVS - Tween 20 as instructed by the company for one hours at a temperature of 37°C.

Incubation with immunoconjugates (antigoat IL-4 production BUL-BIO, NCIPD, Sofia) and antimice IL-10 (SIGMA) diluted 1:400 respectively with RVS - Tween 20 for one hours at 37°C.

After triple washing with PBS - Tween 20 was added O-phenylen-diamine in 0.05 M citrate buffer, pH 5,0 + H2O2 0,01% plaques and incubated for 30 min in the dark. Adjournment of the enzyme reaction by adding 50µl 4N-sulfuric acid to each well.

Before the main testing of serum samples were carried out a series of tests with different antigen concentrations of IL-4 and IL-10 (SIGMA) and anti-human interleukin 4, interleukin-10 to determine the optimal parameters of the reagents on the basis of which it was built standard curve for the recording of the final results.

Each system has substrate and immunoconjugate controls. All extinction values were recorded at a wavelength of 492 nm. with Microelisa Reader 210 (Organon Teknika, Belgium). Patients with concentrations of IL-4 and IL-10 higher than 5pg/ml are considered positive.

Statistical analysis

Statistical analysis completed using EXCEL programs and StatgraphICS + Plus for Windows. Student tests and Wilcoxon used to evaluate differences between groups. Level of statistically significant difference was accepted $p \leq 0,05$.

RESULTS

Results in the levels of IL-4 and IL-10 in sera of patients with various forms of hydatid disease are shown in figures 1, 2, 3 and Table 2. Of all 25 patients tested , higher levels of IL-4 showed 9 (36%) of them, and for IL-10-5 (20%) (Fig.1). Higher levels above the confidence interval (5pg/ml) simultaneously tested for both interleukins were detected in four of the patients surveyed (16 percent). One of them is a very rare location of the cyst - m.psoas.major, and the other three patients with liver cysts (single cysts).

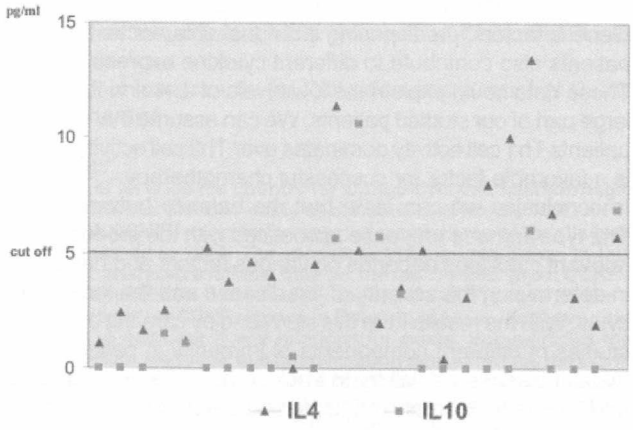

Figure 1. IL-4 and IL10 in serum of hydatid patients evaluated by ELISA in pg/ml

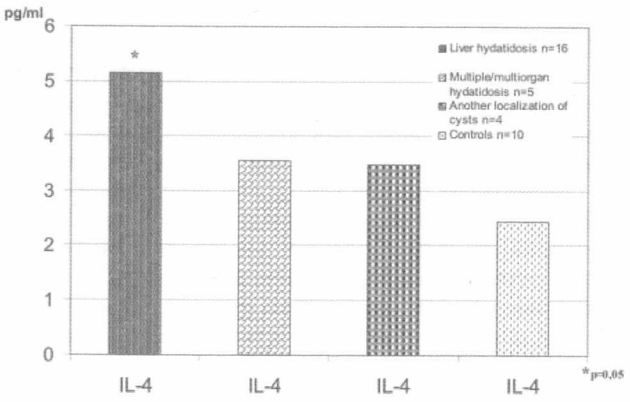

Figure 2. IL-4 in serum of patients with different forms of hydatidosis and healthy controls evaluated by ELISA in pg/ml

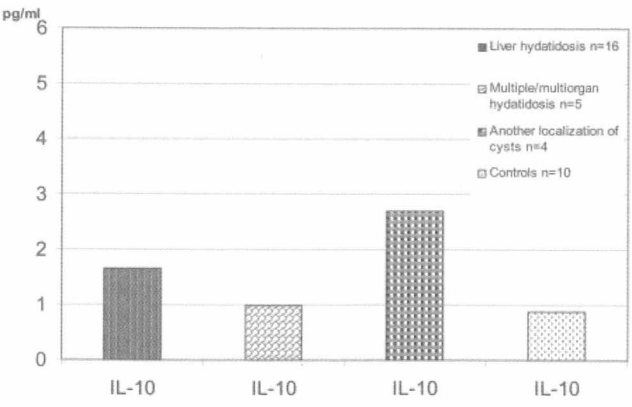

Figure 3. IL-10 in serum of patients with different forms of hydatidosis and healthy controls evaluated by ELISA in pg/ml

Figure 2. shows the average values of IL-4 in all patients tested, divided into 4 different groups - cyst in liver (5,014 pg/ml), multiple /multiorgan cysts (3,55 pg/ml), cysts with another location (3,48 pg/ml), controls (2,44 pg/ml). Significant difference ($p \leq 0,05$) versus control group was found in the group of patients with liver cysts. Fig. 3. shows the average values of IL-10 in all studied patients allocated in the same groups for IL-4. Statistically significant differences ($p \leq 0,05$) for IL-10 were not found in any of the studied groups: liver cyts (1,66 pg/ml), multiple/multiorgan location (0,98 pg/ml), cysts with another location (2,74 pg/ml), controls (0,88 pg/ml), regardless of the three times higher values found in the other location. Higher values above the confidence interval (5pg/ml) were detected in the three groups of patients tested for both IL-4 and IL-10 (Tabl.1). Of 16 tested patients with liver cysts positive for IL-4 were 6 of them (37.5%). Quite impressive is the high percentage (50%) of positive for IL-4 in the group with another locations of cysts. Only one of five tested patients (20%) with multiple/multiorgan cysts proved positive for IL-4, but only one patient (20%) of the same group is also positive for IL-10. Regarding IL-10 the percentage of positive patients is lower than that observed for IL-4: liver cysts (18.75%) cysts with other locations (25%).

Table 1. Clinical profile of patients with hydatidosis

Location sites of hydatid cysts	Number of cysts	Number of investigated patients
Liver	Single	15
Liver	Multiple	2
Spleen-small pelvis	Multiple/multiorgan	1
Mediastinum	Multiple	1
Lung	Single	2
Peritoneum	Multiple	1
Muscular	Single	1
Kidney	Single	1
Kidney	Multiple	1

Table 2. IL-4 and IL-10 positive patients with different forms of hydatidosis

Groups	IL-4 positive n=8	IL-10 n=5	Number of investigated patients n=25
Liver	6 (37,5%)	3 (18,75%)	16
Multiple/multiorgan	1 (20%)	1 (20%)	5
Another localization cysts	2 (50%)	1 (25%)	4

DISCUSSIONS

Activation of helper Th1 and Th2 cells in hydatid disease is result of prolonged stimulation by the larva antigen form of E.granulosus and is accompanied with change in cytokine profile (4.9). Since cytokines affect the relationship between humoral and cellular immune response, most likely they play an important role in host immune response against E.granulosus. Most accurate methods for determining the levels of cytokines used by some authors (1,6,8) are cell cultures, and polymerase chain reaction (PCR) with reverse transcriptase, which is not yet introduced in Bulgaria. This study aims to determine the levels of IL-4 and IL-10 in sera of patients with various forms of hydatid disease before treatment, to seek connection between the levels of these cytokines and the number and organ locations of cysts.

According to Rigano et al.1995, high levels of Th2 cytokines (IL-4 and IL-10) were measured in sera of patients who are not affected by the chemotherapy (14). Similar results have been published by some authors (16.5) for other parazitozes . The analysis of our results showed significant increase of IL-4 in the group of patients with liver cyst (single cysts) $p \leq 0,05$, as well higher values in groups with multiple/multiorgan cysts and cysts with other locations compared to the control group. These data clearly demonstrate the importance of IL-4 in the immune response in hydatid disease and a tendency for a certain relationship with the location. Similar statements from Torcal et al, 1996, are generally consistent with our results. According to these authors central or paramedian location of cysts in the liver was significantly associated with the production of high levels of IL-4, IL-2, IL-1 and IgG, but the study of Torcal et al. is limited to hydatid disease of the liver.

A different trend was found for the levels of IL-10. Only five (20 percent) of all patients tested showed higher levels compared with controls, while four of them were observed at the same time with significantly increased levels of IL-4. Although differences in IL-10 levels in the cysts with another location were not significant, there is a marked tendency for higher levels of IL-10 in this group compared with the other groups. This could explain the survival and growth of cysts in other organs and systems, as IL-10 resulted in inhibition of cell-mediated immunity through their anti-inflammatory action (18). In our study we have determined the levels of IL-4 and IL-10 in sera of patients with various forms of hydatid disease before any treatment was started. Thus relying on the above statements, we can predict the effectiveness of subsequent chemotherapy (14).

Several authors (15, 7) in their studies on the immunity of mice infected with scolices of Echinococcus granulosus, reached the conclusion that in the early stages of infection there is increased production of IFN-γ, while in the later stages high levels of IL-4 and IL-10 are detected. The balance between Th1/Th2 cell activity may be altered in the course of the disease and depends on many factors (11). Antigen B (AgB lipoprotein contained in a cyst fluid) is a factor which, according to Rigano et al. (11) modulates the immunity of host for production of Th2 cytokines (IL-4 and IL-10). These data explain our results - high levels of these cytokines in all tested groups. On the other hand hydatid cyst fluid contains other antigens and AgB is only 10% of them. Genetic factors, predisposing individual differences between patients also contribute to different cytokine expression (13). These data could explain the low levels of IL-4 and IL-10 in a large part of our studied patients. We can assume that in these patients Th1 cell activity dominates over Th2 cell activity, which is a favorable factor for successful chemotherapy.

In conclusion we can state that the balance between Th1/Th2 type immune response associated with the production of relevant cytokines depends on various factors and has a role in determining the severity of the disease and the location of cysts. With the results from this study and by carrying out future studies of different components of immunity in patients with hydatid disease we will try to track some of the mechanisms and factors that largely modulate and determine the immune response in this disease. We believe a continuous study on larger groups of patients is necessary for both IL-4 and IL-10 and other cytokines, stimulating host humoral and cellular immunity in order to shed some light on the mechanisms and factors associated with the development of the disease.

VACCINE AGAINST CONGO-CRIMEAN HAEMORRHAGIC FEVER VIRUS-BULGARIAN INPUT IN FIGHTING THE DISEASE

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SUMMARY

Bulgaria is an endemic country for Congo-Crimean haemorrhagic fever (CCHF) as are also many of the countries on the Balkan Peninsula. A CCHFV vaccine for specific prophylaxis of the disease was developed in 1970. The vaccine is unique until today. In this study, we analyzed available data for the last 5 years about CCHFV vaccine application and officially reported CCHF cases in the 3 most endemic for CCHF regions in Bulgaria (Burgas, Haskovo, and Kardjali) to consider if any relationship exists. Summarizing the available data, we found that immunization tend to reduce the CCHF incidence. The more persons were vaccinated, the less CCHF cases were reported in the same year. In conclusion, after introducing the vaccination program, a 4-fold reduction in the number of annually reported CCHF cases was observed. Also, extrapolating from the 3 most endemic regions in Bulgaria, we concluded that increasing the number of vaccinated persons per year lead to reducing the number of reported CCHF cases for the same year.

Key words: Crimean-Congo hemorrhagic fever (CCHF), incidence, vaccination

INTRODUCTION

Bulgaria is an endemic country for Congo-Crimean haemorrhagic fever (CCHF) as are also many of the countries on the Balkan Peninsula. CCHF is a public health problem in South-East Europe, Africa, Asia and the Middle East.

CCHF virus (CCHFV) is transmitted to humans through the bite of ticks of the Hyalomma genus or through contact with blood or tissues from infected persons or animals (1,2). CCHF causes a severe multisystem disease characterized by profuse bleeding and a case-fatality rate up to 30%.

Specific treatment includes application of specific immunoglobulins. CCHF-Bulin is specific immunoglobulin preparation against CCHFV produced by BulBio-NCIPD (Bulgaria) for intramuscular application. It contains active protein fraction isolated from blood plasma of volunteers, immunized with vaccine against CCHF (3). Concentration of specific antibodies in the blood plasma of the volunteers is 6-10 times higher than in initial plasma thus showing effect of the CCHF vaccine.

For specific prophylaxis of the disease in Bulgaria, a CCHFV vaccine was developed in 1970 (4). The vaccine is unique until today. It contains mouse brain suspension of cultivated CCHF virus, inactivated by chloroform and heating at 58°C, and absorbed on Al(OH)3. The regimen for application includes two doses - at day 0 and 30 days later, and a third dose one year after; and every five years after that. The vaccine is designed for prophylaxis of CCHFV infection in military, medical, agricultural and other personnel living or working in the endemic regions.

In this study, we analyzed available data about CCHFV vaccine application and officially reported CCHF cases in the 3 most endemic for CCHF regions in Bulgaria to consider if any relationship exists.

MATERIALS AND METHODS

Studied regions: Burgas (Eastern Bulgaria), Haskovo and Kardjali (South-Eastern Bulgaria). They represent the most heavily affected by CCHF districts.

Parameters: Incidence of CCHF together with mortality; number of vaccinated people per year in each district.

Time frame: 5 years - from 2004 to 2008.

RESULTS AND DISCUSSION

CCHF was first recognized in the country in 1952 and is a reportable disease since 1953. In 1968 CCHFV was isolated from blood samples of two patients. Results from serological investigations show that approx. 20% of patients living in endemic areas, who reported a tick bite, have antibodies to CCHFV. The seropositivity in animals in the endemic areas is as high as 50% (5). Most cases are reported from south-eastern, northeastern and central regions of the country.

Between 1953 and 1974 (period of 22 years), 1105 CCHF cases had been reported to the Bulgarian Ministry of Health; the fatality rate was approx. 17%. Among them a number of 20 cases were nosocomial infections with 52% fatality rate.

In 1974, an immunization program was introduced in the medical workers and the military personnel in endemic areas. As a result, between 1975 and 1996, again for 22 years, the number of reported CCHF cases was reduced to 279 (4-fold comparing to the previous 22-year period!), with a fatality rate of 11.4%. No infection was reported from the immunized military personnel.

Table 1. Reported CCHF cases before and after immunization with CCHFV vaccine

Time period	Reported CCHF cases	Fatality rate
1953-1974	1105	17%
1975-1996	279	11,4%

Since 1997, up to 20 cases maximum were officially reported to the Ministry of Health. The only exception was the year 2002, when the number of registered cases unexpectedly reached 54. Regardless of this exception, there is a strong trend towards decreasing of CCHF incidence in the last years in Bulgaria (Table 2). The problem is diminishing in Bulgaria and we do believe that vaccination is the main reason.

Table 2. Relative part of reported CCHF cases in the 3 most endemic districts in relation with all number of reported cases in the country

Year (number)	In Bulgaria (number/%)	Officially reported cases In the 3 districts - Burgas, Haskovo and Kardjali
2004	18	11 (61%)
2005	14	6 (43%)
2006	7	4 (57%)
2007	3	1 (33%)
2008	13	5 (38%)
TOTAL	55	27 (49%)

As it is shown in Table 2, the three most endemic regions -

ABBREVIATIONS USED IN THIS PAPER:

CCHF - Congo-Crimean halmorrhagic fever
CCHFV - Congo-Crimean halmorrhagic fever virus

ACCEPTED FOR PUBLICATION: 09. 03. 2009

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Table 3. Relation between number of vaccinated persons and CCHF incidence

A. In the district of Burgas

Year	Vaccinated persons	CCHF cases	Mortality
2004	427	7	3
2005	720	3	-
2006	989	2	1
2007	588	1	1
2008	479	3	-
TOTAL	3203	16	5

B. In the district of Haskovo

Year	Vaccinated persons	CCHF cases	Mortality
2004	137	2	-
2005	23	3	-
2006	29	1	-
2007	14	-	-
2008	11	2	-
TOTAL	214	8	-

C. In the district of Kardjali

Year	Vaccinated persons	CCHF cases	Mortality
2004	28	2	-
2005	14	-	-
2006	30	1	-
2007	101	-	-
2008	64	-	-
TOTAL	237	3	-

Burgas, Haskovo and Kardjali - account for about half of the CCHF incidence in Bulgaria. That is why, we chose these districts to show relation between and tendencies in the number of vaccinated persons and reported CCHF cases per year.

Table 3 represents data showing this relation - Table 3A for Burgas, Table 3B for Haskovo and Table 3C for Kardjali. Summarizing the presented data, one can say that mass-immunization tend to reduce the CCHF incidence. The more people were vaccinated, the less CCHF cases were reported in the same year. More than that, one should keep in mind that only risky contingent is vaccinated. It means that even vaccination of only these people can lead to stable reduction in CCHF incidence. If more people could be included in the vaccination program, then the incidence reduction could be even greater. Nowadays, most of the CCHF cases in our country are due to tick bites and mainly agricultural workers or farmers are affected.

In conclusion, after introducing the vaccination program, a 4-fold reduction in the number of annually reported CCHF cases was observed. Also, as we showed in this report, extrapolating from the 3 most endemic regions in Bulgaria, increasing the number of vaccinated persons per year lead to reducing the number of reported CCHF cases for the same year. In addition, it is proved that immunization with CCHFV vaccine lead to 6-10 times higher concentration of the specific immunoglobulins comparing with the initial plasma, thus showing effect of the CCHFV vaccine.

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NOSOCOMIAL URINARY TRACT INFECTIONS IN UROLOGIC PATIENTS: SHIFT IN PATHOGENS AND THEIR ANTIBIOTIC SUSCEPTIBILITIES

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SUMMARY

Urinary tract infection (UTI) is the most common nosocomially acquired infection. Surveillance data report a shift in the etiology and resistance patterns of uropathogens. The aim of this study was to investigate etiologic agents of nosocomial UTIs in our institution and to study their susceptibility to antimicrobial agents. We evaluated retrospectively 97 nosocomial infections among the 1278 patients admitted to the Department of Urology for one year period from July 1st 2006 to June 30th 2007. The incidence of nosocomial infections (NIs) was 7.59%. NUTIs constitute 91.75% of them. Gram-negative bacteria were responsible for 70% of NUTIs. Two-third of NUTIs were caused by enterobacteria. E.coli, Klebsiella spp. and Enterobacter spp. were isolated in almost equal proportion (range 18 to 24%). The percentage of nonfermenters was relatively low (5.62%). Of the particular note was the emergence of Enterococcus spp. (28%) as important nosocomial uropathogen. Multidrug resistance was observed in 64.51% of gram-negative bacteria. Production of ESBL was detected in 37 (64.91%) of enterobacteria. Resistance to ciprofloxacin and trimethoprim-sulfamethoxazole (TMP/SMX) was observed in 88.7% and 90.3% of isolates respectively. Enterococcus spp. was the only gram-positive bacterium with a high incidence and one third of enterococci were multidrugresistant. The data reveal that gram-negative bacteria are still an important cause of NUTIs, but Enterococcus spp. play the leading role in our urologic population. Etiologic shift in NUTIs and upsurge of antimicrobial resistance of these pathogens are impressive and alarming. Multidrug resistance is a common problem in our Department of Urology. In order to prevent or decrease resistance to antibiotics the use of antimicrobials should be kept under supervision, should be given in appropriate doses and effective control programme for hospital infections should be carried out.

Key words: nosocomial urinary tract infections, uropathogenes, antimicrobial resistance

INTRODUCTION

Urinary tract infection is the most common nosocomially acquired infection. Nosocomial urinary tract infections (NUTIs) constitute 40-50% of all hospital infections (12, 19). It often results in serious complications like secondary bacteraemia and sepsis leading to a rise in the hospital costs and mortality. The rate of NUTIs is determined by the interactions of several factors such as primary disease, duration of hospitalization and treatment, and invasive interventions. NUTIs are most common in urologic settings and are associated with urinary catheters in 80% of the cases (12, 14). Surveillance data report a shift in the etiology and resistance patterns of uropathogens (4, 5, 8, 10). In recent years, bacterial resistance to different antibiotics has been risen dramatically leaving physicians with few therapeutic options. Problematic microorganisms as methicillin-resistant Staphylococcus aureus (MRSA), extended-spectrum β-lactamase (ESBL) producing organisms, vancomycin-resistant enterococci (VRE) and multidrugresistant (MDR) nonfermentors have become the common hospital pathogens (3, 7, 9 .13).

