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EPIDEMIOLOGICAL AND CLINICAL RESEARCH IN CHILDREN WITH PROVEN SHIGELLOSIS

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SUMMARY

We studied the dissemination, age structure, seriousness of the disease and some special symptoms at children in the group of 0 to 14 years old with proven shigellosis. All the children with proven dysentery have been studied retrospectively during the period from 1993 to 1997. The number of children was 1451. 47,96% of them were sick and received clinical treatment. 33.63% were sick and have been treated at home. The rest of them 18.40% were only carriers. The comparison between the average number of patients for the whole country and that ones for Plovdiv and the region was made. The group receiving clinical treatment has been prospectively studied with respect to the basic clinical symptoms of Shigellosis, seriousness of the disease, age, sex and morbidity. Routine microbiological and statistical methods were used. The number of cases with Shigellosis in children from Plovdiv and the region was higher than the rest of the country since 1995. The cases with children up to 1 year old were 19-25 times more than the other ones but those in the group of children from 1 to 3 years old were only 7-8 times more. The most frequently isolated strain were S. flexneri (80.25%), followed by S.sonnei (18.52%), S. boydi and S. dysentery (1.22%). The relatively groups of hospitalized children 0-1 and 1-3 years old were respectively 35.20% and 34.20%. The cases with children 4-7 and 8-14 years old were respectively 15.80% and 14.80%. The seriousness of Shigellosis in the course of disease was as follows: mild in 25.71%, moderate in 45.54%, severe in 27.72% and lethal in 1.02%. Hypotrophy was registered in 58.54% of the breast-fed infants. 56.03 patients were with bloody diarrhea, 68.85% with vomitting and 79.31% with high temperature. Shigellosis is widely disseminated disease among children in Plovdiv and the region. The most frequent cases are among children up to 3 years old and the disease is the most severe and continuous.

Key words: *shigellosis (dysentery), epidemiological research, childhood, symptoms*

Recently Shigellosis becomes the most widely spread bacterial intestinal disease in childhood. The morbidity at children is significantly higher than that one at adults. They suffer from the disease more seriously and most of them are under home treatment that suggest an idea the special symptoms in the course of the diseases to be known by clinical practioners.

We studied the dissemination, age structure, clinical forms and some special symptoms of the disease in the course of the treatment at children with proven shigellosis.

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MATERIAL AND METHODS

The research was divided into two parts:

1. Retrospective epidemiological observation, investigation and analysis at 1451 children in the group of 0 to 14 years old with proven dysentery during the period from 1993 to 1997. Data from Hygieno-Epidemiological Institute - Plovdiv, Regional Center of Health services - Plovdiv and Infectious Clinic at Medical University - Plovdiv have been used for the purpose of the research. The following epidemiological indices have been studied: morbidity, age stucture and lethality. A complete epidemiological method including epidemiological observation, investigation and analysis was used. All cases were microbiologically proven by conventional methods valid for the country. The correlation between the individual serovars in the region for the same period was established.
2. The admitted for treatment in the clinic 696 sick children with proven dysentery were prospectively monitored and examined with respect to their age, sex, seriousness of the disease by the generally accepted in the practice criteria and by fever. Some basic symptoms such as diarrhea, vomitting, etc. Routine statistical methods were used.

RESULTS AND DISCUSSION

The total morbidity rate from dysentery in Bulgaria on 0/0000 population was 54.03 in 1993, 116.38 in 1994, 74.38 in 1995, 39.55 in 1996 and 35.74 in 1997. Regarding to the data from Plovdiv region (Table 1) the following differencies were determined: in the initial two years the morbidity was lower in comparison with the total one for the country, but during 1995 have been increased and reached 87.80 and in the following two years retained permanently higher from the average for the country as decreased in comparison with that one in 1995 for the region. The most frequently sick from dysentery were the children up to one year old. The morbidity in this group by years was 19 to 25 times higher in comparison with the total for the region. Children from 1 to 3 years old have been also sick often but here the morbidity was 7 to 8 times higher than the total. This incidence rate at older children decreased from 1.2 to 2.7 times. Therefore, the morbidity of dysentery at the children up to three years old have been retained steadily high during the studied five years and represented a serious problem for epidemiologists, paediatricians, specialists of infectious diseases and general practitioners. The data obtained from other authors are the same, especially from the developing countries where the dysentery is a great problem for children in childhood and requires continuous regional investigation (12,13,15).

According to the data of Hygieno-Epidemiological Institute in Plovdiv and the region totally for the period the most frequently isolated strain at children was S.flexneri - 80.25%, followed by S.sonnei - 18.52% ($p<0.001$) as S.boydii and S.dysenterie were isolated only in a single cases totally 1.22%. The more of the authors (10,11,12,13) indicated similar relationship of the specified two serovars. The relatively percentage of S.flexneri was increased from 66.80 to 90.15% and S.sonnei decreased from 31.79 to 9.84% ($p<0.001$) - Table 3.

From 1451 children with proven dysentery in Infectious clinic were admitted 696 (47.96%) sick and 267 (18.40%) carriers. The rest of them 488 (33.63%) sick children have been leaved for home treatment. Totally for the period the hospitalized sick children were with high relatively percentage 47.96% (Table 2). The relatively percentages of the children from 0 to 1 and from 1 to 3 years old are high and close to each other, respectively 35.20% and

Table 1. Morbidity of shigellosis on 0/0000 population in Plovdiv and the region for the period 1993-1997

Year	Total morbidity on 0/0000 population	Children from 0 to 14 years old				Adults
		0-12 months	1-3 years old	4-7 years old	8-14 years old	
1993	37.02	872.09	276.85	98.32	83.13	11.36
1994	67.02	1700.13	484.07	184.69	142.91	20.47
1995	87.80	1702.06	611.02	191.32	119.41	41.96
1996	55.55	1062.69	448.62	101.63	70.11	28.34
1997	52.79	1452.96	421.78	103.98	66.28	26.09

Table 2. Distribution of the hospitalized children with shigellosis by age and years for the period of 1993-1997

Year	Total number of children %	From them hospitalized children and %	Hospitalized children from 0 to 14 years old			
			0-12 months	1-3 years old	4-7 years old	8-14 years old
1993	212	122	38	41	23	20
	%	57.54	31.15	33.61	18.85	16.39
1994	372	134	42	55	19	18
	%	36.62	31.34	41.04	14.18	13.43
1995	400	168	68	42	29	29
	%	42.00	40.48	25.00	17.26	17.26
1996	237	145	50	57	19	19
	%	61.18	34.48	39.32	13.10	13.10
1997	230	127	47	43	20	17
	%	55.22	37.01	33.86	15.75	13.38
Total	1451	696	245	238	110	103
	%	47.96	35.20	34.20	15.80	14.80

34.20% ($p>0.05$). The relatively percentages of the age group from 4 to 7 and from 8 to 14 years old were lower in comparison with the mentioned above but also similarly close to each other, respectively 15.80% and 14.80% ($p>0.05$). The relatively percentages for the studied period by years varied in the age of 0 to 1 year old from 31.15% (1993) to 40.48% (1995) ($p>0.05$). For the children in the age from 1 to 3 years old these values were from 25.00% in 1995 up to 41.04% in 1994 ($p>0.05$). The high relatively percentage of hospitalized children (47.96%) towards the total number of sick children indicates indirectly the more seriously passing of shigellosis at children. Similar age structure of the admitted for treatment children with shigellosis has been indicated from almost all authors (4,5,8) and directed attention to the early age from 0 to 3 years old as a risky group subjected to antiepidemical measuires. The partition of male sex at the admitted for treatment children was insignificantly higher - 53.53%.