The aim of this study was to investigate etiologic agents of nosocomial NUTIs in our institution and to study their susceptibility pattern to antimicrobial agents.

ABBREVIATIONS USED IN THIS PAPER:

ESBL - extended spectrum beta-lactamase, NCCLS - National Committee for Clinical Laboratory Standards; NUTIs - nosocomial urinary tract infections

ACCEPTED FOR PUBLICATION: 17. 12. 2008

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MATERIALS AND METHODS

A retrospective study was performed to evaluate the prevalence of nosocomial infections among the patients in the Department of Urology for one year period from July 1st 2006 to June 30th 2007.

A total number of 1278 patients were hospitalized in the ward during the study period. The diagnosis of NI was based on Centers for Disease Control (CDC) definitions.

Specimens and culture: Majority of the studied samples were midstream urine specimens and the others catheter aspirates. Culture was done by the calibrated loop technique delivering 0.001ml of urine and plated on CPS ID 3 chromogenic medium (bio-Merieux). Identification of isolates was done using standard microbiologic techniques.

Antimicrobial susceptibility testing: All isolates were tested for susceptibility to antimicrobial agents on Mueller Hinton agar by the standard disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI), (5).

Detection of ESBL: All gram negative bacilli were tested for ESBL production by a double, disk diffusion method recommended by the CLSI.

Screening for high level aminoglycoside resistance (HLAR): Isolates of Enterococcus species were screened for HLAR using gentamicin 120µg disks.

Multidrug resistance: Multidrug resistance was defined as resistance to three or more classes of antimicrobial agents.

RESULTS

During the studied period 97 nosocomial infections were found in 1278 patients admitted in Department of Urology. The incidence of nosocomial infections was 7.59%. NUTIs constitute 91.75% of them. We obtained clinical information of 89 patients that had nosocomially acquired UTIs. Majority of them were males (73 patients). Mean age was 62.8 (range 21 to 87 years). In all age groups, except those age 21-40 years, males were more frequently affected than females. The prevalence of NUTIs was highest between the 7th and 8th decades (Table 1).

Table 1. Prevalence of nosocomially acquired urinary tract infections according to gender and age

Age (Years)	Male (n=73)	Female (n=16)	Total (n=89)
0 - 20	0	0	0
21 - 30	0	4 (25.00%)	4 (4.49%)
31 - 40	0	4 (25.00%)	4 (4.49%)
41 - 50	3 (4.11%)	2 (12.5%)	5 (5.61%)
51 - 60	11 (15.06%)	2 (12.5%)	13 (14.60%)
61 - 70	28 (38.36%)	2 (12.5%)	30 (33.71%)
71 - 80	28 (38.36%)	2 (12.5%)	30 (33.71%)
> 80	3 (4.11%)	0	3 (3.37%)

The list of uropathogens isolated is shown in Table 2. Gram-negative bacteria were responsible for 70% of NUTIs. Two-third of NUTIs were caused by enterobacteria. E.coli, Klebsiella spp. and Enterobacter spp. were isolated in almost equal proportion (range 18 to 24%). Of the particular note was the emergence of Enterococcus spp. (28%) as important nosocomial uropathogen. The percentage of nontermenters was relatively low (5.62%).

Table 2. Etiological structure of nosocomial uroinfections

Isolated organism	№	Percentage
Gram - negative bacteria	62	69.66
Esherichia coli	21	23.59
Klebsiella spp.	18	20.22
Enterobacter spp.	16	17.98
Morganella spp.	1	1.12
Proteus spp.	1	1.12
Pseudomonas spp.	2	2.25
Acinetobacter spp.	3	3.38
Gram - positive bacteria		
Enterococcus spp.	25	28.09
Other		
Candida spp.	2	2.25
Total	89	100

Antimicrobial susceptibilities of gram-negative bacilli: Only one of the total 62 gram-negative bacterial isolates was sensitive to all antimicrobials tested. Multidrug resistance was observed in 64.51% of isolates. Production of ESBL was defected in 37 (64.91%) of enterobacteria. It ranged from 50% (lowest in Enterobacter spp.) to 80.95% in E.coli. Ceftazidime resistance was detected in 52 of all 62 gram-negative isolates tested (83.9%).

Resistance to ciprofloxacin and trimethoprim-sulfamethoxazole (TMP/SMX) was observed in 88.7% and 90.3% of isolates respectively. Among the aminoglycosides, amikacin showed better activity (resistance rate 30.6%) as compared to gentamicin (resistance rate 82.3%). Overall, the gram-negative isolates were high susceptible to imipenem and meropenem (resistance rate 3.2% and 1.6 respectively).

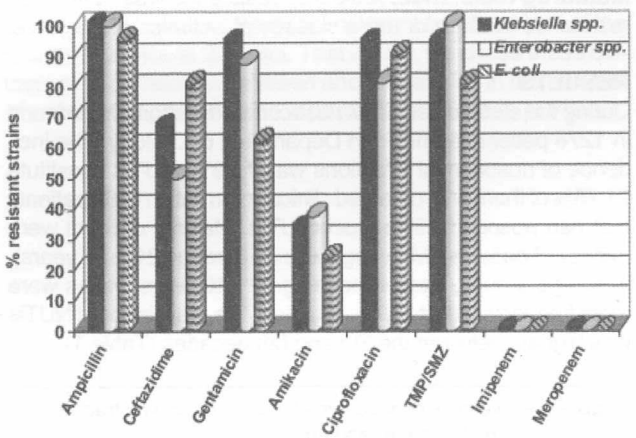

Figure 1. Resistance to antimicrobial agents among Klebsiella spp., Enterobacter spp., and E. coli

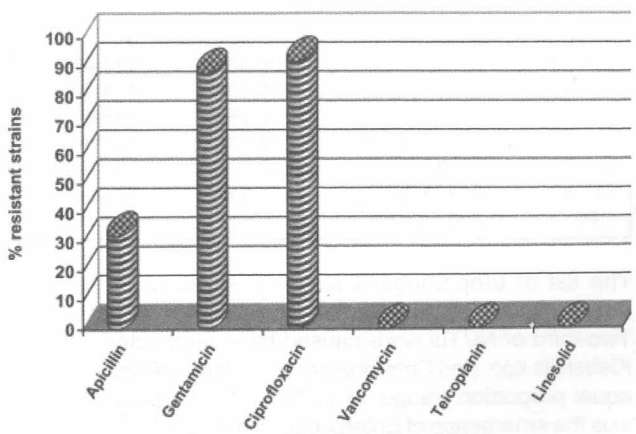

Figure 2. Resistance to antimicrobial agents among Enterococcus spp.

Enterococcus spp. were the prevalent gram-positive isolates. The most of them (68%) were susceptible to ampicillin. HLAR was observed in 22 of the 25 enterococcal isolates (88%). Resistance to ciprofloxacin was observed in 92% of the strains. One third of enterococcal isolates were multidrugresistant. All enterococcal strains were susceptible to glycopeptides and linezolid.

DISCUSSION

The high mean age of our patients reflects the hospital population. The increased prevalence of nosocomially acquired UTIs in the male group after sixty years might be due to the higher incidence of urinary tract pathology, like prostate diseases among this population. The female group has a more uniform distribution, and the elevated incidence between twenties and forties might be caused by the obstetric and gynaecologic causes.

Consistent with the results of numerous studies, this study reveal that gram-negative bacteria are still an important cause of NUTIs (7, 9, 12, 13). Enterococci play the leading role in development of NUTIs in our urologic population consisting of elderly patients with preceding surgical procedures and catheterization. There is a high prevalence of Enterococcus spp. (28%), the number of which doubled in comparison to our previous data (1). This phenomen is due to excessive use of cephalosporins as prophylactic and therapeutic agents in this setting. Our results correlate with data from other studies that report an increase in number of gram-positive bacteria and yeast as nosocomial UTI pathogens (4, 5, 8, 10, 18).

The results of this study show that more than half (53,9%) of NUTIs in the Department of Urology were caused by problematic pathogens (37 ESBLs producers, 3 polyresistant nonfermenters, 7 enterococcal HLAR strains resistant to ampicillin and ciprofloxacin, and one strain of C. krusei). Several studies have indicated that multidrug resistance was usually related to production of ESBL (3, 13, 15).

The percentage of ESBL producers among our isolates of Enterobacteriaceae was 64.91% and was higher compare to other recently published data (3, 7, 9). It reflects the overall epidemiological situation in University Hospital - Pleven with respect to ESBL production and a large hospital outbreak has been described (17). Beta-lactamase production has often occurred in parallel with an increase in resistance to TMP/SMX, ciprofloxacin, and gentamicin as seen in this study.

TMP/SMX was found to be ineffective for UTIs in the present study as all urophatogens showed high degree of resistance to it. A drug considered in the treatment of UTIs because of its concentrating ability in urine and high renal clearance is ciprofloxacin (16). However, a dramatic increase in the prevalence of fluoroquinolones resistance has been reported recently in several European countries (2, 9, 11, 18). The very high rate of ciprofloxacin resistance among both gram-negative (88.7%) and gram-positive (92%) organisms observed in our study warrants special attention. It could reflect the overuse of the quinolones for treatment of community acquired UTIs. The most effective antibiotics against gram-negative bacteria were amikacin, imipenem and meropenem. However, the emerging resistance to carbapenems described in the literature was found in 2 of 5 isolates of nonfermentative bacilli, posing a major problem in the management of NUTIs.

CONCLUSIONS

Etiologic shift in NUTIs and upsurge of antimicrobial resistance of these pathogens are impressive and alarming. Multidrug resistance is a common problem in our Department of Urology. This has important implication as patients in this setting receive cephalosporins, gentamicin, ciprofloxacin

or a combination of these drugs as empirical therapy or as definitive treatment. Thus, on the basis of our results use of mono-drug therapy with these agents should be guided only according to the susceptibility data. Ampicillin may still be considered as an appropriate agent for treatment of enterococcal infections in our department. Inappropriate usage of antimicrobials in surgical perioperative prophylaxis should be prohibited and a close collaboration between physicians, surgeons and microbiologists must be established.

In order to prevent or decrease resistance to antibiotics the use of antimicrobials should be kept under supervision, should be given in appropriate doses for an appropriate time and effective control programme for hospital infections should be carried out.

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A POSITIVE CRIMEAN CONGO HEMORRHAGIC FIVER CONTROL REACTIONS BY DIFFERENT MOLECULAR TECNIQUES

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ABSTRACT

Crimean - Congo hemorrhagic fever (CCHF) is a tick-borne disease caused by the arbovirus Crimean - Congo hemorrhagic fever virus (CCHFV). The disease now occurs sporadically throughout much of Africa, Asia, and Europe and results in an approximately 5-10% fatality rate. Bulgaria takes part of this endemic region and therefore prompt confirmation of CCHF diagnosis of the first suspected case is important. That is why, contemporary molecular techniques as Nested RT - PCR and Real time RT - PCR are needed. In this study, we present the elaboration and establishment of positive control reactions for CCHFV by the mentioned methods for the first time in our country.

Key words: Crimean - Congo hemorrhagic fever (CCHF), Nested RT - PCR, Real time RT - PCR, Positive control

INTRODUCTION

Crimean - Congo hemorrhagic fever (CCHF) is a severe tick-borne acute hemorrhagic fever found throughout Africa, propagating northward to Albania, the former Yugoslavia, Bulgaria, the Crimea, the former Soviet Union, Iraq, and much of the Middle East, as well as eastward to Pakistan and Western China. Clinically, the disease was well described from cases seen in the former Soviet Union. Nosocomial outbreaks were common among hospitalized patients, especially those undergoing surgery, infectious diseases and ear-nose-throat clinics. The case - fatality rate originally described in the Crimea was 15-30% (1); other outbreaks had case - fatality rates as high as 40% (3); in Bulgaria, during the period 1975-1996, mortality decreased to 5, 48% (4). However, in 2002, 54 cases were officially reported. Single cases usually occurred among shepherds or agrarians and small clusters among individuals involved in the slaughter of an infected animal (8). Nosocomial outbreaks emerged in the former Soviet Union, Kosovo, Albania, Bulgaria, South Africa and the Middle East (5). Therefore, the prompt confirmation of CCHF diagnosis of the first suspected case to avoid future spread of the infection is important for Bulgaria, as an endemic region.

The combined use of nested RT - PCR for detection of viral RNA and Complement Fixation Assay (CFA) for detection of specific IgG antibodies is the approach of choice for rapid and specific diagnosis of acute CCHF. We report here the first use in Bulgaria of this combination for diagnostic purposes. It is worth to note, that the Real time RT - PCR assays could be alternatively used instead of conventional nested RT - PCR which is time consuming due to detection of amplicons by gel electrophoresis. In this study, we present the elaboration of positive control reactions for Crimean Congo Hemorrhagic Fever Virus (CCHFV) by different molecular techniques.

ABBREVIATIONS USED IN THIS PAPER:
CCHF - Congo-Crimean halmorrhagic fever

ACCEPTED FOR PUBLICATION: 15. 01. 2009

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MATERIALS AND METHODS

Control materials: Hundred microliters of suspension containing CCHFV V42/81 previously isolated in the laboratories of BUL BIO - NCIPD were inoculated directly in the right part of the newborn mice skulls. One week later, the animals were autopsied, the brains mashed and collected in tubes with saline. The suspension enriched with CCHFV was inactivated, sublimated and stored at -70°C before the RNA extraction procedure.

Materials from patients and ticks: Sera and blood samples from 7 patients included in this study were tested in parallel for the presence of CCHFV. A total of eighteen ticks were collected from an endemic for CCHFV area (vicinity of Gotze Delchev).

RNA preparation: RNA was extracted from the mice brain suspensions, sera and blood from patients and from ticks by QIAamp® Viral RNA Mini kit (QIAGEN, Germany) and Ilustra RNAspin Mini RNA Isolation Kit (GE Healthcare). Viral RNAs were used for synthesis of complementary DNA (cDNA) and further amplification.

Molecular techniques - Polymerase Chain Reaction (PCR): Nested RT-PCR and Real Time RT-PCR: Two different protocols were used for the amplification of a fragment of the S segment of RNA genome from the CCHFV: Nested RT-PCR, using two sets of primers (F2-R3 and F3-R2) (7) and Real-time RT-PCR using one set of primers (F2-R3) (6). The kits used for nested RT-PCR were One-Step RT-PCR Kit, QIAGEN Germany and Illustra Ready-To-Go RT-PCR Beads, GE Healthcare. Conditions for optimal amplification are shown in tables 1 and

Table 1 and 2. Conditions for amplification of the first and second round of nested RT - PCR

Steps	First round
1	Denaturation at 95°C for 15 min.
2	Reverse transcription at 50°C for 30 min.
3	40 cycles
4	Denaturation at 95°C for 30 sec.
5	Annealing of primers at 50°C for 30 sec.
6	Elongation of the chain at 72°C for 45 sec.
Steps	**Second round**
1	Denaturation at 92°C for 2 min.
2	33 cycles
3	Denaturation at 94°C for 30 sec.
4	Annealing of primers at 41°C for 1 min.
5	Elongation of the chain at 72°C for 2min.
6	Final elongation at 72°C for 10min.

Table 3. Conditions for amplification of the real time RT - PCR

Steps	Conditions
1	50°C/10 min Synthesis of complementary DNA
2	95°C/5 min Inactivation of Reverse Transcriptase
3	95°C/10 sec.
4	55°C/30 sec.
5	Plate reading
6	Steps 3-5 are repeated 39 times
7	95°C/1 min.
8	55°C/1 min.
9	Melting curve analysis 55°C to 95°C; 0,5°C/read, 10 sec. hold
10	4°C Forever
11	End

2. For Real-time RT-PCR we used Script One- Step RT-PCR Kit With SYBR® Green (BIO-RAD). Conditions for the real time amplification are shown in table 3. In all cases the final reaction volumes were 50µl with 2µl of each primer.

RESULTS AND DISSCUSSION

To optimize the rounds of nested RT-PCR, we tested two systems for extraction and amplification (QIAGEN and GE Healthcare), both with similar results. Detection of PCR products was achieved by staining with ethidium bromide following electrophoretic separation on 2% agarose gel in 1X TBE buffer for 1 hour at 100V and visualization under UV illumination. Five hundred thirty four (534) bp amplicon size

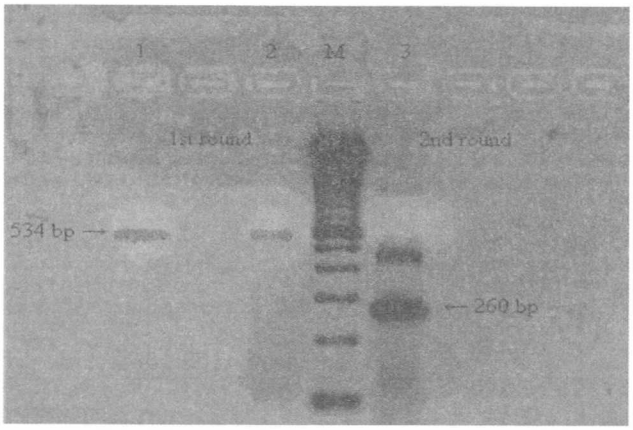

Figure 1. Nested RT-PCR. During the first round (amplicon size 534 bp; starts - 1 and 2) two positive controls were extracted and amplified by different systems. To increase the specificity of the PCR system, a second round was performed (start - 3)

indicated both presence of CCHF virus and the specificity of the primer system (Figure 1). Two hundred sixty (260) bp amplicon size should be expected after the second round

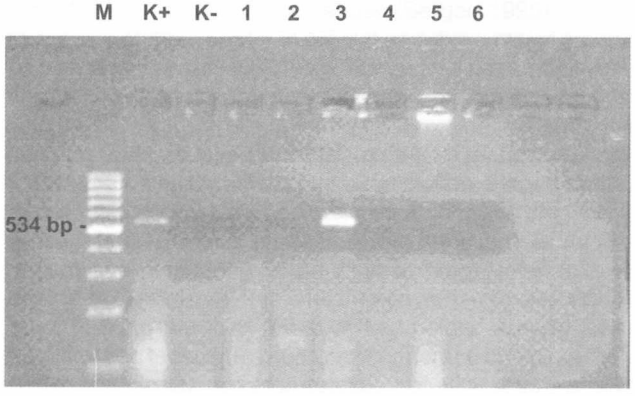

Figure 2. Electrophoretic separation on 2% agarose gel of PCR products from the S segment of CCHFV in mice brains (start 3); Marker (100 bp DNA Ladder, Fermentas Life Sciences; start M); K+ - positive control (start K+); negative control (non-infected mice brains; start K-); viral RNAs used to prove the specificity of the primers for CCHFV in remaining starts: rotavirus (start 1), calicivirus (start 2), flu viruses (starts 4-6)

of the Nested RT-PCR.

To prove the specificity of the primer system, various viral RNAs, kindly provided by the National Reference laboratories of flu and enteroviruses, were tested (Figure 2).

As a second part of this experiment, we describe a system for faster detection of CCHFV by one step real time RT-PCR, applied for the first time in Bulgaria. The method has a number of advantages in comparison with the conventional RT-PCR and nested RT-PCR, including limited possibility for contamination, higher sensitivity, specificity and time-saving in obtaining results.

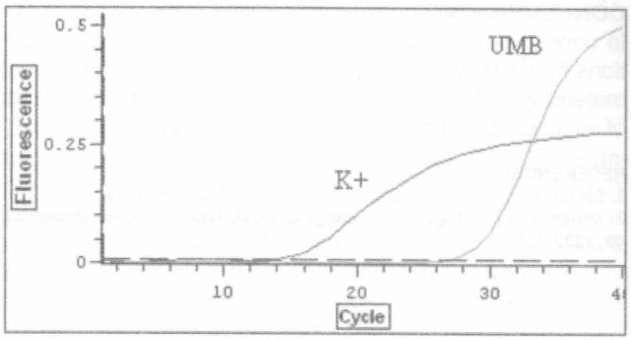

Figure. 3 SYBR Green I analysis of the intensity of the fluorescence. The fluorescent intensity of mismatched oligonucleotides might be recognized falsely as positive control. A melting curve analysis is needed. Positive control - (K+); uninfected mice brains - (UMB)

During the amplification, a detection of fluorescent signal was recorded (Figure 3). The positive control reaction (V42/81 sample) and the nucleic acid extracted from uninfected mice brains showed an increased signal, but in different cycles. We

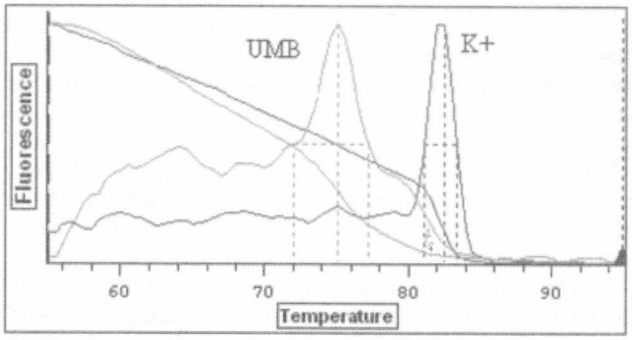

Figure 4. Usage of SYBR Green I melting curve analysis to demonstrate specific (positive control - K+) and non-specific (uninfected mice brains - UMB) amplification products

used a SYBR Green I dye which intercalates in any double stranded DNA (2). Therefore, the observed increase of the fluorescent signal in the healthy brains samples might be due to non-specific amplification products. To distinguish the desired product, a melting curve analysis was performed (Figure 4). Melting curve analysis is a well-established method for characterization of amplicons (9). It is generated by slow heating of the amplicon/probe heteroduplex and measuring the changes in fluorescence that result when the probe denatures. Different by size and GC content amplicons demonstrate different melting curve profiles. Figure 4 demonstrated the difference between the specific amplification products in the positive control and non-specific ones.

The final primers and probe concentrations used are described in the section „Materials and Methods" and were the same for all amplification kits. Sera and blood samples from 7 patients included in this study were tested in parallel for the presence of CCHFV. The results obtained after application of serological tests, nested RT-PCR and real time RT-PCR were discussed together. One of the samples examined was detected as infected with CCHFV only by molecular techniques. However, later in the course of disease, the patient seroconverted and IgG antibodies were detected. In other two samples, CCHFV was proved by both molecular and serological methods. Besides, a total of eighteen ticks were collected from an endemic area for CCHFV and examined along with the positive conventional RT-PCR control reactions. All ticks tested were negative for CCHFV.

CONCLUSIONS

In conclusion, the elaboration of the positive control reactions for CCHFV for the first time in our country by different molecular techniques will allow early differential diagnosis of suspected viral hemorrhagic fever patients.

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SYBR GREEN BASED REAL-TIME PCR ASSAY FOR DETECTION OF POLYOMAVIRUS JC

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SUMMARY

Reactivation and replication of polyomavirus JC (JCV) in the oligodendrocytes results in loss of myelin and causes progressive multifocal leucoencephalopathy (PML). In order that PML is diagnosed, both clinical and laboratory criteria have to be met. Real-time polymerase chain reaction (PCR) assays have been suggested as the method of choice, allowing rapid and specific detection of viral sequences in the tested materials. In order to support the work of clinicians and to benefit the patient's follow up we have introduced and approbated with specimens from the clinical practice a SYBR Green based system for detection of JCV in PML suspected cases.

Key words: PML, JCV, real-time PCR

INTRODUCTION

Lytic replication of the polyomavirus JC (JCV) in the oligodendrocytes causes progressive multifocal leucoencephalopathy (PML), which is a fatal central nervous system demielinating disease. The initial infection is usually subclinical, occurs during the childhood years and establishes a lifelong latency (Kalvatchev, 2007; Imperiale and Major, 2007). As impairment of cellular immunity is crucial for the viral reactivation PML was rarely seen in the pre AIDS era, but, the onset of HIV pandemic lead to an increasing number of PML cases. It has been shown that approximately 5% of all people living with HIV would develop the disease (Berger, 1998).

Diagnosis of the disorder is difficult and three major groups of criteria have been suggested. Although clinical manifestation lacks any specific symptoms, their presences altogether with multifocal lesions appearing on a magnetic resonance imaging (MRI), support a PML possible case. Brain biopsy followed by a histopathological examination shapes up the second group of criteria and can give a definitive diagnosis. Since it is an invasive procedure with serious risks for the patient and monitoring of the disease progression in time cannot be performed, molecular assays have been developed forming the third group of key points. Among them amplification techniques such as polymerase chain reaction (PCR) are largely used for detection of JCV sequences in cerebrospinal fluid (CSF) and quantitative real-time PCR has been suggested as the method of choice (Cinque, 2003).

We describe a SYBR Green based real-time PCR system for efficient detection of JCV in PML suspected cases that can be in support to clinicians in terms of diagnosis and patient follow up.

ABBREVIATIONS USED IN THIS PAPER:

PML - progressive multifocal leucoencephalopathy,
JCV - human polyomavirus JC, PCR - polymerase chain reaction,
CSF - cerebrospinal fluid, MCA - melt curve analysis,
Ct - threshold cycle

ACCEPTED FOR PUBLICATION: 10. 06. 2009

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MATERIALS AND METHODS

Clinical samples and study design

Brain biopsy derived DNA from a patient with post mortem histopathological conformation of PML was used as a positive control in all optimization procedures. Ten CSF samples from people with neurological disorders, which were considered PML possible cases, were tested in order to verify the application of the detection system in the clinical practice. Polyomavirus BK, SV-40, human papillomavirus 16 and human herpes virus 1 DNA were also tested to exclude non-specific amplification. Additionally a template free control was included in each run.

DNA extraction procedures

Aqua Pure® Genomic DNA Isolation Kit (BioRad) was used for isolation of DNA from the solid tissue sample, according to the manufacturer's recommendation. Quality and quantity of the extracted nucleic acid was assessed both with a spectrophotometer and an agarose gel electrophoresis. CSF DNA was extracted, purified and concentrated employing QIAamp DNA Mini Kit (Quiagen) after the instructions of the supplier.

Real-time PCR design

A primer pair named JCT 1 and JCT 2, constructed by MacKenzie et al., 2003 for specific detection of JCV by TaqMan based real-time PCR assay, was used in our SYBR Green system. The oligonucleotide sequence of the 5' primer JCT1 was AgAgTgTTgggATCCTgTgTTTT and gAgAAgTgggATgAAgACCTgTTT for the 3' primer JCT2, corresponding to positions 4298-4320 and 4352-4375 respectively in the JCV (J02226) GeneBank genome. The primers flanked a fragment from the gene encoding the viral regulatory protein large T-Antigen.

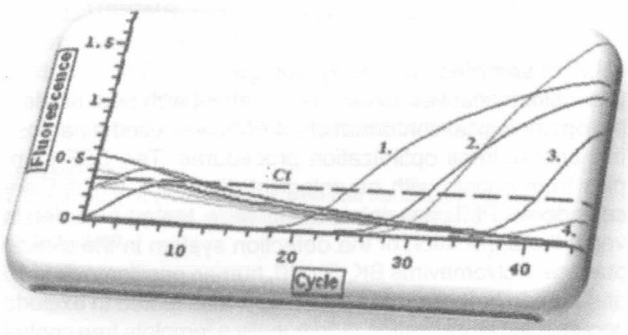
The PCR reaction mixture consisted of 25µl 2X Platinum SYBR-Green qPCR SuperMix-UDG (Invitrogene), 20 pmol of each primer, 1 µl ROX (as a reference dye) and approximately 0.5÷1.2 µg of whole genomic DNA in a 50 µl total reaction volume.

The positive control DNA was tested for amplification of the targeted viral sequence in the range from 55° to 65°C as a common annealing/extension temperature, performed in a DNA Engine Opticon 2 (MJ Research/BioRad). A 50 cycle programme was used with 10 min incubation at 95°C for initial denaturation, 95°C for 15 sec and a plate read at the end of each cycle. As SYBR Green I dye binds to any double-stranded DNA following the amplification protocol a dissociation analysis (melt curve analysis, MCA) was performed. It started with incubation at 95°C for 1 min for a complete separation of the DNA chains followed by 1 min at 55°C. Then the samples were gradually heated up with an increment of 0.3°C in the temperature range of 55° to 90°C and plate reads were taken after each step, resulting in a specific MCA curve and profile.

RESULTS AND DISCUSSION

A real-time SYBR Green PCR assays was designed by modification of a TaqMan system. Initially a highly specific and sensitive primer pair, described to detect as low as 10 starting copies and verified by MacKenzie et al., 2003 was employed, but in contrast to the original system the use of a Taqman probe was avoided. Thus, a reduction in terms of cost and complexity was achieved.

After optimization a distinct fluorescent signal with an earliest threshold cycle (Ct = 24) and best dissociation analysis profile was seen with an annealing/extension temperature of 60°C (Fig. 1).



Figire 1. Optimization PCR fluorescent graph. Curves designated as 1., 2. and 3. represent amplification with a different annealing/extension temperature and 4. is a group of negative controls

As SYBR Green is an intercalating dye it emits light when bound to any double-stranded DNA. In order to compensate the subsequent loss of specificity a MCA was designed and performed after each run. Furthermore, in comparison to TaqMan systems where MCA is not possible, it allows distinction of variances in the targeted sequence. The specific MCA temperature with a maximal decrease in the fluorescent signal (-dI/dT) was determined at 75.80°C (Fig. 2).

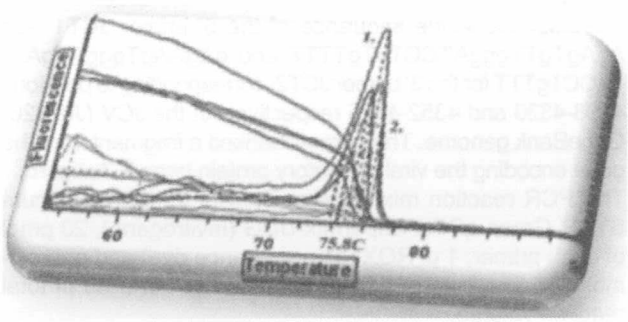


Figure 2. Melt curve analysis profiles of the positive control samples. A distinct peak was obtained for all controls at 75.8°C (2.), but had best characteristics with the control (1.) with an annealing/extension temperature of 60°C

No signal was detected in any of the non-JCV controls, including the blank sample. After the optimal reaction conditions were determined the use of the SYBR Green system was probated with samples from the clinical practice. All clinical CSF specimens were considered positive only when there was a characteristic increase in the fluorescence (Fig. 3) and a MCA profile identical with the one for the positive control was documented.

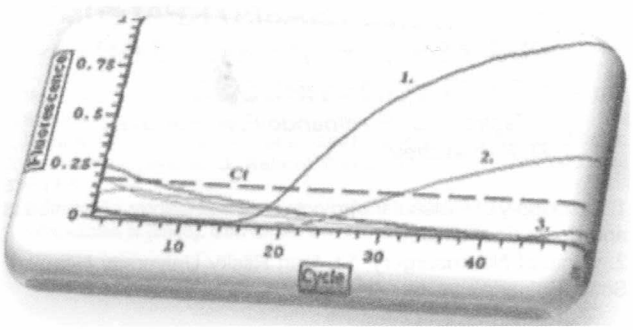


Figure 3. Amplification plot graph representing probation of the system with clinical samples. In increase in the fluorescent signal seen in both the positive control (1.) and a tested CSF derived DNA (2.). Amplification was not detected either in the negative control sample, or with other PML possible specimens (3.)

Out of the 10 samples tested, 2 were found JCV positive. In the case follow up all other 8 were diagnosed different than PML. When JCV was demonstrated in CSF the diagnosis possible PML case had its laboratory conformation, which was considered sufficient for this disorder (Cinque, 2003). All the other 8 samples were JCV negative, but when followed up the patients had a different diagnosis explaining their symptoms. These results indicate that our modified system can be readily introduced in support of the clinicians. Additionally, the qualitative system can be easily transformed to a quantitative requiring the presence of viral standards with known amount of starting copies. We believe that the application of a quantitative real-time PCR can benefit the monitoring of the disease progression and might be of use in determining a prognosis. Moreover, detection of large T-Antigen encoded sequences can be used in further studies concerning the associations of JCV with human malignancies.

ACKNOLEDGMENTS

The authors express their gratitude to the Council for Medical Science at the Medical University of Sofia for the support with a grant to research project No. 15 - 2009.

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HUMAN HEPATITIS VIRUSES B AND C AMONG HIV-INFECTED PATIENTS - CLINICAL AND EPIDEMIOLOGICAL FEATURES

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ABSTRACT

Due to overlapping transmission routes, some individuals living with HIV are co-infected with multiple hepatitis viruses: hepatitis B (HBV) and/or hepatitis C (HCV) virus. Concomitantly with the survival time increase after the introduction of HAART for more than 17 years, hepatitis C-related liver disease has become a leading cause of death in HIV/AIDS patients. Approximately 30% of HIV-infected individuals in the U.S. and Europe are also infected with HCV. Studies indicate that HIV-HCV co-infected patients experience more rapid progression of liver cirrhosis and hepatocellular carcinoma than those with HCV mono-infection. Some researchers have argued that HIV disease is accelerated by HCV-related immune activation, and HIV-mediated immune suppression stimulate HCV replication and impair immune-mediated HCV clearance.

The aim of the study was to point on HIV/HCV and HIV/HBV infected patients, and pose some questions concerning the investigation, treatment and prevention of the diseases.

Among 48 HIV-patients 24(50%) were HCV/HBV negative, 21(43.75%) were infected with HCV, 1(2.08%) - with HBV and 2(4.16%) with HCV and HBV concomitantly. The prevalence of the male young people and I.V. drug users is noted. Because HBsAg is rarely positive, an occult viral hepatitis B might be supposed.

The authors focused on the need of screening, management and counseling for HCV and HBV infected HIV-individuals, immunization of HBsAg negative and adequate treatment of HIV-HCV/HIV-HBV patients.

Key words: Human Hepatitis Viruses, HIV

INTRODUCTION

Due to overlapping transmission routes, viral hepatitis infections are crucial for the morbidity and the mortality among HIV-infected patients. Some of them and especially intravenous drug users (IDUs) are HIV/HCV, HIV/HBV or HIV/HCV/HBV co-infected.

More than 17 years after its introduction, the highly active antiretroviral therapy (HAART) has dramatically reduced AIDS-related illnesses and increased life expectancy for people living with HIV infection. At the same time, viral hepatitis C (VHC) is becoming an increasing problem and now accounts for a large proportion of VHC-related end stage liver diseases and deaths /14/. The HIV/HCV co-infected individuals are almost 30% of all HIV infected patients in USA and Europe /2, 14/.

The co-infected HIV/HCV individuals are faster progressing to cirrhosis and hepatocellular carcinoma versus mono-infected HCV ones /22/. HCV related immune activation leads to acceleration of HIV infection, while HIV mediated immune suppression stimulates HCV replication and worsens the immune-mediated HCV clearance /10, 20/. Some researchers have argued that HCV infection in IDUs occurs more likely after prior HCV-infection and following clearance, than in HCV-naive individuals, implying no increased immunity against further HCV re-infection /1/.

ABBREVIATIONS USED IN THIS PAPER:
HBV - Hepatitis B Virus; HCV - Hepatitis C Virus

ACCEPTED FOR PUBLICATION: 19. 04. 2009

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The very intriguing questions, concerning HIV/HBV interference, and suggesting the possibility for an occult viral hepatitis B in HIV/AIDS patients, as well as the subsequent risk for the AIDS progression and/or HAART increased hepatotoxicity worth discussing /4, 5, 6, 7, 9, 11, 12/.

The aim of this study was to analyze some HIV/AIDS patients infected with human hepatitis viruses B and/or C, and discuss the questions concerning their diagnosis, treatment and prevention.

PATIENTS AND METHODS

Forty-eight HIV/AIDS patients (35 men and 13 women at the age range from 18 to 67 years old) were tested for HBsAg and anti-HCV antibodies. Clinical and epidemiological studies including serological (ELISA) and laboratory (bilirubin, ALT, AST, AP, γ-GT, etc.) tests were done. Abdominal ultrasonography, pulmonal radiography, microbiological tests for opportunistic pathogens and the necessary ophthalmic and psychological consultations were made as well.

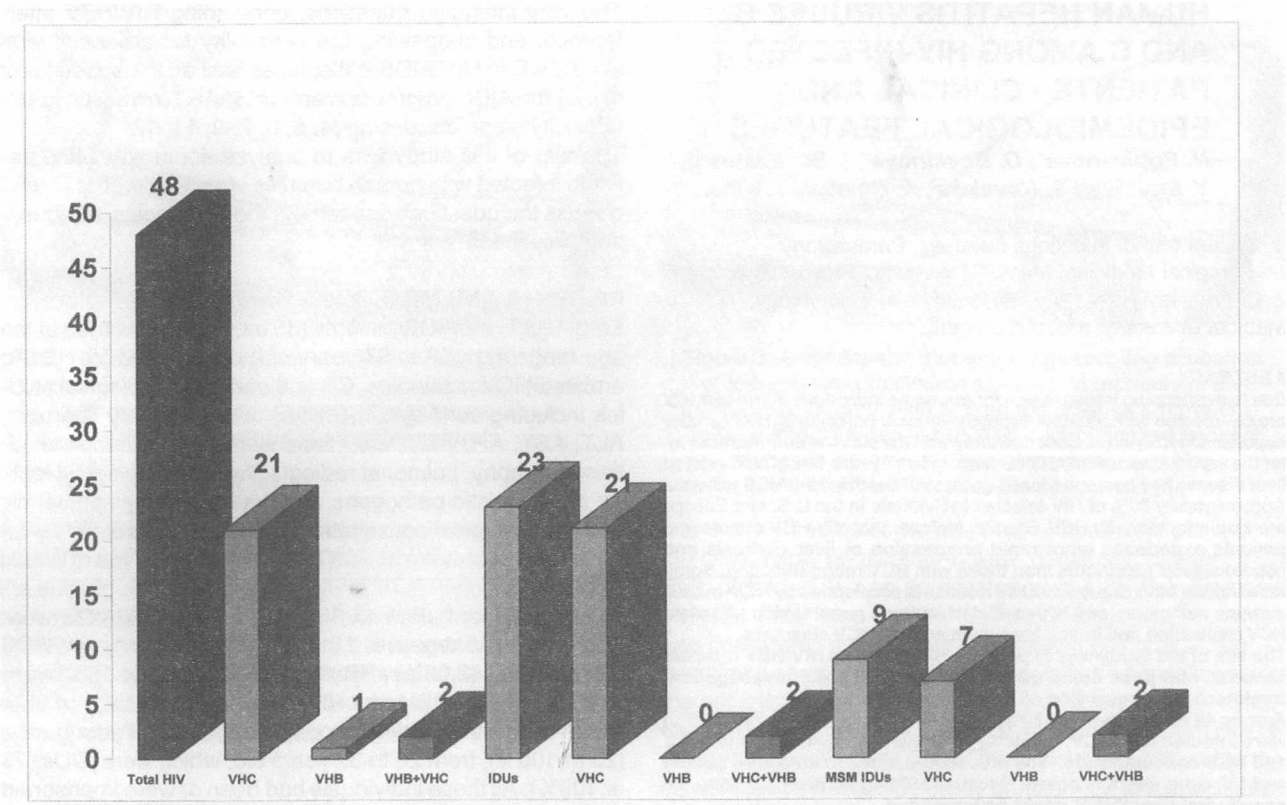
RESULTS

Twenty four, or half of all 48 patients with HIV/AIDS were HCV and HBV negative; 21 or 43,75% were only anti-HCV AB positive; 1 (2,08%) - HBsAg positive, and 2 (4,16%) were co-infected with HCV and HBV (Fig.1.). The cohort of anti-HCV AB positive patients consisted entirely of young men (23 or 100%), from 20 to 33 years old, which were IDUs (23 or 100%). All these individuals had been or were imprisoned at the time of the study and only one patient received ART, accordingly his clinical and monitored medical records. The HIV/HCV positive patients were anicteric and two of them had ALT/AST level twice the normal levels. Hepatomegaly and splenomegaly were usual for most cases in this group. Interestingly, HBsAg was rarely found among the HIV and HIV/HCV infected patients. There were two HBsAg and anti-HCV AB positive individuals, and one just HBsAg positive. The first two were homosexuals and the latter was heterosexual man. One of the HBsAg positive cases undergone a severe icteric form of viral hepatitis. Interestingly, in the study there were no HIV positive women infected with HBV/HCV, respectively there were no confessed IDUs among them.