The distribution of the hospitalized with shigelosis children according the seriousness of the clinical course of the disease (Table 4) indicates statistically authentic the highest relatively percentage 45.54% of those with a moderately form in comparison with those with severe form 27.72% ($p<0.001$) and those with mild one 25.71% ($p<0.001$). The relatively percentages of the clinical forms in different age groups are presented in Table 4: the dysentery passes the most severe at the breast-fed infants 58.54% because of which they are a risky group. The severe forms decreases at children under 3 years old up to 27.46%, at those under 7 years old up to 8.29% and at the oldest ones - up to 5.69%.

7 children were died (5 - breast-fed infants and 2 - under 3 years old). The specific for dysentery changes in the intestines have been found in all of them and hypotrophy II-nd and III-th degree was also registered. The lethality of the admitted children was 1.02%. Compared with the data received from other authors it is low. According some of the authors it could reached up to 19% (3,6,7,9).

Hypotrophy has been found in 138 of 245 breast-fed infants (56.32%). At 124 (17.81%) of children have been established another accompanied diseases - the most often bronchopneumonia followed by tracheobronchitis, rhinopharyngitis, purulent otitis,tonsillitis, uroinfections, etc. Probably the hypotrophy accompanied sufferings, as mentioned above, was the reason for more frequently infectiousness and morbidity of the children from dysentery (1, 2, 14).

Some clinical symptoms at children with shigellosis in % by age are presented in Table 5. There have been registered from 5 to 10 defecations for twenty-four hours

Table 3. Proved shigellosis strains (serotypes) in % at children for the period 1993-1997

Year	S.flexneri	S.sonnei	S.boidii	S.dysenteriae
1993	84.69	11.03	3.91	0.35
1994	66.80	31.79	1.20	0.21
1995	75.00	24.84	0	0.15
1996	84.63	15.12	0	0.24
1997	90.15	9.84	0	0

Table 4. Clinical forms of shigellosis by severeness according to the age

Clinical forms	Total number and %	From them by age			
		0-12 months	1-3 years	4-7 years	8-14 years
Mild	179	34	64	33	48
	25.71	18.99	35.75	18.43	26.82
Moderately severe	317	93	119	61	44
	45.54	29.33	37.54	19.24	13.88
Severe	193	113	53	16	11
	27.72	58.54	27.46	8.29	5.69
Dead	7	5	2	0	0
	1.02	2.04	0.84	0.00	0.00

Table 5. Some clinical symptoms at children with shigellosis in % by age

Clinical symptoms	Age			
	0-12 months	1-3 years old	4-7 years old	8-14 years old
Number of defications for 24 h				
n = 696				
up to 5	30.00	39.23	13.84	16.92
from 5 to 10	40.55	39.17	12.44	7.83
over 10	28.84	30.76	23.07	17.69
Duration of the diarrhea				
n = 696				
up to 3 days	16.52	26.33	25.00	32.14
up to 6 days	30.39	40.19	18.14	11.27
up to 10 days	47.50	36.67	9.16	6.66
over 10 days	60.13	35.81	4.05	0
Bloody diarrhea	30.00	33.84	18.46	17.69
n = 390				
Tenesmus	11.43	22.85	34.28	31.42
n = 35				
Temperature				
n = 552				
up to 38°C	44.79	26.04	18.75	10.41
38-39°C	41.35	40.60	9.77	8.27
over 39°C	23.68	35.96	25.43	14.92
Convulsions n = 27	22.22	48.14	25.92	3.70

at 48.11% of the studied children and more than 10 at 23.06%. Divided by age into bread-fed infants and up to 3 years old children, more than 10 defications were registered for 24 hours, respectively 28.84% and 30.76%. At the older of them from 4 to 7 and from 8 to 14 years old those percentage run down, respectively up to 23.07% and 17.31%.

Diarrhea with different duration has been registered at all 696 children as follows: up to 3 days in 32.18%, up to 6 days in 29.31%, up to 10 days in 17.24% and more than 10 days in 21.26%. Although the conducted treatment the duration of the diarrhea was more than 6 days at 38.50%. The diarrhea continued more than 10 days at 60.13% of the breast-fed infants while at the half of the older children was only 3 days. Bloody diarrhea was registered at 390 (56.03%) from the total 696 children. It was occurred more often in children from 1 to 3 years old (33.84%). It was registered in 30.00% of breast-fed children and in the age between 8-14 years old in 17.69%.

Tenesmus or prolapse of anus was found only in 35 of patients (5.02%). It was occurred more often in the age between 4-7 and 8-14 years children.

477 children (68.53%) from total 696 studied were admitted in the clinic with vomitting. Here has been registered the same dependency. The breast-fed infants vomitted in 34.59% but older children between 8-14 years old in 18.86% ($p<0.05$).

Higher temperature was registered at 552 (79.31%) of the patients. Temperature in the range of 38-39°C was registered in 42.93% and above 39°C in 28.62%. Temperature above 39°C was found in 35.96% of 1 to 3 years old children.

Convulsions have been observed at 27 sick patients (3.88%) and a paralytic ileus at 9 (1.29%).

In conclusion, it should be noticed that the morbidity of shigellosis in children from 0 to 3 years old has been retained high during the studied period. The percentage of admitted for treatment children was high (47.96%) that indirectly confirmed the severe course of the disease. The most severe shigellosis were in breast-fed children as the hypotrophy undoubtedly contributed to this. Following the basic symptoms of the disease we arrive at the conclusion that at breast-fed infants and children up to 3 years old the defecations are more frequent, diarrhea is more prolonged, temperature is higher and vomitting is more often. The percentage of the children leaved on home treatment is high (33.63%). From this follows that it is necessary the more wide circle of specialists to know in details the disease and to pay more attention especially to children up to 3 years old.

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NEUTROPHILIS RESPIRATORY BURST ACTIVITY IN NEONATES: A COMPARISON STUDY OF TWO DIFFERENT METHODS

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SUMMARY

The aim of this study was to compare two different methods: flow cytometric analysis and nitroblue tetrazolium (NBT) reduction test. These methods were used for measuring neutrophils respiratory burst activity (NRBA) in neonates. The study population consist of 19 neonates with clinical and laboratory signs of infection and 9 neonates that are not infected. NBT dye reduction assay was performed with whole blood and smears were observed microscopically for presence of formazan - positive PMN cells. The whole blood flow cytometric method was performed with dihydrorhodamin 123 as an oxidative probe. In our study there is a correlation between the results obtained with NBT test and DHR 123 - flow citometry: ($r=0.865$, $p<0.01$ for noninfected neonates and $r=0.553$, $p<0.05$ for neonates with infection) and it has been more prominent in noninfected neonates compared to infected.

Key words: *neutrophil respiratory burst activity, nitroblue tetrazolium test, flow cytometric dihydrorhodamin 123 oxidative burst assay, neonates.*

Peripheral blood polymorphonuclear leukocytes (PMN) play an important part in the hosts's defense against bacterial infections. Upon activation with appropriate stimuli as bacterial lipopolysacchardes, cytokines at al in normal PMN, oxidase is activated which results in the consumption of oxygen and its conversion into superoxide. Thes reaction, known as respiratory burst, is shown below:

$$NADPH + 2O_2 + H^+ \Rightarrow NADP^+ + 2O_2^- + 2H^+.$$

Much of the superoxide (O_2^-) formed in this reaction spontaneously reacts with water to dismutate into hydrogen peroxide (H_2O_2) and oxygen (O_2). Subsequently, H_2O_2 may be converted by the enzyme myeloperoxidase into highly reactive compounds such as hypochlorous acid (HOCL) or it can be converted with ferrous ion (which acts as reductant) to other reactive oxygen intermediates such as OH^- (1). These products of respiratory burst play an essential role in the host's response to infections, and destroy bacteria inside the phagosome. For example, patients with chronic granulomatous disease who have inherited defects in NADPH oxydase of phagocytic cells fail to produce superoxide and other reactive oxygen species following cell activation (2). They suffer from recurrent pyogenic infections, which is often life threatening. In this study two defferent methods: flow cytometric analysis and nitroblue tetrazolium (NBT) reduction test were used for determination the ability of neonatal phagocytes to produce bactericidal oxygen metabolites. Nitroblue tetrazolium (NBT) reduction test is first described from Park et al. in 1968 (4). It is a classical method, characterized with simplicity and availability, recommended for clinical and laboratory practice as an objective method for the study of leukocyte function. The NBT dye reduction test measures the reduction of the clear yellow water-soluble compound to the deep blue dye formazan by mechanisms related to the metabolic events in the respiratory burst in neutrophils. These events include hydrogen peroxide and superoxide radical formation, increased oxygen uptake, and an increase in the hexose monophosphate shunt (5). Currently, the NBT is used in clinical practice to detect defects in intracellular killing of microorganisms by the neutrophils (4, 5, 6). Flow cytometric analysis is a new, sensitive method for quantitating intracellular respiratory burst activity at a single cell level. It was first described by Bass et al. in 1983 using the fluorescent dye 2'7' - dichlorofluorescin diacetate (DCF) as oxydative probe (8). Recently, generation of reactive oxygen species has been evaluated flow-cytometrically using the oxidative probe dihydrorhodamin 123 (DHR) (9, 10). DHR is freely permeable; it localizes in the mitochondria and after oxidation by H_2O_2 and O_2^- to rhodamine 123 emits a bright fluorescent signal. DHR is the most effective flow-cytometric probe for assessing the oxidative burst in human PMN cells (11). It is intended to investigate the altered oxidative burst activity: rapid and sensitive method for the diagnosis of chronic granulomatous disease, impaired in transplant patients and patients with AIDS and increased in neonates with laboratory signs of infection (12). In this study, the neutrophils respiratory burst activity (NRBA) has been examined. For this purpose two different methods: flow cytometric analysis with DHR 123 and nitroblue tetrazolium (NBT) reduction test has been used. We evaluated the two methods, which are different as techniques, but reflect one at the same process - neutrophil respiratory burst activity.

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ABBREVIATIONS USED IN THIS PAPER:
dihydrorhodamin 123 (DHR), nitroblue tetrazolium (NBT) test, neutrophils respiratory burst activity (NRBA), polymorphonuclear (PMN) cells

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METHODS

Study population. The study population consisted of two groups of neonates: a) 19 with clinical and laboratory signs of infection and b) 9 neonates not infected (no clinical or laboratory signs of infection). Non infected patients were admitted to the unit for reasons other than infection. All studied neonates were admitted to the neonatal intensive care unit at the University Pediatric Hospital in Sofia. NRBA was measured in the same blood specimens taken for routine procedures. Measurements were performed once in each neonate in first 2 days after admission in whole-heparinized venous blood.

NBT day reduction assay. Venous blood with heparin (100 µl) was mixed with 100 µl 0.2 % NBT solution and 100 µl PBS (non stimulated NBT test). After incubation at 37°C for 15 min and at room temperature for 15 min blood smears were prepared. The smears were fixed in methanol and counterstained with Giemsa. The PMN cells were than observed microscopically for presence of the formazan precipitate. Results were expressed as the percentage of PMN cells containing formazan precipitate.

Flow cytometric oxidative burst assay - Bursttest. The quantitative determination of NRBA as % activated PMN cells was realized with *Bursttest* kit (*Orpegen* Pharma). Heparinized whole blood was mixed with PBS (nonstimulated test) and incubated for 10 min at 37°C in a water bath. After that, an oxidation was made by adding substrate solution (DHR 123); then another incubation (10 min at 37°C) followed. Reaction was stopped by adding lysing solution (2 ml for 20 min at room temperature). After this step, the samples were washed with PBS and resuspended with PBS containing propidium iodide (10 min at 0°C) for DNA staining.

Flow cytometry. A FACS Calibur flow cytometer (Becton Dickinson) was used for acquisition and analysis of the data. To exclude cell debris and platelet aggregates from analysis, a gate was set on propidium iodide-stained leukocytes during acquisition in the red fluorescence (575 nm, FL 2 channel). Granulocytes were identified by forward light scatter and side scatter. For each

measurement, 10 000 events were collected in the granulocyte gate. Rhodamine 123 green fluorescence was collected in the FL1 channel and a fluorescence histogram was plotted. The results were recorded as the percentage of fluorescent cells (% positive cells). The regions for negative/positive fluorescence were setting by examination of the histogram of the negative control (healthy adult, as a daily control).

Statestical analysis. For comparison of studied groups Student's test was used. For correlation analysis, Pearson's correlation coefficient (r value) was used. Differences with a P value of less than 0.05 were considered statistically significant.

RESULTS

The results about NRBA, obtained with NBT test (% formazan - positive PMN cells) and with flow cytometyr (% activated PMN cells), are presented on Table 1. They demonstrated that patients with infection had NRBA higher than noninfected neonates: there is a significant difference between the two studied groups ($P<0.05$). Correlation between results from NBT test and flow cytometry (fig. 1 and fig. 2) has been estimated. Pearson coefficient of correlation was $r=0.865$, $p=0.0026$ in noninfected neonates and $r=0.553$, $p=0.0139$ in neonates with infection. Correlation has been more prominent in noninfected neonates compared to infected.

DISCUSSION

In activated neutrophils, reactive oxygen radicals are produced and several different methods have been designed for quantitating reactive oxygen radical production: cytochrome c reduction, luminol-chemilumines cence, NBT dye reduction, flow cytometry et al. (14). In this study we describe experiment in which two different methods were used for measureng oxidative burst activity in neonates with and without infection: NBT test and flow cytometry with DHR as oxidative probe. We measured neutrophilis oxidative burst activity without stimulation, in whole blood. Initially, we evaluated the neutrophil respiratory burst activity as % activated cells in neonates with and without infection. The infected neonates demonstrated the greater % activated cells compared with noninfected neonates with NBT test and flow cytometry. We have found also a correlation between results from NBT test and DHR-based flow cytometric assay in infected neonates and in neonates without infection.

The NBT test is classical, widely used method characterized with simplicity and availability. But in NBT assay many parameters are difficult to control: the smears quality, direct counting by light microscopy may be exclusively subjective, failure of method in patients with leukopenia et al. The DHR-based flow cytometric assay is a new method which is defined by many authors as a sensitive functional assay, which appears to satisfy all requirements for laboratory assay - it can be performed using little whole blood, can be performed on very small numbers of PMN and involves a technically simple and standard procedure, guarantied by the *Bursttest* kit. In this study our results also illustrate the utility of the dihydrorhodamine assay as an alternative to the more widely used NBT test. The method may be useful in identifying state of infection in neonates.

We found also that the coefficient of correlation of the two methods is different: the correlation was lower in infected neonates compared with noninfected. Based on this findeng, we can speculate that in state of infection many factors can influence the result of oxidative burst assay and more sensitive, modern and precise methods can be used.

In conclusion, NRBA is elevated in neonates with infection. It can be measured with different methods and there is a correlation between the results obtained with NBT test and DHR 123 - flow cytometry.

Table 1. Neutrophil respiratory burst activity in neonates with infection and in neonates without infection measured with two different methods: NBT test (% formazan - positive PMN cells) and flow cytometry (% activated PMN cells).