DISCUSSION

From a cohort of 48 studied HIV patients 50% do not carry hepatitis B and/or C markers, i.e. HBsAg and/or anti-HCV AB. All 23(47.91%) HCV positive patients (with two of them HIV/HBV/HCV co-infected), were young men, former or present IDUs that were or had been imprisoned for a different period at the time of examination. This condition could be explained with HCV parenteral rout of transmission among young, lower-class roma-individuals, living in poor neighborhood areas and with low levels of education. Nine individuals from this group were men who have sex with men (MSM) and their number could be even bigger. Only one of these patients had the proper indications and was treated with HHART and methadone for drug withdrawal at the same time. A number of the mentioned patients had HHART indications too, but never presented in the infection clinic word for initiating and following treatment. The two HIV/HCV/HBV positive patients were MSM and IDUs at the same time, which could explain their infection with the two hepatitis viruses. The only HBV/HIV patient was heterosexual man with multiple sex partners.

We searched the available published data to discuss the specificity of the HIV/AIDS individuals infected with hepatitis viruses, as well as the mechanisms of this processes, their treatment and prevention.



HIV/HCV coinfection: Many scientific trials have been carried out in the field of HCV infection among HIV /+/ patients during the last few years. HIV/HCV co-infection is most prevalent among IDUs. The rates of HIV/HCV co-infection approach 90 to 95%, especially in Asia/Pacific region /10/. Our data about HIV/HCV co-infection among the IDUs correlate with the above information. The uncontrolled HIV viremia and the immune deficit associate with the accelerated progression of hepatic disease. The progress to chronic hepatitis reaches more than 90% in these patients and the HCV-RNA viral load is higher especially among patients with increased immuno-suppression /10, 14/. The HCV/HIV co-infected patients are with a rapid train of liver injury progression to cirrhosis, end stage liver disease, and hepatocellular carcinoma /20/. These conditions are the mane causes of death even in cases with an AIDS defining CD4+ T cell count of >200 cells/μL /22/. In addition, co-infected individuals may have altered immunological responses to HAART and are at increased risk of highly active antiretroviral therapy related hepatotoxicity. The standard treatment for HIV-patients with chronic HCV infection includes the combination of pegylated IFN-alfa and ribavirin, which does not differ from that of HCV mono-infected patients /17/. Recent international guidelines for management of HIV/HCV co-infection recommend all these patients should be estimated for treatment of hepatitis C infection /8, 23/. These individuals usually have a complex therapeutic, social and psychiatric status with alterations in their cognitive and mental functions /20/. The sustained viral response to HCV therapy in HIV/HCV patients is often 10-15% lower compared with the HCV mono-infected individuals. It is suggested that the HCV genotype defining, and the HCV-RNA levels as well as the liver injury histological stage determining can contribute to better prediction of treatment issue /21, 22, 23/. This treatment should precede the HAART therapy but not delay or cancel it /17/. Recently, the impact of antiretroviral drugs on response to anti-HCV therapy in HCV/HIV co-infected patients were largely discussed /23/.

HIV/HBV coinfection: HIV/HBV co-infection is due to the shared way of transmission of the two viruses, however HBV is 10 times less efficiently transmitted by sexual intercourse than HIV /15/. Isolated anti-HBc antibodies are more frequently detected in HIV infected individuals /6, 21/. Accordingly to some reports it occurs in 98% of the tested patients /6, 16/. HBV may persist in serum and liver in very small quantities (102-103 copies/mL) in HBsAg-negative patients /4, 5, 18, 24/. This phenomenon called "Occult infection" has frequently been identified in patients with isolated presence of anti-HBc antibodies and was rarely described in cases negative for all HBV markers /4/. It has been documented that liver allograft from HBsAg negative, anti-HBcAB positive donors can transmit HBV to uninfected recipients /9, 25/. Probably the cccDNA serves like a reservoir for reactivation of HBV among patients with immunosuppression due to HIV infection /11, 12, 13, 19/. As a result of an association with the immune down-regulation, the concomitant HIV infection modulates the clinical expression in each stage of the HBV-related disease /15/. It is unclear if the presence of occult HBV infection may lead to progression of the illness, or just increases the risk for HAART hepatotoxicity. For the moment, it is not recommended to treat patients which are only anti-HBcAB positive and with extremely small quantities of HBV-DNA. The role of the HBV-vaccination in individuals with isolated anti-HBcAB is unclear. The isolated presence of anti-HBcAB cannot be interpreted like an evidence for previous HBV infection and HBV immunity since the HIV infected patients reveal an accelerated loss of anti-HBsAB /3/ and this could explain the frequently met existence of isolated anti-HBcAB in these cases. If the level of the protective antibodies after immunization is not satisfactory, HBV-DNA should be examined to determine the presence of occult HBV co-infection /13/. An important step in preventing co-infection and reducing mortality from HBV-related liver disease is immunization against HBV. The use of antiretroviral medications with dual activity against HBV and HIV is hoped to lead to decreasing trends in HBV-related morbidity and mortality in co-infected patients /2, 26/.

CONCLUSIONS

We consider that study as preliminary one, since our investigations among abruptly increasing number of HIV infected patients in Plovdiv and neighborhood regions continues. We focused on the need of screening, management and counseling for HCV and/or HBV infected HIV-patients, immunization of HBsAg negative and adequate treatment of HIV/HCV and/or HIV/HCV/HBV chronically infected patients. For this purpose, HIV-patients need the attention of the infection disease concomitantly with the gastroenterological clinical setting wards.

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PROTECTION OF THE ADULTS AND ADOLESCENTS AGAINST DIPHTHERIA, TETANUS AND WHOOPING COUGH IN BULGARIA

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SUMMARY

The investigation is done during the period 2001-2008 in Sofia. The aim of this study is estimation of immune status of population against diphtheria, tetanus and pertussis. For investigation were tested 9 934 human sera above 7 years of age. The ELISA methods developed in BB - NCIPD, Ltd are used for quantity assay. The comparison during the investigation period showed that the immunity against tetanus in different years in constantly levels. The results demonstrated that the full protected people are about 65%, the people with basic immunity are between 30% and the non protected sera are in up to 6%. The summarized results for diphtheria protection showed the persistence of immunity. The full protected people are more than 65%, the persons with basic immunity are about 10% and the sera with titers less than the protected level are less than 25%. The serological studies for pertussis showed that the best protection had seen in people up to 25 years old (more than 85%). The increase of age in people leads to reduction of the percent of persons with full protection and increase of sera with basic immunity and non-protected people. 55% of the tested sera in age group over 56 years old are non- protected because the vaccination program in Bulgaria started since 1958. The prevalence of pertussis antibody in various age groups in the general population depends on the status of immunization against pertussis in childhood. Present results indicate a good immune status of the population against diphtheria, tetanus and pertussis in Bulgaria. This fact is a result of specific immunoprophylaxis by bacterial vaccines produced by BB - NCIPD, Ltd., Sofia.

Key words: diphtheria, tetanus, pertussis, whooping cough, ELISA, immune status

INTRODUCTION

The diseases caused from Corynebacterium diphtheriae, Clostridium tetani and Bordetella pertussis are still a serious problem in many countries with significant morbility rates (15). The only way to limit it is the immunization program for adolescents and adults following international recommendations. The obligatory specific prophylaxis against the diseases in Bulgaria started in 1959. The specific immunoprophylaxis of humans with diphtheria toxoid, tetanus toxoid and pertussis antigens as a component of mono or combine bacterial vaccine leads to production of specific antibodies that have main role in diseases prevention (16). For evolution of immunization procedures and the vaccine itself, antibody levels against diphtheria toxin, tetanus toxin and pertussis are useful to show the immune status of the population. However, the biological assay of diphtheria and tetanus toxin antibodies in human serum is not useful for seroepidemiological purposes, regardless of its sensitivity and reproductivity (5, 6, 21, 22). Laboratory verification of whooping cough, which is almost exclusively based on isolation of the bacterial strain, is associated with several problems (13, 15, 24). Therefore attempts have been made to develop serological assays (3, 8, 4, 5). However, the agglutination - type assay to study to assess the immune response to pertussis vaccine and Bordetella pertussis infection in human serum is not practical for seroepidemiological and diagnostic purposes, regardless of its sensitivity and reproductivity (6, 12, 18). Direct immunofluorescent antibody staining of nosopharyngeal secretions has been used for diagnosing whooping cough, but this test lacks specificity, and false - positive results of up to 40% have been described (1). The ELISA method is simple, economic and precise and permits to perform rapidly the levels of antibodies in humans (7, 11, 20).

Knowledge of the immune status of population against diphtheria, tetanus and pertussis has big practicable implication (15, 17, 19, 20). It may assist in checking the efficacy of immunization schedules adopted and the persistence of immunity. Seroepidemiological investigations, performed in various European countries (9, 10,14) and, in particular, in Sweden (17) and Russia, have demonstrated that in a high percentage of the population, even in the younger age groups, diphtheria antitoxin titer, tetanus antitoxin titer and pertussis antibody titer is below the level considered to confer protection against these deseases. Similar data have also been reported for the USA (5, 15).

In this paper we present our results of estimation of the immune status of the population by ELISA methods for immunoglobolin G antibodies against diphtheria and tetanus toxin, and whooping cough in human sera.

ABBREVIATIONS USED IN THIS PAPER:

Lf - Limes of flocculation, PBS - phosphate buffered saline, OPD - ortophenilenediamine, PN - protein nitrogen

ACCEPTED FOR PUBLICATION: 12. 04. 2009

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MATERIALS AND METHODS

Population studied

The epidemiological study on tetanus was carried out on 9 934 subjects and for diphtheria and pertussis on 5 887 subjects, selected by Hygiene Epidemiological Inspection, Sofia, Bulgaria. Sera separated from clotted blood were stored at - 20°C in small vials and were tested for diphtheria antibodies using ELISA method. According to person's age serum samples were divided in following groups - young people between 7-15 years old, adults between 16-25 years old, adults from 26 to 35, adults from 36 to 45, adults from 46 to 55, adults from 56 to 65 years old and persons over 65 years. The number of sera are performed on Table 1 and Table 2.

The observed results for diphtheria and tetanus were classified depend on titer obtained in following subgroups: subgroup with antibody level up to 0.001 IU - negative; subgroup with antibodiy level between 0.002 to 0.009 IU- non protected; subgroup with level of antibodies between 0.01 to 0.09 IU- sera with basic immunity and subgroup with level of antibodies more than 0.1 IU - sera with full protection (9, 25).

The obtained results for pertussis were classified depend on the titer of antibody in following subgroups: subgroup of sera with the level of antibody up to 1:80 - group of non protective people; subgroup with level of antibodies against pertussis between 1:81 to 1:160 - sera with basic immunity, subgroup with level of antibodies against pertussis between 1:161 - 1:320 - sera with full protection and subgroup with level of antibodies against pertussis more than 1:321, human sera with titer used as a criteria for disease (13).

Biological assay

The antibodies against diphtheria toxin were tittered by the guinea pigs intradermal test according of the technique of Rommer. For this purpose diphtheria toxin (batch 3, National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria) was tittered against 10 IU of the Anti - diphtheria Standard Serum (Internationally Laboratory of Biological Standards, Copenhagen, Denmark). Serum dilutions were incubated with the toxin for 30 minutes at 37°C and 0.1 ml of the mixture was intradermally inoculated in guinea pigs. Readings were done after 120 h with reference to the standard mixture

in concentration 0.002 IU inoculated at the same time. The highest dilution of toxin - antitoxin mixture, which produce nonnecrotic erythematous area after 120 hours is the titer.

Bacterial Agglutination (BA) Test

The BA test was the first method developed to measure pertussis antibody and it is still the most frequently used method. Bordetella pertussis suspension (serotype 1, 2 and 3), (batch 36, National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria), dued and inactivated with 0.01% thiomersal was used in BA test. Diluting the stock suspensions to opacity 10 OUs made working dilutions. Sera samples were prepared in following concentrations: 1:10; 1:20; 1:40; 1:80; 1:160; 1:320; 1:640; 1:1280; 1: 2560. The level of antibody is measured after 24 hours incubation at 37°C and following incubation for 24 hours at room temperature. The last dilution of human sera with agglutination is a titer of antibody against pertussis.

Toxin - binding inhibition test

The round-bottomed polystyrene (PS) micro-titration plates for preincubation of serum dilutions and antigen mixtures were blocked by filling each well with 150 µl block buffer. Plates were coated and incubated at 37°C for 1 hour. Then wells were washed tree times with washing buffer. All serum samples were prediluted to a concentration of 1:100 in diluent and transferred to the corresponding wells of the PS micro-titration plates (1-10). Columns 11 and 12 are used as positive and negative controls. Well H1 was filled with undiluted control serum with known level of tetanus antibodies. Twofold dilutions of control serum were made in wells H 1 - H 10. Purified tetanus toxoid was diluted to a concentration of 0.3 Lf/ml in PBS and 40 µl were added to all wells except those for negative control (A12 - H 12), where 40 µl PBS were added. Plates were shaken gently, covered and incubated overnight in a humid atmosphere at 37°C.

ELISA

Polystyrene plates with U - shaped 96 wells (Nunc immuno-plates, Denmark) were coated with purified diphtheria toxoid or purified tetanus toxoid or pertussis antigen (1, 2, 23).

Test procedures: 100 µl volumes blocking buffer - PBS containing 1% BSA (Bovine Serum Albumin) were added to the wells and incubated at 37°C for 1 hour. 100 µl volumes of serum dilutions in PBST were added to the wells of sensitized plates, incubated at 37°C for 120 minutes and washed three times in PBST. 100 µl volumes of peroxidase anti - human immunoglobolin G (National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria) diluted in PBST was added to each well, followed by incubation at 37°C for 60 minutes. The plates were washed three times with PBST. 100 µl volumes of a chromogen solution (10 mg of ortophenilenodiamine and 10 µl of 30% hydrogen peroxidase in 50 ml citrate - phosphate buffer pH 5.0) were added to the wells of plates and than were left at room temperature. The reaction was stopped after 30 minutes by adding 100 µl of 1 N H_2SO_4.

The absorption of each wells of plates were directly read at 405 nm in a MicroELISA Minireader Photometer (Bio - Tek). Curve with dilutions of control serum was prepared for each experiments.

RESULTS AND DISCUSSION

The results of protection against tetanus in human sera are performed on Table 3. The 99.36% of persons in age group between 7 to 15 years old are protected. The 87.26% of them showed full protection. In this group the negative and non-protected people are 0.64%.

The patients between 16 to 25 years old showed that 97.62% of human sera had titer of antigen against tetanus more than 0.01 IU. The 27.0% of them have basic immunity. The non protected people are 2.38%.

The sera with protected titers of antibodies in group of age between 26 to 35 are 96.15%, in group of age between 36 to 45 are 96.75%, in group of age between 46 to 55 are 96.85% and in group of age between 56 to 65 are 95.82%. The human sera with titer up to 0.009 IU in group of age between 26 to 35 are 3.85%, in group of age between 36 to 45 are 3.25%, in group of age between 46 to 55 are 3.45% and in group of age between 56 to 65 are 4.18%. The full protected people are among 58-87% in different groups.

The persons over 65 years old demonstrated good titers of antibodies against tetanus. The obtained results showed that only 5.31% of sera are negative and non-protected. The tested sera with full protection is 58.27% and with basic immunity are 36.42%.

The comparison during the investigation period showed that the immunity in different years in constantly levels. The results performed on Figure 1 demonstrated that the full protected people are about 65 - 75%, the people with basic immunity are between 25-35% and the non protected sera are in up to 6%.

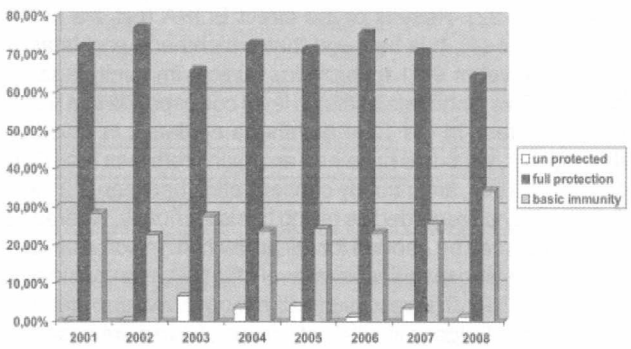

Figure 1. Determination of antibodies level against tetanus in human sera during the period 2001-2008

The present results of immune status of population against tetanus has big practicable implication and demonstrated the efficacy of immunization schedules applied in the country.

The results of immunity against diphtheria in human sera are performed on Table 4. All persons of age group between 7 to 15 years old have full protection. In this group the negative and non-protected people were not found.

The patients between 16 to 25 years old have a good protection against diphtheria. The titers obtained after assay showed that 86.45% of tested humans had titer of antigen more than 0.01 IU. In 13.55% the titer was less than 0.009 IU. 79.0% of the people on this group have full protection. Only 7.45% of sera have basic immunity.

The analyses of immune status of the population in rage from 25 to 65 years old showed decrease of the protection against diphtheria disease connected with the increase of the age. The sera with titer of antibodies more than 0.01 IU in group of age between 26 to 35 are 81.15%, in group of age between 36 to 45 are 76.75%, in group of age between 46 to 55 are 62,7% and in group of age between 56 to 65 are 36.55%. The human sera with titer up to 0.009 IU in group of age between 26 to 35 are 18.55%, in group of age between 36 to 45 are 23.25%, in group of age between 46 to 55 are 38.25% and in group of age between 56 to 65 are 63.45%. The full protected people are among 27-79% in different groups.

The persons over 65 years old have very low titer of antibodies against diphtheria. The obtained results showed that only 25.7% of serums have protection against diphtheria and 73.3% are negative and non-protected. Only 6.85% of tested sera are full protected.

The summarized results during the period 2001-2008 performed on Figure 2 showed the persistence of immunity. The full protected people are between 55-75%, the persons with basic immunity are in range between 8-10% and the sera with titers less than the protected level are less than 25%.

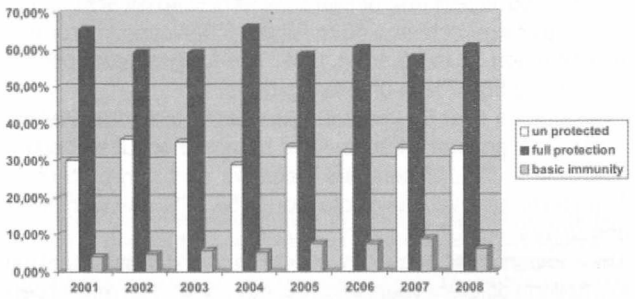

Figure 2. Determination of antibodies level against diphtheria in human sera during the period 2001-2008

The ELISA involves the blinding of antigen to polystyrene tubes. Exotoxins, like diphtheria toxin or toxoid, that have highly lipophilic moiety in their molecule, coat the tubes efficiently (8, 22). Results of the direct ELISA test are highly reproducible (4). It is believed that a diphtheria and tetanus antitoxin level of 0.01 IU provides clinical immunity against disease. This diphtheria antitoxin level corresponds to a negative Shick test. In the 1984 diphtheria epidemic in Sweden, the patients, who died have had antitoxin titers less than 0.01 IU (4, 17). Thus, an antibody concentration between 0.01 and 0.09 IU may be regarded as giving basic immunity, whereas a higher titer may be needed for full protection. In some studies that used in vitro techniques, a level of 0.1 IU was considered protective (3, 7). The results of estimation of immune status of population in Bulgaria in different groups of age were based on the same criteria. The data, presented on Table 4, showed that the best protection against diphtheria had seen in children and teenagers younger than 15 years old. The increase of age in people leads to reduction of the percent of protected persons, excpecialy in adults older than 45 years and to increase the percent of non-protected people. Independently of this, the percent of protected population in groups of age between 16 to 65 years old is better than some European countries and of the USA (3, 7, 17, 22). The patients over 65 years old had a very low levels of antibodies against diphtheria and in about 73% lacked protection against diphtheria disease.

The results of direct ELISA for pertussis on human sera of age between 7 to 15 years old demonstrated that 30.7% of persons have full protection, 62.5% have basic immunity and the antibody's level against pertussis in 4.5% of human sera is high than 1:321. The non-protected people in this group are 2.45%.