Method	Neonates with infection mean ± SD n = 19	Neonates without infection mean ± SD n = 9	P
NBT test	25.84 ± 9.4	17.33 ± 9.7	< 0.05
Flow cytometry	9.85 ± 4.9	6.34 ± 3.9	< 0.05

P - Neonates with infection vs. neonates without infection

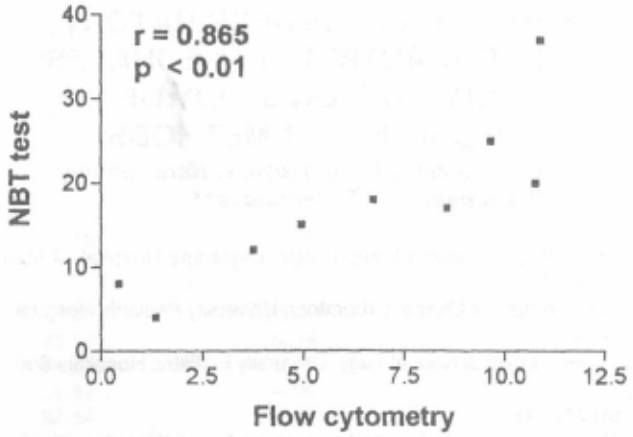

Figure 1. Correlation between the results obtained with two different methods - NBT test (% formazan - positive PMN cells) and flow cytometry (% activated PMN cells) in neonates without infection.

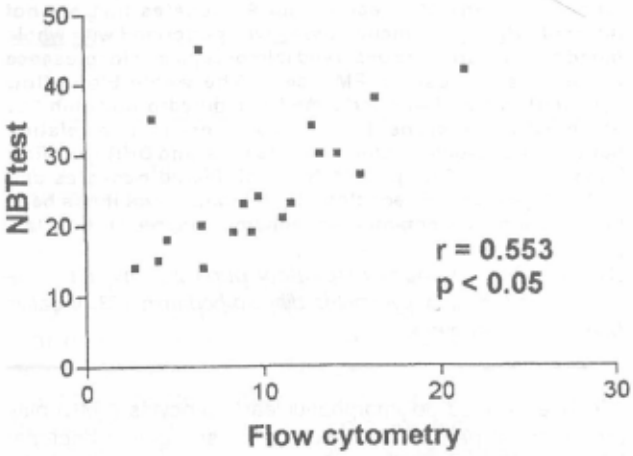

Figure 2. Correlation between the results obtained with two different methods - NBT test (% formazan - positive PMN cells) and flow cytometry (% activated PMN cells) in neonates with infection.

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DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR ESTIMATION OF TETANUS ANTIBODIES IN HUMAN SERA

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SUMMARY

The level of tetanus antibodies after immunization is measured traditionaly in vivo by toxin neutralization test. However, the test is time consuming and requires laboratory animals, thus challenging us to develop more rapid and simple alternative in vitro test. The goal of this report is to develop an enzyme linked immunosorbent assay (ELISA) for detection of IgG tetanus antibodies.

Key words: *tetanus, tetanus toxoid, immunoprophylaxis, sera reactivity*

The disease caused from Clostridium tetani is still health problem for many countries all over the world, mainly because of its high lethality. The only way to limit it, is the specific immunoprophylaxis with tetanus toxoid. After introducing in humans it leads to production of specific antibodies that have main role in tetanus prevention.

The obligatory specific prophylaxis against tetanus in Bulgaria started in 1959. Nowadays the incidence of the disease in our country is 0.06 ‰ (1997) and the conducted seroepidemiological study reveals that these patients were not completely immunized or were not immunized at all (1, 2, 3, 4). The immunity against tetanus is humoral and depends on the level and the ability of specific antibodies to neutralize tetanus toxin in shortest period of time.

The level of tetanus antibodies after immunization is measured traditionaly in vivo by toxin neutralization test. However, the test is time consuming and requires laboratory animals, thus challenging us to develop more rapid and simple alternative in vitro test. The goal of this report is to develop an enzyme linked immunosorbent assay (ELISA) for detection of IgG tetanus antibodies (6, 7).

Knowledge of the immune status of population against tetanus may have practical implication (8, 9). It may assist in checking the efficacy of immunization schedules adopted and the persistence of immunity. It may also be helpful to clinicians in hospital emergency rooms in the choice of correct antitetanus prophylaxis for patients at risk of developing tetanus. So, the determination of tetanus antitoxin in human sera may be useful both for seroepidemiological surveys and for clinical purposes.

MATERIALS AND METHODS

Serum samples

Fifty eight human sera seronegative for HIV, HBV and Lyme borreliosis were tested for tetanus antibodies using laboratory developed ELISA. According to person's age serum samples were divided in three groups - children and teenagers younger than 20 years, adults from 20 to 55 years old, and persons over 55 years. All samples were stored at 20°C in small vials until use.

Enzyme linked immunosorbent assay

Plates for micro ELISA with 96 wells (Nunc immunoplates, Denmark) were used in this research. Tetanus toxoid prepared in NCIPD with antigen activity about 450 Lf/ml and protein content 40 mg% protein N was used as antigen. 50 ml were added in each well at a concentration of 0.9 Lf/ml after diluting in carbonate buffer (pH 9.6). Plate was coated and incubated at 4°C overnight. Then wells were washed tree times. The buffer used for all washing procedures was *phosphate buffered saline* (PBS) containing 0.05 % Tween 20 (PBS/T). 50 ml blocking buffer - PBS including 1% bovine serum albumin were added after washing cycles in each well. Plate was incubated at 4°C for 1 hour.

Human sera were prepared to a working concentration 1:100 just before use, using 1% *PBS*/T/albumin. Four control serum samples were used - one highly positive (30 UI/ml); one moderately positive (16 UI/ml); one weakly positive (5 UI/ml) and one negative (below protective level of tetanus antibodies at 0.1 UI/ml). One well with 1% PBS/T/albumin without serum sample served as blank. All serum samples and controls were added to the plate in volume 50 ml and incubated at 37°C for 1 hour. After three washing cycles 50 ml of peroxidase binding anti - human IgG (Dako, Dakopatts, Denmark) diluted 1:4000 were added and plate was incubated at 37°C for 1 hour. After washing procedure 50 ml of substrate chromogen o-phenylenediamine (OPD) were added. The reaction was stopped by adding of 1N HCl and the optical density was read at 492 nm (Uniskan, Labsystem). Standard curve with four points according to measured optical densities of used control serum samples was prepared each time (chart 1).

The results were evaluated by following criteria: strong positive reaction - same and higher than positive control sample with 16 UI/ml concentration of tetanus antibodies; moderately positive reaction - between 5-16 UI/ml; slow positive reaction between 0.1-5 UI/ml and negative reaction - below 0.1 UI/ml.

RESULTS AND DISCUSSION

Fifty eight human sera seronegative for HIV, HBV and Lyme borreliosis were estimated by laboratory developed ELISA.

Development of laboratory ELISA for estimation of tetanus antibodies.

Titration of antigen. Tetanus toxoid with antigen activity 450 Lf/ml was used. The protein content was determined according to Keldal method (5) and found to be 40 mg% PN. Various dilutions of antigen were prepared and results can be viewed on chart 2. For coating of immunoplates antigen concentration of 0.9 Lf/ml was used.

Titration of serum samples. After determining of antigen concentration, check-board titration of human sera was made. Working concentrations with sera dilutions at 1:100 and 1:200 were found as most useful for ELISA. The results obtained after titration of serum samples are present on chart 3.

Two control serum samples - one with known high level of tetanus antibodies and one with low antibody titer were used for all previously described titrations.