The patients between 16 to 25 years old have a good protection against pertussis. The titers obtained after assay showed that 74.7% of human sera had titer of antigen defended a basic immunity, 16.55% of the tested sera on this group have full protection and in 4.5% of the people the titer of antibody is on the levels, which used as a criteria of passed disease. Only 4.25% of sera are non-protective.

The analyses of immune status of the population 25 to 55 years old showed decrease of the titers of antibody against whooping cough compared with the younger groups. The sera with titer of antibodies from 1:81 to 1:160 in group of age between 26 to 35 are 33.55%, in group of age between 36 to 45 are 57.7%, and in group of age between 46 to 55 are 54.8%. The human sera with titer from 1:161 to 1:320 in group of age between 26 to 35 are 38.75, in group of age between 36 to 45 are 21.65% and in group of age between 46 to 55 are 16.65%. The non-protected sera in group of age between 26 to 35 are 20.3%, in group of age between 36 to 45 are 16.1% and in group of age between 46 to 55 are 19.5%.

The persons over 56 years old have very low titer of antibodies against whooping cough. The obtained results showed that only 43.71% of sera have protection against pertussis and 53.25% are non-protected.

Summarized results of serological studies for eight years, presented on Figure 3, showed the persistence of immunity and that the best protection against pertussis had seen in people up to 25 years old (more than 85%). The increase of age in people leads to reduction of the percent of persons with full protection and increase of sera with basic immunity and non-protected people. 55% of the tested sera in age group over 56 years old are non-protected because the vaccination program in Bulgaria started since 1959. The prevalence of pertussis antibody in various age groups in the general population depends on the status of immunization against pertussis in childhood.

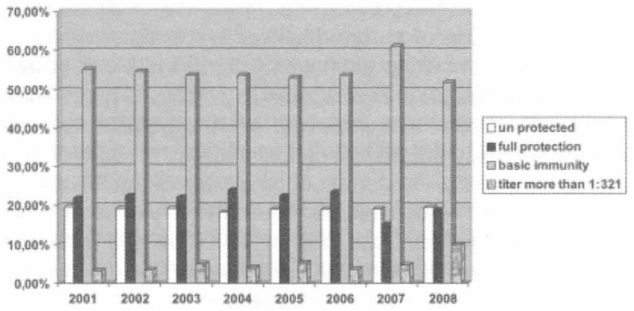

Figure 3. Determination of antibodies level against whooping cough in human sera during the period 2001-2008

The antibody response in unvaccinated patients was with increased the IgG titers. The low percent of children with titer more than 1:321 in 4% of human sera demonstrated that disease has been effectively controlled by vaccine prophylaxis. All of sera have good level of protection against whooping cough and non-protected patients were not found. Previous pertussis vaccinations of patients with whooping cough may interfere with the antibody response to the natural disease (21). Granstorm et al. (10, 11) found that the antibody response in unvaccinated people different in their vaccinated counterparts: unvaccinated children had an early increase in Ig M titers and late Ig G response (more than 1:131), whereas most vaccinated children and adults had a secondary type response with an early increase in Ig A and the level of Ig G antibody is until 1:320 (12, 13).

The ELISA involves the blinding of bacterial cells to polystyrene tubes. Results of the direct ELISA test are highly reproducible (4). It is believed that a pertussis antibody level of 1:81 provides protection against disease. The titers of antibody from 1:161 to 1:320 showed full protection of people against whooping cough and titers over 1:321 are used as a criteria of disease or used as a criteria of passed disease (7). The results of estimation of immune status of population in Bulgaria in different groups of age were based on the same criteria.

The epidemiological situation observed in Bulgaria during the investigation period showed that in result of vaccination program the protected people are about 55% and the humans with basic immunity in amount of 20%. The un protected sera are about 25%.

Present results indicate a good protection against diphtheria, tetanus and whooping cough in Bulgaria. This fact is a result of specific immunoprophylaxis by mono and combined bacterial vaccines. The high levels of diphtheria toxin antibodies in population up to 45 years old (more than 60% with full protection) is a result of revaccination of adults till the age of 35 years as recommended by the Bulgarian immunization program.

Table 1. Number of human sera tested for tetanus protection

Age groups	2001	2002	2003	2004	2005	2006	2007	2008
7 - 15	0	155	179	73	53	118	69	25
16 - 25	187	338	370	82	211	325	218	44
26 - 35	217	665	710	108	178	285	196	31
36 - 45	74	480	639	63	148	126	242	33
46 - 55	53	310	280	35	115	84	126	35
56 - 65	18	429	310	28	92	64	147	10
66 - 75	16	226	188	27	75	58	52	6
More than 75	4	175	221	10	68	84	48	4
Total				9 934				

Table 2. Number of human sera tested for diphtheria and whooping cough protection

Age groups	Number of human sera
7 - 15	609
16 - 25	1 977
26 - 35	1 766
36 - 45	758
46 - 55	581
More than 56	496
Total	5 887

Table 3. Estimation of immune status of population against tetanus in period 2001-2008

Age groups	2001-2008			
	Number of sera	% un protected people < 0.01 AU/ML	% full protection > 0.1 AU/ML	% people with basic immunity 0.01-0.09 AU/ML
7 - 15	672	0.64	87.26	12.10
16 - 25	1 775	2.38	70.62	27.00
26 - 35	2 390	3.85	65.46	30.69
36 - 45	1 805	3.25	55.91	40.84
46 - 55	1 038	3.45	73.42	23.13
56 - 65	1 098	4.18	61.72	34.10
Over 66 years old	1 156	5.31	58.27	36.42
Total		9 934		

Table 4. Estimation of immune status of population against diphtheria in period 2001-2008

Age groups	2001-2008			
	Number of sera	% un protected people < 0.01 AU/ML	% full protection > 0.1 AU/ML	% people with basic immunity 0.01 - 0.09 AU/ML
7 - 15	609	-	100.0	-
16 - 25	1 977	13.55	79.0	7.45
26 - 35	1 766	18.85	76.0	5.15
36 - 45	758	23.25	73.85	2.9
46 - 55	581	38.25	60.95	1.75
56 - 65	496	63.45	27.55	9.0
Over 66 years old	185	73.3	6.85	19.85
Total		5 887		

Table 5. Estimation of immune status of population against pertussis in period 2001-2008

Age groups	2001 -2008				
	Number of sera	% full protection 1:161 - 1:320	% people with basic immunity 1:81 - 1:160	% unprotected people < 1:80	% > 1:320
7 - 15	609	30.7	62.55	2.45	4.3
16 - 25	1 977	16.55	74.7	4.25	4.5
26 - 35	1 766	38.75	33.55	20.3	7.4
36 - 45	758	21.65	57.7	16.1	5.05
46 - 55	581	16.65	54.8	19.5	8.55
Over 56 years old	506	0.6	43.65	53.25	3.5
Total			5 887		

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ESTIMATION OF IMMUNE STATUS OF THE CHILDREN UP TO 6 YEARS OF AGE AGAINST DIPHTHERIA, TETANUS AND WHOOPING COUGH IN BULGARIA

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SUMMARY

The investigation is done during the period 2001-2008 in Sofia. The aim of this study is estimation of immune status of infants and children up to six years of age against diphtheria, tetanus and pertussis. The ELISA methods developed in BB - NCIPD, Ltd are used for quantity assay. The comparison during the investigation period showed that the immunity against tetanus in different years in constantly levels. The results demonstrated that the full protected children are about 86%, the people with basic immunity are more than 13% and the non protected sera are in up to 0.26%. The summarized results for diphtheria protection showed the persistence of immunity. The full protected children are more than 96%, the persons with basic immunity are about 0.25% and the sera with titers less than the protected level are less than 3.6%. The serological studies for pertussis showed that more than 53% of persons have full protection against whooping cough and in more than 43% of children the obtained titters showed basic immunity. In 2.88% of patients the titter of antibody is more than 1:321, which titter is used as a criteria of disease of human sera. Only in 0.25% of tested sera the level of antibodies is not enough for protection. Present results indicate a good protection against diphtheria, tetanus and pertussis in Bulgaria in children till 6 years old. This fact is a result of specific immunoprophylaxis of infants and children up to 2 years of age by DIFTETKOK (DTP) combined bacterial vaccine, produced by BB - NCIPD Ltd., Sofia Bulgaria.

Key words: diphtheria, tetanus, pertussis, whooping cough, ELISA, immune status

INTRODUCTION

Whooping cough - or pertussis - is an infection of the respiratory system caused by the bacterium Bordetella pertussis (or B. pertussis) (21). It's characterized by severe coughing spells that end in a "whooping" sound when the person breathes in. Before a vaccine was available, pertussis killed 5,000 to 10,000 people in the United States each year. Now, the pertussis vaccine has reduced the annual number of deaths to less than 30. But in recent years, the number of cases has started to rise. By 2004, the number of whooping cough cases spiked past 25,000, the highest level it's been since the 1950s (10). The diseases caused from Corynebacterium diphtheria and, Clostridium tetani are still a serious problem in many countries with significant morbility rates (6, 14, 24). The only way to limit it is the immunization program for adolescents and adults following international recommendations. The obligatory specific prophylaxis against the diseases in Bulgaria started in 1959. The morbidity rates of whooping cough in Bulgaria reduced from 12.90‰ children in 1955 to 3.25‰ in 2008. It's mainly affected infants who are younger than 6 months old before they are adequately protected by their immunizations, and kids who are 11 to 18 years old whose immunity has faded. Although whooping cough can occur at any age, it's most severe in un immunized children and in infants under 1 year of age (early immunization can usually prevent this serious disease in babies) (12). But more cases have been reported in teens and adults, because their immunity has faded since their original vaccination. The morbidity rates of diphtheria in Bulgaria reduced from 42.0‰ in 1952 to 0 in 2008 and for tetanus from 4.8‰ in 1955 to 0 in 2008.

If whooping cough is suggested as a diagnosis it is natural to ask how it can be proved or disproved. Unfortunately there is no easy way. The usual way is to try to detect the causative organism (Bordetella pertussis) in the back of the nose (11). This usually involves passing a swab on a wire through a nostril to the back of the throat and sending it to a medical lab to culture the material. This may take 5 to 7 days. If Bordetella pertussis grows this is usually taken as proof that it is whooping cough (13). Unfortunately the organisms are delicate, killed easily by many antibiotics and have often been eliminated from the body by natural defenses by the time the diagnosis is suspected. It is easiest to find it in the first 2 weeks but very unlikely after 3 weeks. But the patient has often had it for 3 weeks before whooping cough is suspected. So it is unusual to get a positive culture in whooping cough. In other words, if a swab is negative, the patient can still have whooping cough (11).

A better and more modern way of detecting the diseases is by an enzyme - linked immunosorbent assay (ELISA) that measures the level of antibody response against microorganisms by detection of IG antibody (15, 18). The high sensitivity and specificity of the ELISA allowed for antibody determination in small amounts of sera for short time. Antibody tests are done by some laboratories on blood samples taken after several weeks of illness. By looking at IgG and IgA antibodies it is possible to say whether it is likely the patient has had passed disease (6, 19).

Knowledge of the immune status of population against diphtheria, tetanus and pertussis has big practicable implication. It may assist in checking the efficacy of immunization schedules for infants and children till six of age, adopted and the persistence of immunity. Therefore was necessary to make a screening for level of the protection against these diseases of the population in Bulgaria.

In this paper we present our results of estimation of the immune status of the children up to 6 years old by ELISA methods for immunoglobolin G antibodies against diphtheria, tetanus and whooping cough in human sera during the eight years investigation period.

ABBREVIATIONS USED IN THIS PAPER:

Lf - Limes of flocculation, PBS - phosphate buffered saline, OPD - ortophenilenediamine, PN - protein nitrogen

ACCEPTED FOR PUBLICATION: 12. 04. 2009

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MATERIALS AND METHODS

Population studied

The epidemiological study on immunity was carried out on 499 subjects between 0-6 years old, selected by Hygiene Epidemiological Inspection, Sofia, Bulgaria. Sera separated from clotted blood were stored at - 20°C in small vials and were tested for diphtheria antibodies using ELISA method.

The observed results for diphtheria and tetanus were classified depend of titer obtained in following subgroups: subgroup with antibody level up to 0.009 AU - non protected; subgroup with level of antibodies between 0.01 to 0.09 AU- sera with basic immunity and subgroup with level of antibodies more than 0.1 AU - sera with full protection.

The obtained results for pertussis were classified depend of the titer of antibody in following subgroups: subgroup of sera with the level of antibody up to 1:80 - group of non protective people; subgroup with level of antibodies against pertussis between 1:81 to 1:160 - sera with basic immunity, subgroup with level of antibodies against pertussis between 1:161-1:320 - sera with full protection and subgroup with level of antibodies against pertussis more than 1:321, human sera with titer used as a criteria for disease (5).

Biological assay

The antibodies against diphtheria toxin were tittered by the guinea pigs intradermal test according of the technique of Rommer (7). For this purpose diphtheria toxin (batch 3, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria) was tittered against 10 IU of the Anti - diphtheria Standard Serum (Internationally Laboratory of Biological Standards, Copenhagen, Denmark). Serum dilutions were incubated with the toxin for 30 minutes at 37°C and 0.1 ml of the mixture was intradermally inoculated in guinea pigs. Readings were done after 120 h with reference to the standard mixture in concentration 0.002 IU inoculated at the same time. The highest dilution of toxin - antitoxin mixture, which produce nonnecrotic erythematous area after 120 hours is the titer.

Bacterial Agglutination (BA) Test (13)

The BA test was the first method developed to measure pertussis antibody and it is still the most frequently used method. Bordetella pertussis suspension (serotype 1, 2 and 3), (batch 36, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria), dued and inactivated with 0.01% thiomersal was used in BA test. Diluting the stock suspensions to opacity 10 OUs made working dilutions. Sera samples were prepared in following concentrations: 1:10; 1:20; 1:40; 1:80; 1:160; 1:320; 1:640; 1:1280; 1: 2560. The level of antibody is measured after 24 hours incubation at 370C and following incubation for 24 hours at room temperature. The last dilution of human sera with agglutination is a titer of antibody against pertussis.

ELISA

Polystyrene plates with U - shaped 96 wells (Nunc immunoplates, Denmark) were coated with purified diphtheria toxoid or purified tetanus toxoid or pertussis antigen (1, 2, 23).

Test procedures: 100 µl volumes blocking buffer - PBS containing 1% BSA (Bovine Serum Albumin) were added to the wells and incubated at 37°C for 1 hour. 100 µl volumes of serum dilutions in PBST were added to the wells of sensitized plates, incubated at 37°C for 120 minutes and washed three times in PBST. 100 µl volumes of peroxidase anti - human immunoglobolin G (National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria) diluted in PBST was added to each well, followed by incubation at 37°C for 60 minutes. The plates were washed three times with PBST. 100 µl volumes of a chromogen solution (10 mg of ortophenilenodiamine and 10 µl of 30% hydrogen peroxidase in 50 ml citrate - phosphate buffer pH 5.0) were added to the wells of plates and than were left at room temperature.The reaction was stopped after 30 minutes by adding 100 µl of 1 N H_2SO_4.

The absorption of each wells of plates were directly read at 405 nm in a MicroELISA Minireader Photometer (Bio - Tek). Curve with dilutions of control serum was prepared for each experiment.

RESULTS AND DISCUSSION

Exotoxins, like diphtheria or tetanus toxoid, that have highly lipophilic moiety in their molecule, coat the tubes efficiently (9). Results of the direct ELISA test are highly reproducible (6, 20). It is believed that diphtheria or tetanus antitoxin level of 0.01 IU provides clinical immunity against disease (16). This diphtheria antitoxin level corresponds to a negative Shick test. In the 1984 diphtheria epidemic in Sweden, the patients, who died have had antitoxin titers less than 0.01 IU (3, 7). Thus, an antibody concentration between 0.01 and 0.09 IU were regarded as giving basic immunity. Higher titers may be needed for full protection. In some studies that have used in vitro techniques, a level of 0.1 IU was considered protective (3, 8).

The results of immunity against diphtheria in children are performed on Table 1. The full protected sera in the investigation period of eight years are more than 95%. The sera with titer less than 0.009 AU are between 3.55-4.55%. The summarized results performed on Figure 1 showed the persistence of immunity. The full protected children are 96.2%, the children with basic immunity are 0.25% and the sera with titers less than the protected level are 3.55%.

The observed titers of tested sera against tetanus presented on Table 2 demonstrated the good protection against disease in infants and children up to six years of age. The human sera titers more than 0.01 AU in all years included in the investigation are more than 99%. The rate of basic immunity are between 0-29% and of full protection are between 71-86%. The non protected children are rare less than 1%. The comparison during the investigation period showed that the immunity in different years in constantly levels. The results performed on Figure 2 demonstrated that the full protected children are 86.56%, the children with basic immunity are 13.18% and the non protected sera are 0.26%.

It is believed that a pertussis antibody level of 1:81 provides protection against disease (10). The titters of antibody from 1:161 to 1:320 showed full protection of people against whooping cough and titters over 1:321 are used as a criterion of disease or used as a criterion of passed disease (11). The results of estimation of immune status of population in Bulgaria in this group of age were based on the same criteria. The tested sera, of group of children till six years of age, showed that 53.75% of persons have full protection against whooping cough - Table 3. In this group the non-protected people are only 0.25% and with basic immunity are 43.13% of tested sera. The children with protective titter are more than 96%.

Summarized results of serological studies for eight years, presented on Figure 3, showed a good protection against pertussis (more than 96%). The prevalence of pertussis antibody in the general population depends on the status of immunization against pertussis in childhood especially for Bulgaria till two years of age.

The antibody response in unvaccinated patients was with increased the IgG titers. The low percent of children with titer more than 1:321 in 2.88% of human sera demonstrated that disease has been effectively controlled by vaccine prophylaxis.The ELISA involves the blinding of toxoids or bacterial cells to polystyrene tubes. Results of the direct ELISA test are highly reproducible. The good protection levels in infants and children up to six years old for diphtheria (96.45%), for tetanus (99.74%) and for whooping cough (96.87%) as a result of the specific immunoprophylaxis with combine vaccine DTP following the shame recommended by the Bulgarian immunization program.

This results confirmed the efficacy of immunization schedules adopted and the persistence of immunity. Previous pertussis vaccinations of patients with whooping cough may interfere with the antibody response to the natural disease. Granstorm et al. (12) found that the antibody response in unvaccinated people different in their vaccinated counterparts: unvaccinated children had an early increase in Ig M titters and late Ig G response (more than 1:131), whereas most vaccinated children and adults had a secondary type response with an early increase in Ig A and the level of Ig G antibody is until 1:320. Populations in other countries that have also been studied in this way appear to be somewhat heterogeneous on the basis of these criteria (4, 5). However, according to our opinion, in most cases the population can be considered comparable and from this comparison, the epidemiological situation observed in Bulgaria appears more favorable than that of some European countries and of the USA. In Sweden (3), for instance, 56.9% of population were found to lack protective immunity against diphtheria, in Germany 52.2% of population was found unprotected against diphtheria (22) and in Denmark 36% of the subjects has neutralizing antitoxin titer <0.01IU ml-1 (17).

Table 1. Estimation of the immune status of children against diphtheria

Year	Number of human sera	2001 - 2008 % Full protection	% Basic immunity	% Non-protected
2001	18	95.45	0	4.55
2002	19	96.0	0	4.0
2003	200	96.0	0	4.0
2004	42	95.45	0	4.55
2005	110	95.0	1.0	4.0
2006	78	96.0	0	4.0
2007	20	96.0	1.0	3.0
2008	12	96.7	0	3.3
Total	499	96.2	0.25	3.55

Table 2. Estimation of the immune status of children against tetanus

Year	Number of human sera	2001 - 2008 % Full protection	% Basic immunity	% Non-protected
2001	1	100	0	0
2002	98	84.28	15.72	0
2003	78	81.35	17.87	0.78
2004	32	93.75	6.25	0
2005	14	71.43	28.43	0.14
2006	56	90.3	9.7	0
2007	102	85.4	13.58	1.02
2008	12	84.28	15.6	0.12
Total	393	86.56	13.18	0.26

Table 3. Estimation of the immune status of children against whooping cough

Year	Number of human sera	% Full protection 1:161-1:320	2001 - 2008 % Basic immunity 1:81-1:160	% Non-protected Less than 1:80	% of children with titter more than 1:321
2001	18	53	43	0	4
2002	19	54	44	0	4
2003	200	51	45	0	4
2004	42	53	44	0	3
2005	110	55	41	0	3
2006	78	55	42	1	2
2007	20	56	43	0	1
2008	12	54	43	1	2
Total	499	53.74	43.13	0.25	2.88

In Sweden high proportion of children had experienced pertussis infection by age 10, in Czechoslovakia the percent of sero-positively decreased from 81% in persons 15 to 19 years old to 16% in persons 30 to 34 years and in Poland the children to one year the proportion with titer 1:40 or higher was 60%, while the proportion with a titer 1:160 or higher was 29%. Only 7% of person aged 15 years in Poland had a titer of 1:160 or higher. In the United States in 1998 there were 7,405 reported cases. There were 5 reported deaths. In 1999 there were 7,288 cases. In 2000 there were 7,867. The provisional number of deaths for 2000 is 12. Only 82% of children in the US are fully immunized against pertussis (11). In England and Wales about 3 deaths per year are recorded. In 2004 there were 504 notifications. Probably the number of cases occurring is probably 50 times greater. The pertussis vaccine is estimated to be 63% to 94% effective in the DPT. The 2.88% of children in Bulgaria with titer more than 1:321 demonstrated the efficacy of administrated vaccine in the country.

Despite a very high vaccination rate in the world, thousands of cases occur. WHO reported that a growing number of pertussis cases are occurring in vaccinated adults. Often adults and teenagers can have atypical whooping cough and only exhibit symptoms similar to a bad cold or flu (13). The undiagnosed adult and teenage carriers of whooping cough, most of who have been fully vaccinated, spread the disease to vulnerable newborn infants and young children.

The 47 years of experience with the application of the bacterial vaccine DTP in Bulgaria and the testing of the immune status of the population give grounds for the following conclusions: The bacterial vaccine Diftetkok, produced by BB-NCIPD Ltd., has a high level of immunogenicity. The antibodies' level against diphtheria, tetanus and pertussis in tested human sera is enough to protect people from these diseases. The bacterial vaccine is effective preparation with a good safety profile for active immunization and indicate a good prevention against whooping cough in Bulgaria.

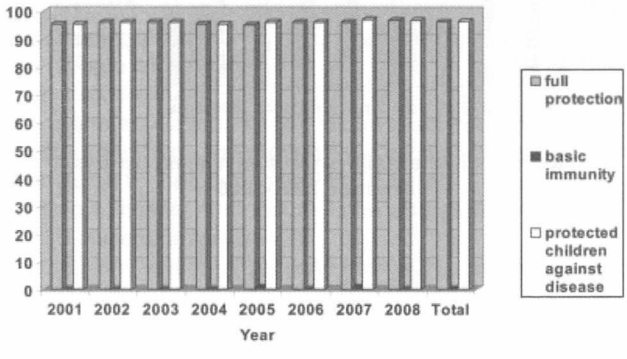

Figure 1. Children with protection against diphtheria in period 2001-2008

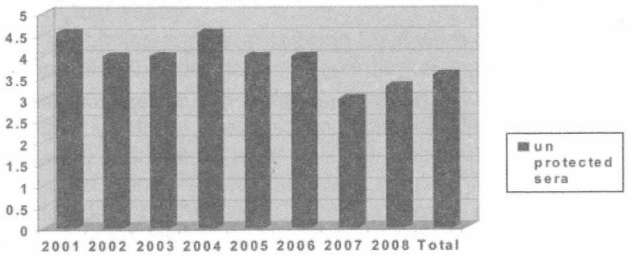

Figure 2. Un protected children against diphtheria in period 2001-2008

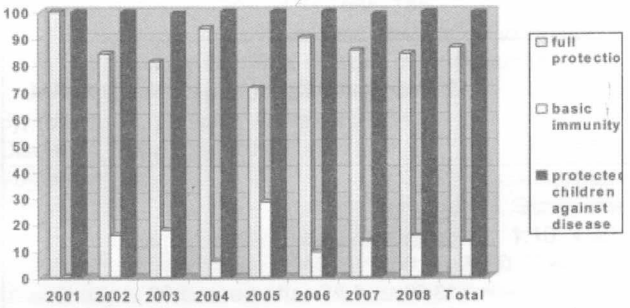

Figure 3. Children with protection against tetanus in period 2001-2008

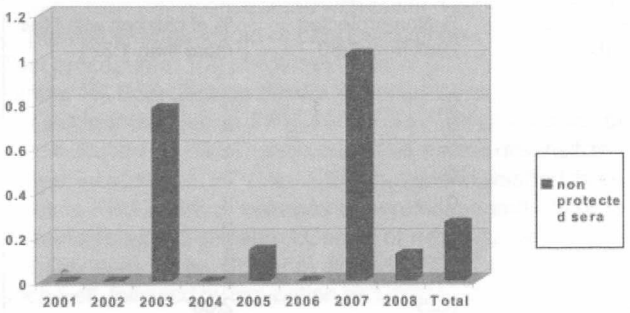

Figure 4. Un protected children against tetanus in period 2001-2008

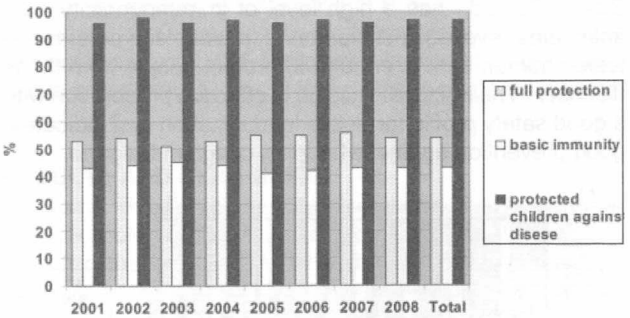

Figure 5. Children with protection against pertussis in period 2001-2008

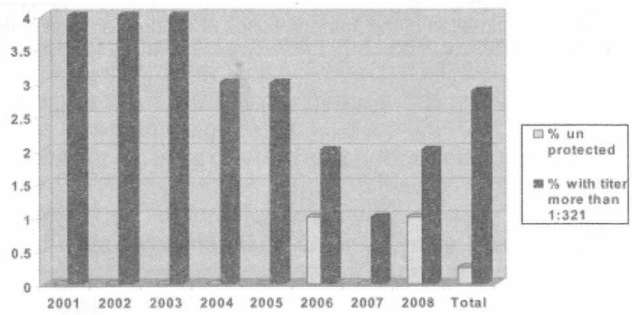

Figure 6. Un protected children and sera with titter of antibodies against pertussis more than 1:321 in period 2001-2008

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CONTAINMENT OF TUBERCULOSIS IN PRISONS - A KEYPOINT OF THE GLOBAL POLICY

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SUMMARY

Prisons play a crucial role in epidemiology of tuberculosis (TB): an air-born infection, with a higher prevalence among lower socio-economical strata, HIV-infected and addicts. Conditions in prisons, incl. overcrowding, facilitate the spread of disease to other prisoners, personnel and visitors. Relative rate of MDR- and XDR- TB, and the conversion of latent TB to an active, is much higher than in society. Released prisoners before the accomplishment of treatment become a source of TB in the society.

In Bulgaria the legislation for TB-care in prisons is acting since 2003 and the National Program for Prevention and Control of TB (supported by the WHO, EuroTB, ECDC and the Global Fund), was accepted in 2007.

The item 4 under the Action Plan focuses on prisons, aiming at identification of TB cases, strengthening the infrastructure of hospitals and medical centers with the prisons. Another important goals are the delivering of modern medical apparatus, the associating of Healthcare sector and facilities of the Ministry of Justice to the information TB system, and the building of an equip to plan, coordinate, manage and control these activities.

The control of TB in prisons is difficult and complex. It requires a prompt identification of disease upon the entrance (screening), isolation, treatment (DOTS) and control of cases upon release out of prison. Microbiology diagnosis should be as rapid as possible, through real-time PCR, FISH diagnostic and other contemporary methods, incl. for susceptibility testing. The questions regarding the Infection Control in prisons are open: new or renovating facilities are required, with negative ventilation (Air-born Infection Isolation Rooms), air-born personal protective equipment (such as N95 respirators) etc. Care should be taken for the health of guards, the medical personnel and to the environment. The total program in prisons will require much more organization, education, coordination, ensuring appropriately trained staff and well equipped facilities, and obviously, more resources, than previously assumed.

Key words: tuberculosis, prisons, infection control, microbiology diagnosis

INTRODUCTION

Resurgence of tuberculosis (TB) nowadays is a significant health-care problem (1-3). TB now occupies a special place among Infectious Diseases with ~ 8 million new cases and 2 million of deaths yearly. The disease (pulmonary TB) is a typical example of air-born infection, fairly transmitted at low infectious dose (1-10 bacteria) - by infectious nuclei 1- 5 mm, generated during cough, sneeze or speech. About 10% of all infected people develop an active disease, as a result of lower immunity, higher exposure, and higher virulence of Mycobacterium tuberculosis. The resurgence of TB is related to several major events:

- Malnutrition and poverty;
- Lack of immunity (BCG vaccine is available);
- Growing a HIV-infected population in which TB is rapidly and badly progressed.

Another important feature of the today's epidemiology of disease is the higher rate of multiply drug resistant (MDR) strains, selected in inappropriate regimens and extensively drug resistant (XDR) - TB, which are difficult to cure/contain.

PRISONS AND TUBERCULOSIS

Recently the particular role of prisons in epidemiology of TB has been established (2-4). It was found, that the incidence and prevalence of TB in prisons is much higher than in society (32000 cases vs. 232 per 100000 persons in Europe, 2004). Furthermore, the rates of MDR TB are up to 30% and up to 50% in previously treated cases. Investigations revealed that in some facilities up to 75-80% of cases are HIV-co-infected (5).

Why the burden by TB and MDR-TB in correctional facilities is higher?

- It is well known, that the prisoners usually belong to the lower strata of society. Typically, these people are lacking food, hygiene; often they escape: education, health-care, incl. vaccination and treatment. A significant part of them are alcohol- and drug abusers. They may be immigrants from countries with higher rate of TB; may be recidivists that have been attracted TB previously in prison, may be HIV-positive, or may be mentally disadvantaged.
- Conditions in prisons usually support disease through over-crowding, lack of ventilation, lack of quality hygiene and health-care, lack of quality food, as well as because of the particular ways of living and rules in the closed groups of prisoners inside the walls of prison.

Moreover, the role of prisons in TB spread in the society has been emphasized. Figure 1 presents a scheme of prisons as a central unit accelerating TB in the society.

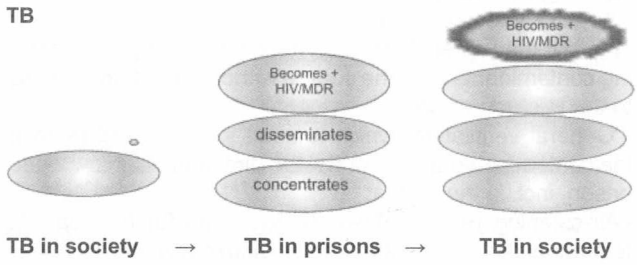

Figure 1. The role of prisons in the spread of TB (upon the WHO Status Paper on TB in Prisons, 2007)

TB IN PRISONS FROM THE POINT OF VIEW OF INFECTION CONTROL

One crucial point is understanding/miss-understanding the epidemiology of disease in prisons and the general conditions that favorite its spread.

First of all, the overcrowding, the poor ventilation, combined with a delayed diagnosis and a failure to adhere to the recommended standards, are proven risk-factors that have already contributed to the poor control of TB in jails.

Therefore, essential measure to be undertaken to contain the TB dissemination are (3):

- Early identification of persons both with active and latent TB infection (LTBI)
- Prompt isolation of contagious people;
- Appropriate use of air-born precautions;
- Contact investigations;
- Successful completion of treatment.

ABBREVIATIONS USED IN THIS PAPER: TB - Tuberculosis

ACCEPTED FOR PUBLICATION: 13. 02. 2009

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Upon entry, an inmate with signs of TB should be immediately masked and housed in negative pressure respiratory isolation. He should be evaluated with TST, chest radiograph, sputum smear and culture for M. tuberculosis.
Those inmates, who will be incarcerated for at least 2 weeks, should have TST.
The inmate with a history of TB should be obligatory evaluated. Also, the new employees should undergo TST. People with a positive TST should be managed, while those with negative test should be re-tested at least annually.

CDC RECOMMENDATIONS FOR PREVENTION AND CONTROL OF TB IN PRISONS

SCREENING

Several medical societies and international organizations, such as WHO, CDC, ECDC, ESM have pointed out the role of screening for limiting TB in prisons. According to their recommendations, the early identification of TB cases is the most effective tool. The guidelines of CDC suggest that:
- Newly arrived inmates should not be housed with the others until screened by health-care professionals for symptoms of TB; those with suggestive symptoms should receive a thorough medical evaluation: a tuberculin skin test (TST) or a Quantiferon TB-Gold test (QFT-G), a chest radiograph (Ro), and if indicated, a sputum examination (within 7 days). HIV-infected should have chest-radiograph;
- Persons with data for inadequate treatment should be immediately placed in air-born isolation room (AII). They should remain there until treatment renders them noninfectious;
- Suspicious for MDR-TB may remain in AII room until negative culture result;
- Facilities without AII room should have a written plan for referring the patient to a facility that is equipped.

ENVIRONMENTAL CONTROL

The next important step is the environmental control. The basic requirements include the presence in the prison of the following conditions:
- Air-born Infection Isolation Room;
- Local Exhaust Ventilation, e.g. hoods, capturing and removing contaminants near the source without exposing persons to the microorganisms;
- General ventilation, which is used to dilute and remove the contaminated air; to control the direction of airflow in the prison, incl. in the rooms;
- Air-cleaning methods. They are necessary for the area with formation of aerosols - AII cells, sputum collection and other procedures rooms: HEPA filters should be introduced before returning the air to the general ventilation;
- Respiratory protection: a personal protective equipment (PPE) - N95 particles respirators are required for the protection of medical and other staff - the personnel should be trained and the PPE should be fit-tested.

MICROBIOLOGY EXAMINATION

Microbiology examination is a key diagnostic procedure for identifying:
- Persons with pulmonary TB (Ro) and suggestive symptoms;
- Persons with Ro finding suggestive for previous infection;
- HIV-infected with any symptom;
- Persons with planed bronchoscopy (sputum test before the procedure).

According to the international recommendations (6-8), the specimen collection procedure requires: - at least three sputa, collected 8-24 hour apart, at least one early morning, to be collected in a sputum induction booth/AII room/outdoors. The procedure should be conducted after an instruction and should be supervised. Inhalation of warm hypertonic saline might induce sputum expectoration for persons without expectoration.
Positive result for acid fast bacilli (AFB) is predictive for an increased infectiousness; while the negative smear result does not exclude TB. Smears positivity is 50-65% of all culture-positive specimens.
Classical culture of sputum provides a positive result within 28 days (using recommended rapid methods: liquid culture and susceptibility), e.g. it takes too much time.
Testing sputum with nucleic acid amplification (NAA), e.g. real-time PCR and PNA-FISH offers a diagnosis in hours and is required for MDR/XDR- TB (9-12).
The risk for lab-workers is particularly high because of aerosol-formation during procedures - bio safety cabinets with exhausting air outdoors should be used (13).
For different reasons, both current conditions in prisons and microbiology diagnosis of TB are suboptimal. Table 1. gives an example for the microbiological diagnosis of TB cases from the Medical Institute - Ministry of the Interior, where specimens from the Sofia-prison are being submitted.

TREATMENT OF TB - DOTS

The most preferred regimen in the directly observed treatment (DOTS) is:
- An initial phase: 2-months isoniazid, rifampicin, pyrazinamid and ethambutol, followed by:
- Isoniazide and rifampicin for > 4 moths, but minimum therapy 6 months;

Persons with cavity TB, and positive culture after the completion of 2 month-therapy should receive a longer, 7-month continuation, total, 9 months.

Table 1. TB workload of Microbiology laboratory - Medical Institute, Ministry of the Interior (MIMI)

Year	Specimen processed	all specimens	type specimen	No of positive: patients
2003	total 842	10	sputum 7, BAL 2, br s-t 1	5
	MIMI hospital 649	3		3
	from prison 193	7		2
2004	total 811	10	sputum 10	4
	MIMI hospital 563	3		1
	from prison 248	7		3
2005	total 835	16	sputum 9, BAL 3, br s 2, pl p 1, ln 1	10
	MIMI hospital 687	6		6
	from prison 148	10		4
2006	total 585	14	sputum 12, BAL 2	8
	MIMI hospital 446	8		3
	from prison 139	6		5

Legend: br s-t, bronchial secret, pl p, pleural puncture, ln, lymph node

For patients with HIV- or MDR-TB, an expert advice should be asked.
Considerations for the treatment of LTBI should not be ignored:
- Staff or inmates with TST > 5 mm, if they are: HIV-infected, or have a recent contact with TB patient, or have on chest Ro data for previous TB; patients with organ transplantation or other immunosupression, equivalent of > 15 mg/d prednisone for > 1 month
- All others should be considered if their TST > 10 mm or have a positive QFT-G result.

DISCUSSION

Recent data about the TB burden show the following for:
• The world: 2006:
- 9.2 million new TB cases; 139 per 100000 inhabitants;
- 1.7 million deaths; 0.7 million co-infected with HIV.
• Europe: 2006:
- 422 830 cases; 48 per 100000 inhabitants;
- EC - 89032 cases; 17 per 100000 inhabitants;
- Romania 127 per 100000 per 100000 inhabitants;
- Baltic countries - 34-75 per 100000 inhabitants;
- Former USSR republics - 306 887 cases; 110 per 100000 inhabitants.
• the Balkan peninsula: 2006:
- 26911 cases; 76% in Turkey: 28 per 100000 inhabitants; Bosnia and Herzegovina: 46 per 100000 inhabitants.
• Bulgaria:
- 2006 - 3011 new cases; 37 per 100000 inhabitants;
- 2007 - 2853 new cases;
- MDR ~ 50 cases/year; 33% of HIV-positive persons have TB.
Bulgarian National Program for TB prevention and control (14), incl. in prisons, is the official state document, developed with the support of the WHO, Euro TB, ECDC and the Global Fund. In brief, Bulgarian TB program 2006 - 2015 consists of eleven components to strengthen and further develop the organization to contain TB: an infrastructure, a diagnosis, a treatment, transmissibility, a specific prophylaxis, a specific focus on TB in prisons and in some populations.
The program part 2007 - 2011 aims at decreasing TB burden from 39 per 100000 people to 36 per 100000 and at increasing successful treatment from 80% to 85%. About 84 million BG leva will be delivered through the help of the international organizations, such as the WHO, the Euro TB, the Global Fund and others. In 2007 the Ministry of Health provided ~ 1 million BG leva for patients' treatment; 90000 leva for laboratories and 16000 leva for treatment of LTBI.
As it was already mention, the item 4 of the Program focuses on decrease of TB spread in prisons:
- identification of TB cases,
- an amelioration of infrastructure of medical care in prisons for better treatment and control,
- a building an effective information TB system
- and monitoring and evaluation.

However, it should be underlined, that a concrete plan and funds to enable the correct Control of Infection in prisons and rapid microbiological service have not been developed up to now. Actually, the real time detection of TB and especially, of MDR-TB is much more indispensable for the diagnosis of disease in the correctional facilities, than in the other settings. The medical society and the society should be aware of the role of prisons to spread, multiply and aggravate the severe disease.

CONCLUSIONS

What should be done to better contain TB?
The current data suggest that an appropriate legislation about TB care in prisons exists, but the care is far away from the accepted standards.
- As it became evident, there are several important steps in containment of TB in prisons: early identification, appropriate isolation, prompt controlled treatment, planned discharge with preliminary arrangements to guarantee the treatment continuation. Regular provision of tuberculostatics; medical personnel, adequately trained in TB diagnosis and treatment; good quality of Microbiology laboratories are of paramount significance.
- One issue appears to be underestimated until now: the Control of infection, including buildings with appropriate ventilation, All rooms, rooms equipped with appropriate devices for air purification/removal, PPE, rules in procedures and transport.
- Biologically safe laboratories with ability for rapid diagnosis (real-time PCR, PNA-FISH, important in MDR (which cases have been already proven in prisons)/XDR-TB) require more efforts and funding.