Titration of peroxidase binding anti-human IgG. Peroxidase binding anti - human IgG (Dako, Dakopatts, Denmark) was used. Five different dilutions of the conjugate (1:2000-1:32000) were checked using five serum samples with different concentration of tetanus antibodies. The observed results are presented on chart 4. As most useful was found conjugate dilution at 1:4000.

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ABBREVIATIONS USED IN THIS PAPER:

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

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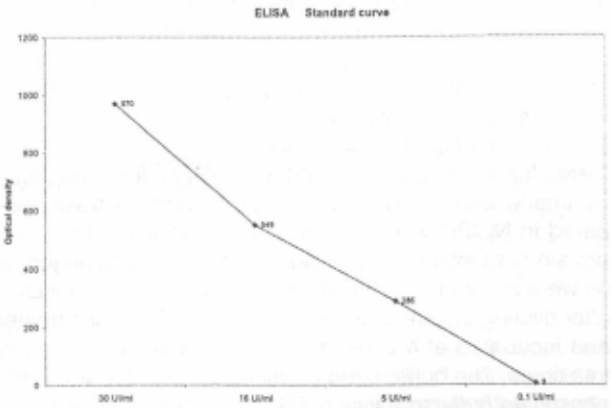

Chart 1. Concentration of tetanus antibodies in used control serum samples

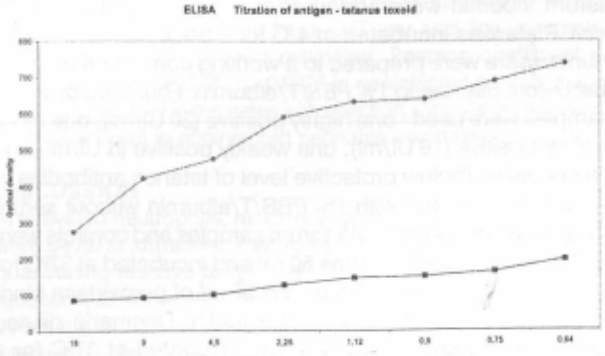

Chart 2. Antigen dilutions in Lf/ml
Legend:
■ serum sample with low concentration of tetanus antibodies
● serum sample with high concentration of tetanus antibodies

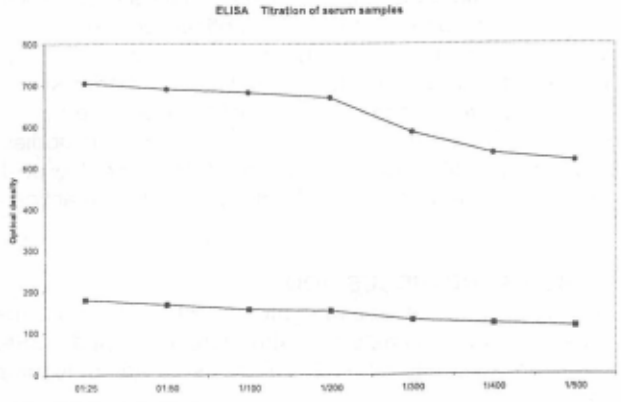

Chart 3. Sera dilutions
Legend:
■ serum sample with low concentration of tetanus antibodies
● serum sample with high concentration of tetanus antibodies

Seroreactivity of human sera. Serum titer at 0.1 UI/ml and higher has been established as protective and reliable enough to prevent tetanus. Serum samples were separated in three groups according to patient's age as described in materials and methods. Three groups according to the observed level of tetanus antibodies in examined sera were made.

Laboratory developed ELISA test showed following results - strong ELISA reactivity was observed in 4.64% samples, moderately positive reaction in 11.6% of cases and weakly positive in 17.4% of serum samples.

The seroreactivity of serum samples obtained by laboratory developed ELISA test is presented on table 1.

The table shows that children and adults up to age of 55 have good protective level of immunity against tetanus and that in older ages this level shows propensity to decrease. This tendency may be a result from absence of compliance between doctors and their patients and also from conservative view of the manipulation - vaccination of old people.

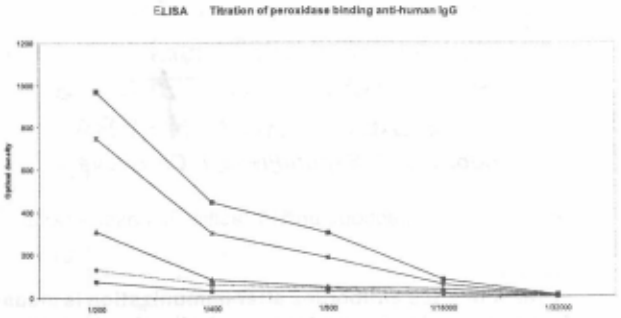

Chart 4. Dilutions of peroxidase binding anti-human IgG
Legend:
■ control serum sample with 30 UI/ml concentration of tetanus antibodies
★ control serum sample with 16 UI/ml concentration of tetanus antibodies
▲ control serum sample with 5 UI/ml concentration of tetanus antibodies
◆ control serum sample with 0.1 UI/ml concentration of tetanus antibodies
● PBS

Table 1. Seroreactivity of serum samples obtained by ELISA

Age groups Titer	Up to 20 years	21- 55 years	Over 55 years	Total
weakly positive ELISA reactivity	3.48%	5.8%	8.12%	17.4%
moderately positive ELISA reactivity	8.12%	2.9%	0.58%	11.6%
Strong positive ELISA reactivity	3.48%	1.16%	——	4.64%

Tetanus toxoid is low reactogenic and several epidemiological surveys conducted in our country showed that it has good tolerance among old people and they must be included in obligatory immunization schedule functioning in Bulgaria.

CONCLUSIONS

An immunoenzyme assay for detection of tetanus antibodies in human sera was developed. The described in this report ELISA test is economical in terms of time and reagents and introduces a new method for estimating of tetanus antibodies. ELISA is sensitive, precise and is reliable enough to replace the currently used high cost in vivo toxin neutralization test.

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TETANUS ANTIBODY INDEX IN EVALUATION OF INTERTHECAL ANTIBODY PRODUCTION IN PATIENS WITH LYME NEUROBORRELIOSIS

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SUMMARY

Neuroborreliosis is the most frequent late manifestation of Lyme borreliosis. The best current indicator for Lyme neuroborreliosis is detection of intrathecal antibody synthesis estimated by specific antibody indices. A new technique for calculation of tetanus antibody indexbased on tetanus toxoid ELISA was developed. Paired cerebrospinal fluid (CSP) and serum samples from 12 patients with clinical manifestations of Lyme neuroborreliosis were examines by B. burgdorferi and tetanus toxoid ELISA and specific IgM/IgG - and tetanus antitoxin - B. burgdorferi antibody indices were determined. When only serum samples were tested by B.burgdorferi ELISA, 7/12 samples were found seropositive. Testing of CSF samples in addition, followed by calculation of specific antibody indices, determined 11/12 CSF-positive patients thus showing higher diagnostic value of CSF testing. Calculation of tetanus antibody index was as sensitive as calculation of IgM/IgG antibody index for detection of intrathecal antibody production. Both techniques revealed prevalence of IgM - over IgG antibody response in CSF despite the stage of the desease.

Key words: *Lyme neuroborreliosis, Borrelia burgdorferi, interthecal antibody production, tetanus toxoid, tetanus antibody*

Neuroborreliosis is one of the most frequent and serious manifestations of Lyme borreliosis - a multisystem tick-borne disease caused by the spirochetes of B. burgdorferi s.l. group - species B. burgdorferi s.s., B. garinii, B. afzelii and B. bisettii.

Our previous investigations (1) showed that neuroborreliosis is the most common late manifestation of Lyme borreliosis in Bulgaria and predicts the outcome of the disease.