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INFLUENCE OF DRUG PRODUCTS ON ANTICOAGULANT BAITS EFFICIENCY

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Summary

The experiment aimed at achieving an express deratization effect in areas of infectious diseases. In order to reach this goal the possibility of activation of rodenticide baits based on bromadialon in two concentrations-0,0025% and 0,005% was examined. The rodenticide baits were combined with Calcium antagonist drugs- Nifedipine and non-steroidal anti-inflammatory drug Nimesulide.

Drug dosage was within the range of the recommended treatment dosages-mg/kg.m.

Deratization effect was carried out with white mice and rats. 100% mortality time decrease was observed in white mice. From 12 days the mortality time shortened to 8 days. However, the effect on the rats was different. The mortality time difference between the control and the experiment groups was not essential. This required new correlations between the drug dosages.

Key words: Anticoagulants, drug products, rodents

INTRODUCTION

One part of the infectious diseases is spread by rodents too. They can be sources of infections and at the same time natural reservoirs of different pathogenic bacteria. To interrupt the mechanisms of transmission of morbidity microorganisms, we need express deratization measures for decreasing the number of harmful rodents and protect human health.

A contemporary chemical method for rodent control is the use of anticoagulant rodenticides of II generation.

Some researchers have proved good results by including some antibiotics (such as Indometacinum and Ibuprofenum) to anticoagulats /1/. According to the data ascertained by other scientists, it has been found that using Nimesulide in repeating dosages causes reproductive toxicity in rats. Moreover, the simultaneous usage of Nimesulide with cumarin anticoagulants brings a cumulative effect /2, 3/.

The present work offers preliminary investigation on the potentiating effect of rodent baits in combination of Nifedipine and Nimesulide with anticoagulant on bromadialone base.

MATERIALS AND METHODS

Two experiments were carried out in laboratory conditions.

I experiment

Forty white male mice (line ICR) with average weight of 18 g were used for the experiment. The mice were divided into 4 groups, each containing 10 mice. The mice were given 50g of wheat baits.

To increase the acceptability of the bait, 16g of sugar powder was used for each experimental group /Table 1/.

II experiment

The laboratory test was done with white male rats of Wistar breed. The average weight of the rats was 182,6 g. The animals were divided into five groups. Each group consisted of 10 rats. All the groups were provided with 200 g wheat bait /Table 2/.

Table 1. Drugs used in the baits in combination with bromadialone

Groups	Dosages (mg/ kg) for white mice Drugs	Bromadialone
I	0.05 Nifedipine	0.0031
II	2.00 Nifedipine	0.0031
III	Without drugs	0.0031
IV (control)	Without drugs and bromadialone	

Table 2. Drugs in combination with bromadialone used in the bait (to white rats)

Groups	Dosages (mg/kg) for white rats Drugs	Bromadialone
I	0.67	0.025
II	0.67	0.0013
III	2.00	0.025
IV	Without drugs	0.025
V (control)	Without drugs and bromadialone	

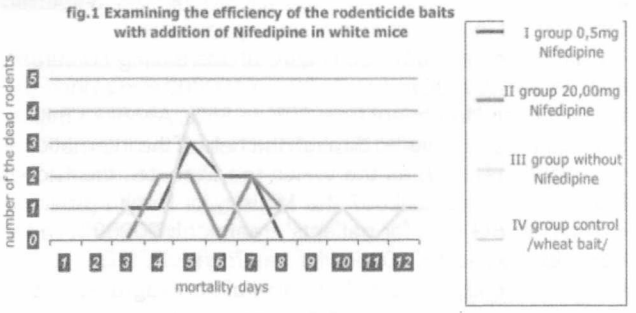
Drug doseages of Nifedipine and Nimesulide are in accordance with singe therapeutic dose for kg/m which is applied to 5g of wheat bait per mouse daily; to 20g wheat bait per rat daily. Bromadialone was used as liquid in two working concentrations 0,005% and 0.0025%. Those baits were used once in both experiments.

After the experimental rodents had eaten the baits they were given supplementary food (forage a pellets) and water. Control rodents were fed only on forage and water.

RESULTS

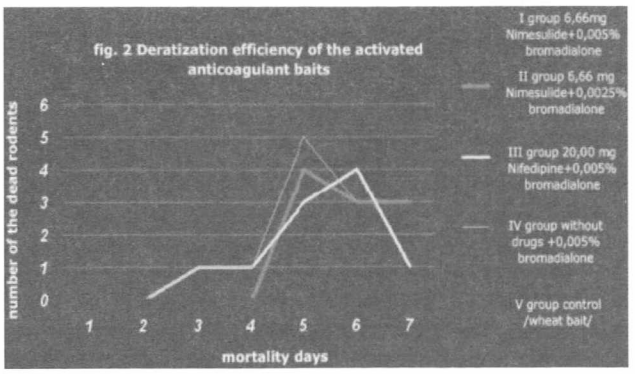
I experiment

Examination of the efficiency of rodenticide baits with addition of Nifedipin to laboratory mice /Fig. 1/.



II experiment

Deratization efficiency of activated anticoagulant baits with Nimesulide and Nifedipine to rats /Fig. 2/.



ABBREVIATIONS USED IN THIS PAPER:

ACCEPTED FOR PUBLICATION: 24. 04. 2009

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Nimesulide and Nifedipine exhibited high affinity to plasma proteins. Interacting with them on metabolic level, mediated through enzymes, they helped for the activation of the anticoagulant - bromadialone. This led to enzyme inhibition causing higher levels of toxicity in the laboratory rodents. The synthesis of vitamin K was suppressed. This prolonged the duration of the prothrombin time /blood clotting indicator/. Intensive hemorrhagic effect /both internal and external/ was observed.

During the first experiment potentiating effect of the baits was observed on the fifth and the sixth day. The rates measured showed that 90% of the rodents in the first group died on the day 8. 70% of the rodents in the second group died on day 6 and 30% of them on day 9. While the animals in the third group /without Nifedipine/ started to die after the third day to day 12 inclusive.

High mortality rate and high rodenticide efficiency of the bait were observed during II experiment in the rodents of I group. There was a difference only in II and III group, where mortality began to occure on day 7.

It was determined that during the days of their death all white rats within I, II, and III group exhibited clear blood clots both external and internal around the anal opening, belly area, head - nose, eyes, mouth.

The previously obtained study results revealed that in order to achieve a potentiating effect in the anticoagulant baits for white rats, it was necessary to look for new correlation between Nifedipine and Nimesulide dosages.

CONCLUSIONS

1. Nifedipine 0,5 and 20,00 mg/kg highly activated the efficiency of bromadialone in rodenticide baits. Mortality time for the mice was shortened from 12 to 9 days.
2. Nimesulide 0,66 and Nifedipine 2,00 mg/kg didn't show enough potentiating effect on bromadialone in rodenticide baits used for white rats. Some minor differences in the mortality time were observed.

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LAMBLIA (GIARDIA) INTESTINALIS SPECIFIC GSA 65 ANTIGEN IN FECAL SAMPLES FROM CHILDREN WITH ASYMPTOMATIC LAMBLIOSIS AND ITS DIAGNOSTIC VALUE

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Abstract

Stool specimens from 90 children were examined using two methods: with lugol's solution (for Lamblia [Giardia] intestinalis cysts) and ELISA - GSA 65 (for stool lamblia antigen). The specimens tested with lugol's solution were positive in 22 (24.4%) of the cases, and negative - in 68 (75.6%) cases. However, when the same samples were tested with GSA 65, it confirmed all positive results, and reveled other 10 positive cases in the group of 68 (14.7%) found as negative with the lugol's solution test. The results obtained illustrate a higher sensitivity and specificity of the ELISA test using monoclonal antibody to detect GSA 65.

Key words: Lamblia (Giardia) intestinalis, lugol's solution, lamblia antigen, GSA 65, immunodiagnostics

INTRODUCTION

Lamblia (Giardia) [L. (G.)] intestinalis is still one of the most common intestinal protozoa found in children's contingents. Over the last five years, the average incidence of lambliosis in Bulgaria ranged from 1.03% to 1.34% (1, 2). The causing agent is isolated in two morphological forms: cystic form in asymptomatic carriers of the parasite, and vegetative form in the cases with clinical presentation and diarrhoeal symptoms.

In routine practice, the diagnosis is made by testing stool specimens with native preparation, stained with lugol's solution. The efficacy of the method is low: it depends on the way the specimes are collected and prepared for the investigation, and the number of times the specimens are tested. In addition, efficacy depends on the experience of the investigator. (3).

The presence of L. (G.) intestinalis in the intestinal tract has oriented investigators to develop alternative methods to detect antigens of the parasite in bowel contents. Communications were published reporting the use of ELISA to prove the presence of lamblia antigen marked as GSA 65 (Giardia Specific Antigen) (4, 5, 6, 7). This makes it possible to diagnose carriers, irrespective of their clinical state, which is important in view of the fact that carriership without symptoms in lambliosis is common.

The aim of the study is to compare the efficacy of the method, proving the presence of GSA 65 antigen in stool specimens with the efficacy of a routine method of diagnosing lambliosis by using a native preparation stained with lugol's solution, detecting cyst of the parasite.

ABBREVIATIONS USED IN THIS PAPER:

OD - Optical density GSA- Giordia Specific Antigen

ACCEPTED FOR PUBLICATION: 10. 05. 2009

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MATERIALS AND METHODS

Stool specimens from 90 children aged 3 to 7 years were investigated. The specimens of 5-10 grams were placed in chemically clean containers. Each s specimen was then concentrated by a formalin-ether method. Light microscopy was performed to detect intestinal protozoa by investigating of three native preparations prepared from the sediment and stained with lugol's solution. The result from the test was considered negative if no cysts of L. (G.) intestinalis were detected in a specimen tested three times.

The specimens were then divided into two groups, according to the results obtained from the test with lugol's solution, and were investigated for GSA 65. Group 1 included specimens positive for cysts of L. (G.) intestinalis, and group 2 - negative specimens.

The stool specimens of both groups were tested for fecal lamblia antigen using ELISA test "Giardia EZ Microplate Assay", according to instructions of the producer (REMEL Inc., USA). This test proves the presence of L. (G.) intestinalis GSA 65 specific antigen, and the result is measured by spectrophotometry or visually, by the intensity of coloration of the investigated and control specimens (5).

Description of the method

The following steps were taken:

1. Stool specimens 200 µl, homogenized with a Specimen Dilution Buffer (buffered solution with rabbit serum and 0.02% trimezol) were pipetted into the wells, pretreated with anti-GSA 65 monoclonal antibodies. A positive control was added to one well and a negative - to the another. (Controls are provided with the test itself, and are prepared from human stool specimens, to serve as reagents and controls in the procedure.)
2. The specimens and controls were incubated at room temperature of 20°C for 60 min.
3. A threefold washing is performed with 10x concentrated buffered solution with 0.01% trimezol to remove the unbound material.
4. Enzyme conjugate (Peroxidase labeled monoclonal anti-GSA with 0.01% thimerosal) of 200 µl was added into each well, and the specimens were incubated at the same conditions for 30 min.
5. After a five-fold washing, 200 µl Color Substrate - TMB in buffer was added to each well.
6. The reaction was terminated after 10-minute incubation at 20°C, using 50 µl of a stop solution (1.0 N Sulfuric acid).

Results were interpreted visually or spectrophotometrically at 450 nm wavelength. Visual interpretation was based on the intensity of yellow coloration of the wells examined, compared to the color table provided with the test. The result was considered positive for the presence of GSA 65 in the stool specimen if the yellow coloration intensity is at least 1+. A colorless reaction was considered negative, indicating the absence of GSA 65 in the stool specimen examined.

Spectrophotometric interpretation was based on optical density (OD), measured in extinctions (E). If the difference between the values of OD for the specimens tested and the negative control was equal to greater than 0.05 E, it was assumed that specimens were positive for GSA 65. In case OD was less than 0.05 E, specimens were considered negative for GSA 65.

RESULTS AND DISCUSSION

The study was carried on a group of 90 children, of which 36 girls (40%) and 54 boys (60%), aged 3-7 years. The results obtained are shown on Table 1.

Table 1. Comparative results from investigation for lambliosis

Type of investigation							
	Lugol's solution			ELISA for GSA 65			
No of subjects investigated	Positive	Negative	Total of positives from lugol's solution test	Positive for GSA 65	Total of negatives from lugol's solution test	Positives for GSA 65	Negatives for GSA 65
	No %	No %		No %		No %	No %
90	22 24.4	68 75.6	22	22 100	68	10 14.7	58 85.3

The three-fold parasitological tests of 90 children revealed the presence of cysts of L. (G.) intestinalis in stool specimens of 22 children (24.4%). The specimens from 68 children (75.6%) were found negative for cysts of the parasite.

The investigation of stool specimens of all the 22 children, positive for cysts of L. (G.) intestinalis with ELISA for GSA 65 antigen proved that they were also positive for L. (G.) intestinalis antigen, i.e. the sensitivity of the ELISA test was 100%.

The ELISA testing of the specimens found negative from tests with lugol's solution revealed the presence of GSA antigen in 10 specimens (14.7%). The test was negative in 58 of the specimens investigated, i.e. the specificity of the test was 85.3%.

The interpretation of the reaction in the ELISA test, the optic density of the specimens tested, expressed as extinction (E) varied from 0.055 E to 3.0 E in positive samples, and from 0.000 E to 0.012 E in the negative samples.

Nowadays, diagnosing lambliosis is based on microscopic detection of cysts of L. (G.) intestinalis in native preparation, stained with Lugol solution, and vegetative forms of the parasite in specimens, collected by duodenal probing. Duodenal probing is traumatic, especially for children, and has limited application in routine practice. Because of irregular excretion of stools with lamblia cysts, microscopic investigation of preparations stained with Lugo's solution has low sensitivity: it helps to detect 50 to 70% of the patients with lambliosis (7). To increase the efficacy of the microscopic examination, it is necessary to investigate the stool specimens three or four times at intervals of three to seven days. Repeated investigations increase the sensitivity of the test to 85-90%. However, in routine practice this is inconvenient, because the need to see a doctor several times usually makes patients refuse to be examined (3, 6).

ProSpectT Giardia EZ Microplate Assay (REMEL Inc., USA) is sensitive and specific for diagnosing lambliosis. The test utilizes monoclonal antibodies against GSA 65 antigen, specific for L. (G.) intestinalis, which prevents cross-reactions (5).

According to the literature, the sensitivity of the method is close to 100% and its specificity is 95% in investigations of fresh stool specimens (4, 6, 8). The test has demonstrated high sensitivity and specificity even in testing stool specimens preserved in 10% formalin solution for two months (6). Our study showed that the test for GSA 65 can detect carriers in a group of patients who had been diagnosed as negative by the test with lugol's solution.

The detection of lamblia antigen in stool specimens using ELISA is not common in Bulgaria and there is no data regarding its application, notwithstanding that this parasitosis is among the most widely spread protozoon invasion in the country. The detection of stool lamblia antigen can be useful in all cases in which the diagnosis is difficult to make, especially in the presence of clinical manifestation of the disease. The test could also help evaluate the effect of treatment, as well as solve major problems regarding epidemiology and control of lambliosis.

CONCLUSIONS

1. Testing for lambliosis using the routine method with native preparation and lugol's solution is not effective enough in view of the fact that the cysts are excreted cyclically and this necessitates repeated examinations.
2. Using the alternative method to detect specific lamblia antigen GSA 65 with monoclonal antibody increases diagnostic possibilities in lambliosis, and can be applied in cases proved negative by the lugol's solution test.
3. Although the test detecting lamblia antigen is highly efficient, it is considerably more expensive as compared to the test with lugol's solution. Therefore, it is preferable to first use the lugol solution test, and apply the alternative method for detecting GSA 65 antigen in cases of clinical and epidemiological indications.

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EMERGING ANTIBIOTIC RESISTANCE AND THE THERAPY OF URINARY TRACT INFECTIONS

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SUMMARY

The aim of this work was: 1) to survey the resistance of most frequent pathogens, 2) to analyze the antibiotic usage and 3.) to evaluate the current antibiotic therapy for urinary tract infections (UTI). Methods: Study design: A retrospective 3-year analysis (2003-2005) of the etiologic structure of UTI and antimicrobial resistance. Microbiology was performed according to the Manual of Clinical Bacteriology, ASM, 2003; Antimicrobial susceptibility testing was done according to the CLSI. Antibiotic usage was calculated in DDD/100 patient/day (ABC Antibiotic Calc, D. Monnet). Results. *E. coli* strains (2193), CoNS (510), *K. pneumoniae* (417), P. aeruginosa (304), *Enterococcus* spp (300), *Proteus* spp (241) and *Enterobacter* spp (155) represented the most frequent pathogens during the study period. The following rates of resistance to ciprofloxacin and co-trimoxazole (usual agents in the empiric therapy for UTI) were registered in 2005: 22% and 27% in *E. coli*, 27% and 31% in *K. pneumoniae*, 5% and 28% in *Proteus* spp and 20% and 40% in *Enterobacter* spp respectively. ESBL-producing strains of Enterobacteriaceae emerged and accounted for 2003, 2004 and 2005 for 2%, 3% and 6% of *E. coli*, while for 15%, 20% and 17% of *K. pneumoniae*. Poly-resistant *P. aeruginosa* strains exhibited resistance to the most useful antibiotics: amikacin (from 41% to 50%), ceftazidime (38% to 53%), ciprofloxacin (28%-58%), carbapenems (3% to 10%). During the last 5 years ceftriaxone, recommended by the national UTI guidelines, was used widely for complicated UTI; cephalosporins of 3rd generation accounted for 33% of this hospital cephalosporins usage (13- to 17.4 DDD/100 patient/day) and total antibiotic consumption was 45.9-, 42.8- and 46.2 DDD/100 patient/day for the last 3 years. Conclusions: A re-evaluation of cephalosporins of 3rd generation use is needed, because of the rapid emergence and dissemination of ESBL-producing *Enterobacteriaceae* strains. The problem of poly-resistant *P. aeuginosa* requires special and complex attention (therapeutic and Infection control).

Key-words: antibiotic usage, antibiotic resistance, antibiotic therapy

INTRODUCTION

Antibiotic usage and overuse is now considered one of the main driving forces for the development of antibiotic resistance (1). Experts recommend the performance of antimicrobial resistance surveillance, the calculation of antibiotic usage, the careful assessment of antibiotic policies and infection control measures (2-4). Among the pathogens of urinary tract infections (UTI), the most frequent and important are Gram-negative bacilli (*Enterobacteriaceae* and sometimes in hospital setting - *Pseudomonas aeruginosa*) (5-8). Important resistance mechanisms emerge during the last years, especially extended-spectrum beta-lactamases and metallo-beta-lactamases which prevent the therapy of infections (9-10).
The aim of this work was: 1) to survey the resistance of most frequent pathogens 2) to analyze the antibiotic usage and 3) to evaluate the current antibiotic therapy for urinary tract infections (UTI).

ABBREVIATIONS USED IN THIS PAPER:
UTI - Urinary tract Infections

ACCEPTED FOR PUBLICATION: 17. 04. 2009

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MATERIALS AND METHODS

Study design: A retrospective 3-year analysis (2003-2005) on the etiologic structure of UTI and antimicrobial resistance. Microbiological part of investigation was performed according to the Manual of Clinical Bacteriology, ASM, 2003; Antimicrobial susceptibility testing was done according to the CLSI. Antibiotic usage was calculated in DDD/100 patient/day (ABC Antibiotic Calc, D. Monnet).

RESULTS AND DISCUSSION

As it was expected, the most frequent pathogens in UTI were *Escherichia coli* in all studied settings (hospital, H, and ambulatory, A). In hospitalized male patients the relative rate of *Enterococcus* spp., *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Serratia marcescens*, the late three, representing typical nosocomial pathogens, was prevalent (Table 1):

Table 1. Top UTI isolates 2003-2005

Rank of pathogens	Number	Distribution: Hospital/Ambulatory; Female/Male
1. *E. coli*	2188	FA > FH > MH > MA
2. *CoNS*	447	FA > FH > MH > MA
3. *K. pneumoniae*	420	FA > MH > FH > MA
4. *E. faecalis*	309	MH > FH > FA > MA
5. *P. aeruginosa*	304	MH > MA > FH > FA
6. *Proteus* spp.	232	FH > MH > FA > MA
7. *Enterobacter* spp	158	MH > MA >FH = FA
8. *C. freundii*	73	MA > MH > FH = FA
9. *S. aureus*	72	MA > FA >FH > MH
10. *S. marcescens*	28	MH > FH = MA

FA, ambulatory female patients; FH, hospital female-; MH, hospital male-; MA, ambulatory male- patients

Emerging antibiotic resistance to the cephalosporins of third generation in *E. coli* is presented on Figure 1.

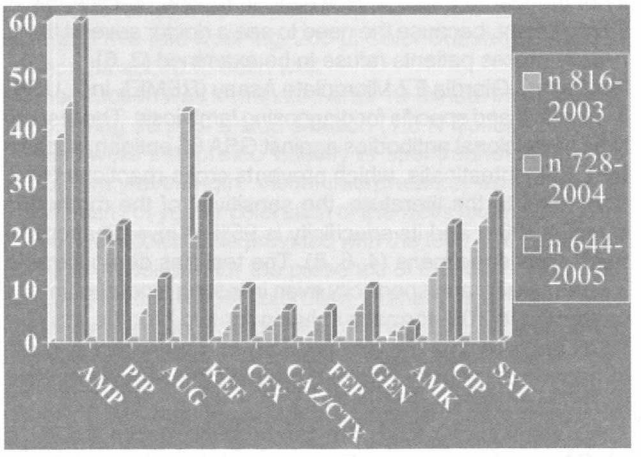

Figure 1. Antibiotic resistance rates in *E. coli* 2003-2005 AMP, ampicillin, PIP, piperacillin; AUG, amoxicillin/clavulanic acid; KEF, cephalothin; CFX, cefuroxime; CAZ, Ceftazidime; CTX, Cefotaxime; FEP, cefepime; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; SXT, co-trimoxazole

Figure 2. illustrates the emerging resistance to the third generation cephalosporins among the strains of *K. pneumoniae*.

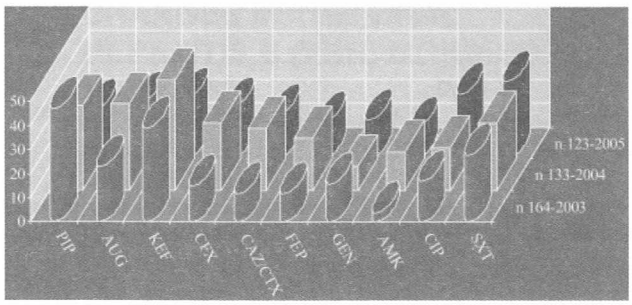

Figure 2. Antibiotic resistance rates in *K. pneumoniae* 2003-2005

Similar augmentation iwwn the rate of resistance towards extended-spectrum cephalosporins can be seen in another species of Fam. *Enterobacteriaceae* - e.g. *Enterobacter* - Figure 3, and particularly, *Serratia*, Figure 4.

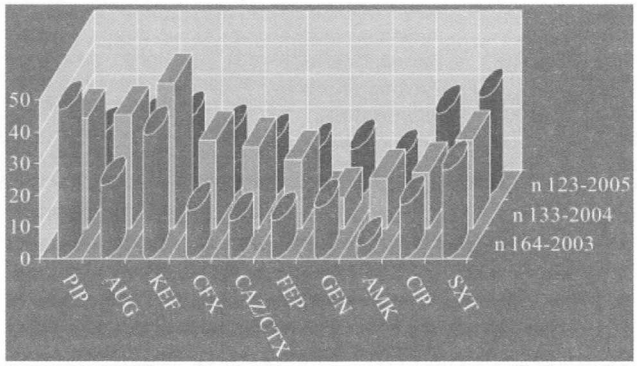

Figure 3. Antibiotic resistance rates in *Enterobacter* spp. 2003-2005

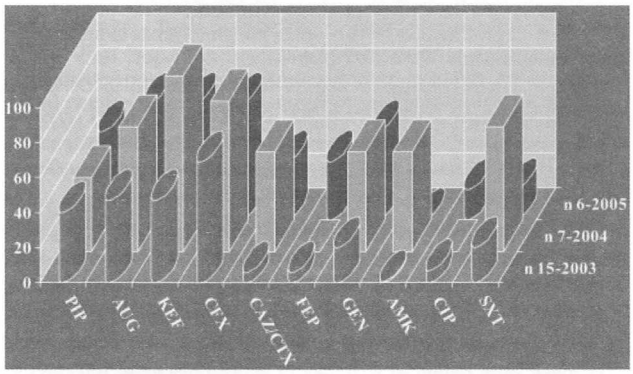

Figure 4. Antibiotic resistance rates in *Serratia marcescens* 2003-2005

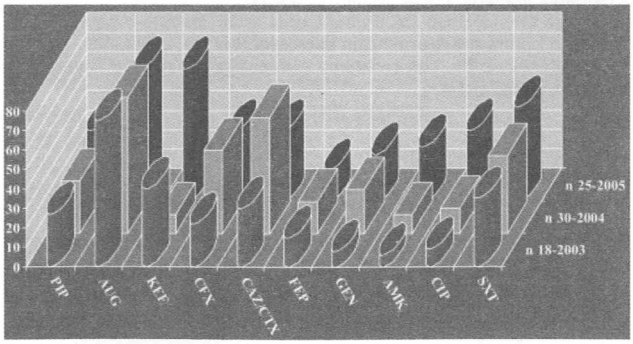

Figure 5. Antibiotic resistance rates in *Citrobacter freundii* 2003-2005

While the emergence of resistance to the cephalosporins of third generation, due to the production of extended-spectrum beta-lactamases is obvious in enterobacteria, another important mechanism of resistance becomes notorious among the strains of Pseudomonas aeruginosa - the resistance to carbapenem antibiotics, which is due to variety of reasons: e.g. changes in antibiotics pumps, permeability or production of inactivating enzymes (metallo-beta-lactamases (10), oxacillinases and others (Fig. 6).

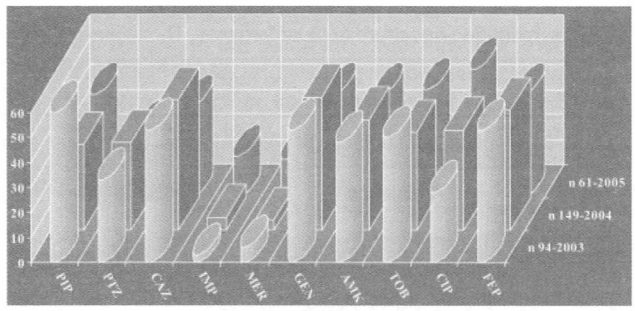

Figure 6. Emerging mechanisms of resistance among Pseudomonas aeruginosa UTI isolates

After having reviewing these data, we can conclude, that the most important mechanisms of antibiotic resistance among Gram-negative pathogens in UTI are:

- for Fam. Enterobacteriaceae: the extended broad spectrum beta-lactamases, ESBL, that confer resistance to the penicillins, cephalosporins of all generations, aztreonam, and frequently, a concomitant resistance to fluoroquinolones, co-trimoxazole and aminoglycosides (Table 2);
- and the resistance to carbapenems in 75 strains of P. aeruginosa in 2005 increased to 15%.

In the literature there is a lot of information, that cephalosporin antibiotics are a powerful driving mechanism for resistance development (11). We checked the current national recommendations for antibiotic treatment of infection. Evaluation of guidelines has shown that cephalosporins were recommended for the Antibiotic prophylaxis in surgery, Empiric therapy for pyelonephritis, Empiric therapy for CAP in hospitals, and that cefrtiaxone was the most prescribed extended-spectrum cephalosporin - from the Empiric therapy for sepsis to any infection. Ciprofloxacin and other fluoroquinolones were recommended for Empiric therapy for lower UTI and for Antibiotic prophylaxis in urology. It is well known, that the overprescription of cephalosporins in hospitals/community selects for MRSA, MRCoNS, *Enterococcus* spp, ESBL-producing *Enterobacteriaceae, P. aeruginosa, Candida* spp, because of their inheritant resistance to cephalosporins.

A review of antibiotic consumption in Medical Institute - Ministry of the Interior showed 45.9-, 42.8- and 46.2 DDD/100 patient-day respectively for 2003-, 2004- and 2006. Table 3 presents information about the antibiotic consumption.

As it can be seen from the table, the relative rate of usage of cephalosporins is high, while other important classes of antibiotics, such as penicillins (broad-spectrum penicillins) and macrolides are used in smaller proportion. We should note that the usage of fluoroquinolones is similar to that in the other European countries, and that the use of carbapenems increases. It is important to note that during the last years ceftriaxone has been recommended by the national UTI guidelines and was used widely for complicated UTI; cephalosporins of 3rd generation accounted for 33% of our hospital cephalosporins usage (13- to 17.4 DDD/100 patient/day). Also, in the antibiotic market in our country some antibiotics, used world-wide and recommended by expert guidelines for UTI (6, 8) (such as nitrofurantoin) are not available.

Table 2. Emergence of ESBLs among UTI Enterobacteriaceae strains

Microorganism	% ESBL			% R CIP			% R AG			% R SXT		
	2003	2004	2005	2003	2004	2005	2003	2004	2005	2003	2004	2005
E. coli	2	4	6	12	14	22	3	6	8	17	21	27
K. pneumoniae	12	26	18	12	16	26	12	24	15	24	26	28
Enterobacter spp	9	21	35	8	10	14	7	12	22	17	18	33
S. marcescens	6	57	33	13	14	11	50	57	33	27	57	33
C. freundii	22	53	36	58	14	27	26	14	20	53	38	50

ESBLs, extended-spectrum beta lactamases; R, resistance; CIP, ciprofloxacin; AG, aminoglycosides; SXT, co-trimoxazole

Table 3. Antibiotic consumption in Medical Institute, Ministry of the Interior, 2001-2005

Year	Antibiotics	DDD/100pt-d	Year	Antibiotics	DDD/100pt-d
2001	Penicillins	6.8	2001	Aminoglycosides	6.2
2002	Penicillins	45.9	2002	Aminoglycosides	5.3
2003	Penicillins	14.0	2003	Aminoglycosides	4.0
2004	Penicillins	11.1	2004	Aminoglycosides	4.3
2005	Penicillins	13.4	2005	Aminoglycosides	4.1
2001	Cephalosporins	9.3	2001	Quinolones	6.2
2002	Cephalosporins	13.1	2002	Quinolones	5.3
2003	Cephalosporins	13.9	2003	Quinolones	4.0
2004	Cephalosporins	17.4	2004	Quinolones	3.1
2005	Cephalosporins	19.7	2005	Quinolones	4.8
2001	Macrolides	2.7	2004	Co-trimoxazole	0.8
2002	Macrolides	5.7	2005	Co-trimoxazole	0.3
2003	Macrolides	5.3			
2004	Macrolides	7.1	2004	Carbapenems	0.1
2005	Macrolides	4.9	2005	Carbapenems	0.4

CONCLUSIONS

1. The analysis of pathogens from UTI revealed the prevalence of *E. coli* in each studied setting, while among the hospitalized male patients the relative rate of particular hospital pathogens: *Enterobacter* spp, *P. aeruginosa* and *S. marcescens* was higher.
2. Antibiotic resistance continues to increase, and emerging resistances represent ESBL-producing Enterobactreiaceae and carbapenem-resistant P. aeruginosa.
3. Antibiotic usage in our institution is high and should be better balanced.
4. It is time to re-assess the guidelines for the antibiotic therapy of urinary tract and other infections according to the current resistance rate and emerging resistance; the role of cephalosporins in infective pathology should be re-evaluated.
5. Professional body for a rational, easy and quick antibiotic licensing in Bulgaria should be established: flucloxacillin/nafcillin, nitrofurantoin, colistin, and other important antibiotics are not at the market yet.

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CHANGES IN THE VIRAL HEPATITIS B (VHB) MORBIDITY 1987-2006

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ABSTRACT

Hepatitis-B virus is one of the more important human pathogens responsible for an enormity of chronic diseases worldwide. Availability of an effective and safe vaccine for VHB prevention is of considerable help for decrease morbidity/mortality of the disease.

Aim of the study was to evaluate the number of VHB patients in different age groups, as well as the dynamics of the morbidity of VHB in Plovdiv-city and suburbs for 1987-2006.

For better compare the results the investigation time was divided into 4 periods of five years each: pre-immunization (1987-1991), intermediate (1992-1996), early post-immunization (1997-2001) and late post-immunization (2002-2006). There are evidences of substantial decrease in the overall HBV-morbidity from the first toward the last monitoring period (1534 patients vs. 578 patients). Particular HBV-morbidity decrease in the age groups up to 1 year (2.41% vs. 0.86%), to 3 years (4.88% vs. 1.55%) and to 7 years (10.36% vs. 0.69%) was marked. It is clear that when people receive HBV-vaccine, the burden of disease decreases significantly over time.

Key words: Viral Hepatitis B

INTRODUCTION

Despite multi-annual medical community increasing efforts, despite availability of preventive vaccine, hepatitis B-viral infection (HBVI) remain global health problem /8/. Almost 40 yrs after its discovery, hepatitis virus B (HVB) continues to be one of the most important human pathogens, responsible for huge number chronic infectious conditions worldwide. WHO reports from 2 billion people infected with hepatitis virus B, more than 350 millions had chronic HBV infection /10/. Clinical specter of this infection performs wide range: during the acute phase - from sub-clinical to self-limiting symptomatic acute viral hepatitis and rarely fulminant hepatitis; during the chronic phase - from inactive HBsAg carriage state, to chronic hepatitis and liver cirrhosis with its complications; some patients with chronic clinical course develop liver cancer /4/. It is expected about 15-40% of patients with chronic HBV-infection to develop cirrhosis and terminal stage liver diseases /3, 5, 9/. Patients with chronic infection have about 25% risk of lethal issue resulting of HBV-related liver cancer or cirrhosis. The last two terminal stages of chronic HBV-infection kill about a million people annually /10/. There are many extra-hepatic demonstrations of the mentioned before etiologic related diseases /6/. All this present the problem of HBVI-prevention especially important.

Aim of the study was to evaluate the number of VHB patients in different age groups, as well as the dynamics of the morbidity of VHB in Plovdiv-city and suburbs for 1987-2006.

ABBREVIATIONS USED IN THIS PAPER:

HVB - Hepatitis virus B

ACCEPTED FOR PUBLICATION: 10. 01. 2009

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METHODS

For collected data comparative analysis, the study period was presented by four stages (5 years each of them): pre-immunization /1987-1991/, intermediate /1992-1996/, early post- immunization /1997-2001/ and late post- immunization /2002-2006/. 3688 patients with acute VHB infection were diagnosed according clinical and epidemiological data, as well as routine laboratory constellations for HBV antigens and HBV antibodies. Radio-immune assay (RIA) was used during the first stage mainly, and ELISA during the following years.

Criteria for epidemiologic diagnosis were:

- Proving HBsAg in the same time with HBcIgM antibodies
- Proving only HBsAg and tracing its following clearance.

It is difficult to reject in some minority of cases HBV co-infections with HAV, HCV and HDV, affecting some patients during all stages of the study, but this hardly influence results of this investigation.

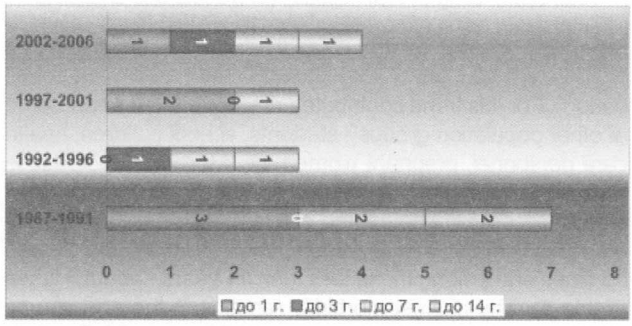

Figure 1. Children acute viral hepatitis death number during the study periods

RESULTS

The morbidity dynamics and the relative incidence of VHB among up to 14 years old children during the investigation period are present on Table 1 and on Figure 1:

DISCUSSION

A great privilege is an effective and safe HBV-vaccine availability and this makes HBV-infection preventive. Hepatitis B-immunoglobulin /HBIG/ with high titer of anti-HBs antibodies was produced as well. In 1991 WHO recommend vaccination of all children and 116 countries, incl. Bulgaria added this vaccination in their routine immunization programs. Thanks to organizations like Global Alliance for Vaccines and Immunization /GAVI/ and Global Fund for Children's Vaccines /GFCV/ this immunization became accessible for poorest countries too /10/. Nevertheless, it is necessary the following contingents to be vaccinated as well: those exposed to family and sexual contact; health care personnel; patients on hemo-dialysis and these on recurrent hemo-transfusions and transfusions of other biologic products; HIV positive persons; drug addicts; individuals who made contacts in HBV-endemic regions. Immune antibodies are found 10 years after vaccination, but the immunity is longer. Despite the vaccination would not treat chronic hepatitis, it is 95% effective in preventing development of chronic infection and is the first vaccine against one of the most dangerous human carcinoma. According certain studies vaccine-responses demonstrate reverse correlation with age and body mass index and are influenced by genetic factors such as specific HLA haplotypes and environmental factors (smoking etc.) who could affect the persistence of vaccine induced immunity.

The obligatory vaccination of newborn for HBV decreased general HBV morbidity in Plovdiv's region from 21.9‰ (for pre-immunization period) to 8.25‰ (for post-immunization

Table 1. Acute HBV infection age group morbidity monitoring, divided in five-year periods until and after HBV-immunization program starting in Bulgaria

Age/period	1987-1991	1992-1996	1997-2001	2002-2006	1987-2006
Up to 1 year	37-2.41%	4-0.41%	2-0.32%	5-0.86%	48
Up to 3 years	75-4.88%	28-2.91%	7-1.13%	9-1.55%	119
Up to 7 years	159-10.36%	81-8.42%	29-4.71%	4-0.69%	273
Up to 14 years	208-13.55%	135 -14.04%	110-17.88%	27-4.67%	480
Up to 24 years	455-29.66%	352-36.62%	237-38.53%	273-47.23%	1317
Up to 44 years	367 -23.92%	279-29.03%	186-30.24%	169-29.23%	1001
Up to 60 years	137-8.93%	63-6.55%	33-5.36%	54-9.34%	287
Over 60 years	96-6.25%	19-1.97%	11-1.78%	37-6.40%	163
Total HBVI	1534	961	615	578	3688
Severe forms	429-27,96%	211-21,95%	86-13,98%	75-12,97%	801-21,7%
VH[1] mortality	58-3,78%	39-4,05%	26-4,22%	28-4,84%	151-4,09%
AVH[2] mortality	46-2,99%	24-2,49%	19-3,08%	24-4,15%	
Morbidity	21.9‰	13.7‰	8.78‰	8.25‰	

[1] Total death rating of viral hepatitis (VH) acute and chronic forms
[2] Total death rating of acute viral hepatitis (AVH) forms - acute liver failure (ALF)

period). For this trend contributed obviously the immunization of other population groups - students, school children, health care personnel, pregnant women etc. Morbidity decrease is noted especially among children less than 14 years of age: among breast fed babies and children less than 3 years the reduction is about 3 times; among children less than 7 years - 15 times and among these less than 14 years - about 3 times (Table 1). The highest morbidity remains in the age groups 15-24 years, followed by the groups to 44 years old. Our data is in accordance with the data concerning the whole country /1, 2, 7/. Not only morbidity is decreased, but also the segment of the cases with severe clinical HBVI-course as well (Table 1.) That could be explain with the lesser exposure to home contacts with HBV-affected children; immunization of certain adult age groups; with the biological products strictly HBV-testing; sterilization of instruments for surgical intervention etc. On Table 1 the total viral hepatitis (VH) lethality count during the observation periods is presented and on Figure 1 the death number among children up to 14 years old is shown. Because HBsAg could not be always proved in ALF (acute liver failure) cases, also there are very rare ALF cases caused by HAV or by HCV alone, we presented VH lethality in general, no matter etiological factor and clinical form of hepatitis. Epidemiological study however revealed data supporting HBV-infection among non immunized children, rather than other human hepatitis virus infection. Despite decrease of the general number of cases, lethality remains high with certain tendency for percentage increase from the first to the four periods (Table 1).

Our study revealed the effects of a vaccination program in Plovdiv hyperendemic area. It is clear that when people receive HBV-vaccine, the burden of disease decreases significantly over time.

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