The best current marker for neuroborreliosis is B. burgdorferi - specific antibody synthesis in cerebrospinal fluid (CSF). Patients with meuroborreliosis develop specific intrathecal antibody response. However, esimation of intrathecal antibody production via antibody indices has been introduced (4, 5). The most often applied is IgM/IgG antibody index. Our 5-year experience with evaluation of this index shows good correlation and confirmation of Lyme neuroborreliosis cases.

Theoretically calculation of specific antibody index using tetanus antitoxin posses an advantage over total immunoglobulin antibody index because antibodies appeared after immunization and normally are not detected in CSF. The goal of this study is to develop a system for comparison of CSF/serum ratios of tetanus antibodies and B. burgdorferi antibodies, calculation of tetamus antibody index and investigation of its correlation with total IgM/IgG antibody index.

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ABBREVIATIONS USED IN THIS PAPER:
CSF - cerebrospinal fluid, B. burgdorferi - Borrelia burgdorferi

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MATERIAL AND METHODS

Patents: paired CSF and serum samples, collected on the same day, from 12 patients with clinical manifestations of Lyme neuroborreliosis were used for the study.

Controls: paired CSF and serum samples from 10 patients with aseptic meningitis and clinically defined multiple sclerosis served as controls. All serum and CSF samples were stored at - 20°C until use.

B. burgdorferi ELISA: Antibodies against B. burgdorferi were detected using the ELISA as described previosly (6). Briefly, B. garinii strain was used as antigen in protein concentration 10 μg/ml. Serum samples were diluted 1:200 and CSF samples 1:2. Horseradish peroxidase conjugated antihuman IgM and IgG antibodies (Dako, Dakopatts, Denmark) and ophenylendiamine were used to reveal antigen-antibody reaction.

Tetanus toxoid ELISA: Recently developed ELISA for estimation of tetanus antibodies (7, 8) was applied. Tetanus toxoid antigen (0,9 Lf/ml), serum dilution 1:200 and CSF dilution 1:2, and horseradish peroxidase conjugated antihuman sera were used as described.

IgM/IgG antibody index: Antibody index (AI) using total immunoglobulins (Ig) was calculated according to Reiber et al. (9) by the following equation.

$$AI = \frac{AbCSF}{Abser} : \frac{IgCSF}{Igser}$$

where Ab are antibodies against B. burgdorferi in CSF or serum respectively and Ig are total IgM or IgG immunoglobulins in CSF or serum.

Intrathecal antibody production is confirmed when AI ≥ 2.0, i.e. CSF/serum ratio of specific antibodies exceeds CSF/serum ratio of total immunoglobulins (IgM or IgG) at least two times. Quantity of total IgM or IgG was expressed in mg/dl. Antibody level was expressed in IU/ml, where maximal optical density of known positive control serum was equal to 100 U/ml.

Tetanus antibody index: To calculate tetanus antibody index, CSF/serum ratio of teranus antibodies was used instead of CSF/serum ratio of total immunoglobulins and compared with ratio of B. burgdorferi-specific antibodies.

$$AI = \frac{AbCSF}{Abser} : \frac{TeCSF}{Teser}$$

wher Ab are antibodies to B. burgdorferi and Te are antibodies to tetanus toxoid in paired CSF and serum sample resp.

Intrathecal antibody production is considered when AI ≥ 2.0. Levels of B. burgdorferi-specific and tetanus antibodies were expressed in IU/ml, where highest optical density of both serum positive controls were equal to 100 IU/ml.

RESULTS

Routine Lyme borreliosis serology. Routine B. burgdorferi ELISA detected specific antibodies in serum samples from 7/12 patients with clinical manifestation of neuroborreliosis. In 4 of 7 patients IgM antibodies were detected, in 2 patients - both IgM and IgG antibodies and in 1 patient - only IgG antibodies. Thus prevalence of IgM serum antibodies to B. burgdorferi over IgG antibodies was clearly defined.

Among control serum samples, only one serum sample from patient with multiple sclerosis was found positive.

IgM/IgG antibody index. When paired CSF and serum samples were tested, more patients with neuroborreliosis were detected.

At least 2-times higher CSF/serum ratio of B. burgdorferi-specific IgM antibodies in comparison with the total IgM ratio was detected in 9/12 patients with Lyme neuroborreliosis. In 3 of the 9 patients intratecal synthesis of specific IgG antibodies (i.e. AI ≥ 2.0) was revealed together with IgM antibody synthesis. In addition, 2 of 12 patients had only IgG antibody synthesis in CSF.

Prevalence of IgM over IgG antibody response was found in CSF as in serum despite long lasting of symptoms in some of the patients.

None of the samples from the control group of patients gave evidence for intrathecal antibody preduction to B. burgdorferi.

Tetanus antibody index. Recently developed ELISA for tetanus antibodies and modification of the technique for antibody index calculation were applied to determine tetanus antibody index.

CSF/serum ratio of B. burgdorferi-specific antibodies exceeds two or more times the CSF/serum ratio of tetanus antibodies in 11 of 12 patients. The same patients that were confirmed by IgM/IgG antibody index were detected by tetanus antibody index. The only defference was that in almost all cases CSF/serum ration of total IgM/IgG was remarkably higher than CSF/serum ratio of tetanus antibodies (table 1).

Table 1. Comparison of different indices for detection of intrathecal production of antibodies to B. burgdorferi and ELISA testings of serum samples of 12 patients with Lyme neuroborreliosis.

Patient No	IgM ELISA	IgG ELISA	IgM index	IgG index	Te IgM index	Te IgG index
1	+	+	6.2 (+)	5.5 (+)	9.6 (+)	7.8 (+)
2	+	+	4.7 (+)	6.7 (+)	6.4 (+)	9.3 (+)
3	+	-	11.1 (+)	4.1 (+)	16.1 (+)	6.2 (+)
4	+	-	7.3 (+)	1.1 (-)	6.1 (+)	0.9 (-)
5	+	-	3.4 (+)	1.2 (-)	4.5 (+)	1.6 (-)
6	+	-	2.7 (+)	0.9 (-)	3.2 (+)	1.1 (-)
7	-	+	0.7 (-)	8.2 (+)	0.9 (-)	7.3 (+)
8	-	-	0.6 (-)	4.4 (+)	0.8 (-)	4.2 (+)
9	-	-	5.1 (+)	0.7 (-)	7.6 (+)	1.1 (-)
10	-	-	7.2 (+)	0.9 (-)	3.6 (+)	0.8 (-)
11	-	-	5.3 (+)	1.5 (-)	7.7 (+)	1.6 (-)
12	-	-	0.8 (-)	0.9 (-)	0.7 (-)	0.9 (-)
Total	**6**	**3**	**9**	**5**	**9**	**5**
Positive	7		11		11	

DISCUSSION

Detection of specific intrathecal antibody production to B. burgdorferi requires estimation of antibody indices in order to preclude possible penetration of serum antinbodies into CSF through impaired blood-brain barrier. Among various techniques used for calculation of the indices, IgM/IgG antibody index is the most commonly applied. These techniques have been routinely used in our laboratory for the last 3 years. However, theoretically estimation of intrathecal antibody production by CSF/serum ratio of tetanus antibodies is more precise. Such antibodies are not synthesized in CSF representing better substance. On the other hand, intrathecal antibody synthesis is observed in various neurological disorders - neurosyphilis, multiple sclirosis, subacute sclerosing panencephalitis and miningoencephalitis.

Our comparative data on paired CSF/serum samples from 12 patients with neuroborreliosis showed that both indices, total IgM/IgG antibody index or tetanus antibody index, give similar results and can detect specific intrathecal antibody production to B. burgdorferi in CSF. Tetanus antibody index probably has higher potential to differentiate among negative and positive cases because of its higher values in comparison with IgM/IgG index. This hypothesis should be proven by testing of a large series of samples. Nevertheless, high concordance of the results using both indices indicates that detection of tetanus antibodies is a good alternative of routine requirement for measurement of total IgM, IgG and albumin in CSF and serum samples.

The next interesting finding of our investigation is restricted ability of serum antibody testng to detect Lyme neuroborreliosis. One third of all patients tested had antibodies only in CSF but not in serum. It is very plausible that kinetics of antibody response is different in CSF and serum. Antibody response in serum seems may start later than antibody response in CSF and decline sooner or both. Thus, the only confirmation of neuroborreliosis is to detect intrathecal antibody production against B. burgdorferi.

As a confirmation of this hypothesis is our finding that IgM antibody response prevails over IgG antibody response in such patients. IgM antibodies were detected even in patients with long lasting history of neurological desorder. So, the dynamics of antibody response in CSF is different. One can speculate that antigen particles or shole B. burgdorferi cells are hidden in the host organism and stimulate immune system. Extremely important for the treatment strategy is differentiation between both possibilities - antigen presence or autoimmunity.

In conclusion, in niuroborreliosis there is a pronounced B. burgdorferi - specific IgM and IgG B-cell response confined to the CSF. Due to its independence of blood brain barrier leakage, estimation of CSF/serum antibody indices increases diagnostic reliability of specific CSF antibody detection. Calculation of tetanus antibody index is promising being as sensitive as the routinely IgM and IgG index for detection of intrathecal antibody synthesis.

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PHENOTYPIC CHARACTERISATION OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM NASAL CARRIERS

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SUMMARY

Objective: **To compare *S. aureus* nasal isolates from healthy nasal carriers and out-patients with recurrent furunculosis on the basis of their phage-type and antibiotic resistance phenotype.** ***Methods:*** **Forty-six medical staffs working in an intensive care unit, 27 healthy volunteers and 25 out-patients with recurrent furunculosis were cultured from the anterior nares. Fifty-five *S. aureus* strains were isolated and phage-typed using an international basic set of 23 bacteriophages. The antibiotic susceptibility testing was performed by disc diffusion technique, oxacillin resistance was determined phenotypically by oxacillin screen-agar method and genotypically by PCR detection of *mec*A gene.** **Results: The data of the present study showed higher prevalence of phage group II among S. *aureus* nasal isolates from out-patients with recurrent furunculosis. The most frequently isolated staphylococcal strains from the nose of medical staff and healthy volunteers were III and mixed lysotype groups respectively. The majority of *S. aureus* isolates showed comparatively high sensitivity to antimicrobial agents. Not one multiply resistant strain was found among nasal isolates from medical staff and one was detected in each of the rest two group of strains.** ***Conclusions:*** **The data presented in this study observe some correlation (in case of staphylococcal furunculosis) between the kind of host infection and the phage-type of the nasal strain. This was shown with the higher prevalence of group II among nasal isolates from out-patients with recurrent furunculosis, compared with the lysotypes of nasal strains from healthy persons. No comparable correlation was found with the antibiotic resistance phenotypes. It was not found significant difference in resistance phenotypes among nasal strains from medical staffs and from out-patients with furunculosis.**

Keywords: *Staphylococcus aureus, nasal carriage, phage-type, antibiotic resistance phenotype*

Large number of studies have consistently documented that the carriage of *S. aureus* in the anterior nares is an important human reservoir for *S. aureus*, from where the organisms can spread to other parts of the body (1). Infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. The nasal carriage of *S. aureus* in the normal adult population is in the range 10 - 40% (2). Two patterns of *S. aureus* nasal carriage can be distinguished: individuals who almost always carry *S. aureus* are called stable or persistent nasal carries and such persons who harbours the microorganism intermittently are called intermittent nasal carriers.

Some groups of people have been shown to be more frequent carriers in comparison with general population, such as people with chronic dermatitis, other chronic lesions or allergic rhinitis, patients with insulin dependent diabetes mellitus and intravenous drug addicts, also hemodialysis patients, and those with HIV/AIDS. The reasons for differences in colonisation patterns and the factors that lead to the establishment of *S. aureus* carrier state are incompletely understood but it is known now that nasal carriage of *S. aureus* plays a key role in the development of staphylococcal infections (1). It has been clearly established that it is a major risk factor for the development of infection in certain groups of patients (e.g. patients on hemodialysis and CAPD, patients undergoing surgery or with intravascular devices and HIV (3).

The aim of the present study was to characterise *S. aureus* isolates from the nose of healthy individuals and out-patients with recurrent furunculosis on the basis of their phage-types and antibiotic resistances.

MATERIALS AND METHODS

Subjects and bacterial cultures

Forty-six medical staffs working in an intensive care unit, 27 healthy volunteers and 25 out-patients with recurrent furunculosis were cultured from the anterior nares. The nasal cultures were taken by cotton swabs, after several times rotation in each nare and then plated directly on 5% sheep blood agar. Blood agar plates were examined after 24 hours of incubation at 37°C.

Species identification

Isolates were identified as *S. aureus* if they were gram-positive cocci in clusters and were catalase, tube coagulase and clumping factor positive.

Detection of oxacillin resistance

1. Phenotypic detection of oxacillin - resistant *S. aureus* strains was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations, using screen agar plates (BBL, Becton Dickinson, Heidelberg, Germany), containing 6mg of oxacillin per litre and 4% of NaCl (4).

2. PCR detection of *mec*A gene was performed according to Ünal et al. (5) using the oligonucleotide primers: 5'-GAC GA AAC AAT GTG GAA TTG GCC - 3' and 5'- CAC CTT GTC CGT AAC CTG AAT CAG C - 3' (Roth, Karlsruhe, Germany). The reaction mixture (30 µl) contained 1µl primer 1 (10 pmol), 1µl primer 2 (10 pmol), 0.6 µl dNTP (10 mM; MBI Fermentas, St. Leon Rot, Germany), 3.0 µl 10 x thermophilic-buffer (Promega/Boehringer, Ingelheim, Germany), 1.8 µl $MgCL_2$ (25 mM, Promega/Boehringer), 0.2 µl Taq DNA polymerase (5U/µl; Promega/Boehringer) and 21.4 µl aqua dest. Finally 1 µl DNA preparation was added to each 0.2 µl - reaction tube (Biozim). The DNA preparation was performed with the QIAamp Tissue Kit as described by the manufacturer (Qiagen, Hilden, Germany). The DNA could be stored at -20°C. The amplification was carried out using the following program: precycle 1 x 2 min at 94°C , 30 x 30 sec at 94°C, 30 sec at 55°C, 2 min at 72°C and final extension incubation at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 8 µl of the reaction product in a 1.0% agarose gel with Tris acetate electrophoresis buffer TAE (40 mM Tris HCl, 1 mM EDTA), 1.14 µl/L glacial acetic acid (pH 7,6) and a 100 bp DNA ladder (Gibco BRL Life Technologies, Eggenstein, Germany) as molecular marker.

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ABBREVIATIONS USED IN THIS PAPER:
CAPD - continuous ambulatory peritoneal dialysis, PCR - polymeraze chain reaction

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Table 1. Characterisation of 17 *S. aureus* strains isolated from nasal carriers - medical staff.

Number of isolates (n=17)	Antibiotic resistance phenotype	Phage type	Phage group
3	Not typeable	-	P
1	Not typeable	-	P, T
1	Not typeable	-	-
1	83A/53/54/75/77/85/95/47	III + misc	P
1	83A/53/54/75/77	III	P
1	83A/53/75/77	III	-
1	83A/85/54/47	III	-
1	83A/71/55/75/54	I + III	P
1	53/54/77	III	-
2	3A/55	II	P
1	52/79	I	P
2	96	V	P
1	94	V	P

Table 2. Characterisation of 14 *S. aureus* strains isolated from nasal carriers - healthy volunteers.

Number of isolates (n=14)	Antibiotic resistance phenotype	Phage type	Phage group
1	Not typeable	-	-
2	Not typeable	-	P
2	83A/52A/29/80/52/95/3C	I+II+III+ misc	P
1	83A/52A/53/85/47/71	I + II + III	P, E
1	83A/53/54/75	III	P, T, Cm, E
1	52A/80/52/95	I + misc	-
1	3C/55/77/47/53/75/6/85	II + III	-
1	3C/55/3A/71/85	II + III	P, T
1	55/3A	II	P, T
1	71	II	P
1	54	III	P
1	53	III	P

Phage typing

An international basic set of phages (group I - 29, 52, 52A, 79 and 80; group II - 3A, 3C, 55 and 71; group III- 6, 42E, 47, 53, 54, 75, 77, 83A, 84 and 85; group V - 94 and 96; miscellaneous, 81 and 95) was employed using standard methods. The test was performed by routine test dilutions (1 : 100). Phages that produced a strong reaction as defined in international rules (6) determined the phage-type.

Antibiotic susceptibility testing

The antibiotic susceptibility was determined by agar diffusion method as recommended by the NCCLS (Standard M-2 A6 1997) with the following discs: ampicillin (A-10 μg), oxacillin (O-1μg), chloramphenicol (Cm-30 μg), ciprofloxacin (C-1μg), erythromycin (E-15 μg), gentamicin (G-10 μg), rifampin (R-5 μg), tetracyclin (T-30 μg), (BBL, Becton Dickinson, Heidelberg, Germany) and mupirocin (M-5 μg), vancomycin (V-30 μg) (Oxoid Limited, Hampshire, England).

RESULTS

Fifty-five *S. aureus* strains were isolated from nasal carriers: 17 - from medical staff, 14 - from healthy volunteers and 24 - from patients with recurrent furunculosis.

The lysotype group distribution of *S. aureus* isolates from medical staff was the following: group III (23, 59%), group V (17, 65%) and 11, 76% for group II and mixed group. The staphylococcal isolates from healthy volunteers belonged more frequently to mixed lysotype group (42, 85%), followed by group III (21, 43%) and II (14, 28%). In contrast, 83, 33% of the nasal strains isolated from patients with recurrent furunculosis gave a strong reaction with lytic group II phages. An absence of oxacillin resistance and a similarity of antibiotic resistance patterns among nasal isolates from healthy individuals and out-patients with chronic furunculosis was found.

S.aureus nasal isolates from medical staff and from volunteers showed higher sensitivity to antimicrobial agents compared with *S. aureus* from patients with skin infections. There were 23,52% of isolates from medical staff sensitive to all antibiotics tested, 21 ,42% and 12,83% from healthy volunteers and patients with skin infections respectively. The commonest resistance phenotype among staphylococcal nasal strains from medical staff (70 ,05%), from healthy volunteers (50,00%), and from patients with skin infections (58,33%) was resistance to penicillin. One strain (5,89%) among isolates from medical staff was found to be resistant to penicillin and tetracycline simultaneously, 14, 44% from volunteers and 2,83% from patients with skin infections showed the same pattern of resistance. Penicillin together with erythromycin resistance showed one isolate from healthy volunteers (7, 14%) and two from patients with skin infections (8, 33%). The results are presented in Table 1, Table 2 and Table 3. Not one of the nasal isolates from medical staff was multiply resistant and one only strain was multiply resistant in each of the rest two groups.

DISCUSSION

The factors that lead to establishment of carrier state are not well elucidated. We still have little insight in the basic determinants of successful long-term colonisation by *S. aureus*. Presently, no mechanisms underlying persistent or intermittent interaction between host and bacterium and no differences between strains in their colonisation ability have been proposed. However, the significance of nasal carriage of *S. aureus* in the epidemiology of infection has been comprehensively established. The anterior nares provide the principal reservoir for *S. aureus* dissemination to other body sites, thus predispose patients to auto-infection. Some authors suggest that *S. aureus* mainly leads to infection among persistent carriers and that nasal carriage could be used to identify patients more susceptible to these infections (7).

The data of the present study demonstrated a higher prevalence of phage-group II among *S. aureus* nasal isolates from patients with recurrent furunculosis (83, 33%), whereas the carrier strains from medical staff and healthy volunteers mainly belonged to III and mixed I+II+III phage-groups, respectively. The lower prevalence of group II staphylococcal strains among healthy carriers was presented previously in a study by Eriksen et al. (7). The authors further suggest that the lower prevalence of phage group II *S. aureus* strains could be caused by probably low ability of these strains to colonise but a higher ability for establishing infection. Faber et al. (1996) reported an increasing of phage group II strains among *S. aureus* invasive isolates in Denmark hospitals during the years 1971-1990 (8). Moreover, it was found a dominance of *S. aureus* strains of phage group II among isolates from chronic furunculosis (9, 10, 11). Hedstrum presented data for identical phage-types of staphylococcal cultures from the nares, perineum and concomitant lesions. These results lead authors to suggest that group II staphylococci rather than group I strains tend to disseminate over the body surface (10). On the other hand, our previous study, based on pheno- and genotypic methods revealed identity between *S. aureus* isolates from anterior nares and furuncles of one and the same patient (11). If so, we could then consider that the knowledge of the antibiotic phenotypes of nasal strains can guide physicians when selecting empiric therapy to a more rational use of antibiotics. The high prevalence of phage group II strains found either in the furuncles and in the nose seems to be essential for strains causing staphylococcal skin infections. In addition, the rate of *S. aureus* nasal carriage

Table 3. Characterisation of 24 *S. aureus* strains isolated from nasal carriers with recurrent furunculosis.

Number of isolates (n=25)	Antibiotic resistance phenotype	Phage type	Phage group
1	Not typeable	-	-
1	71/55/3C/3A/29/52/52A	I + II	-
4	71/55/3C/3A	II	P
2	71/55/3C/3A	II	P, T
1	71/55/3C	II	P, T
1	71/55/3C	II	P
1	71/55/3C	II	P, E
3	71/3C	II	P
1	3A/3C	II	P
3	3C	II	P
1	3C	II	P, T, E
1	3C	II	P, E
1	3A	II	P, T
1	71	II	P
1	53/54/47	III	P, T
1	83A/54/47	III	-
1	96/84/52A	I + III + misc	P

was shown to be higher among patients with skin infections than in general population (12, 13, 14). It seems the nasal carriage of *S. aureus* could be accepted as a risk factor for development of staphylococcal skin infections.

The data of the present study revealed higher sensitivity of *S. aureus* nasal isolates from healthy individuals (including medical staff) to all antibiotics tested than the nasal strains from out-patients with recurrent furunculosis are (Table 1, Table 2 and Table 3). However, the patterns of antibiotic resistance did not differ significantly among nasal isolates from healthy volunteers and out-patients with recurrent furunculosis. The data presented in this study observe some correlation between the type of host infection and the phage-type of the nasal isolate. This was shown above with the higher prevalence of phage group II among nasal isolates from patients with recurrent furunculosis compared with the lysotypes of nasal strains from medical staff and healthy volunteers. No comparable correlation was found with the antibiotic resistance phenotypes. The prevalence of phage group III strains isolated from the nares of the medical staff did not associate with multiple antibiotic resistance, as it was suggested by others (15). Although Lacey et al. reported a decrease in virulence of *S. aureus* in chick embryos associated with the acquisition of plasmids for streptomycin, tetracycline, chloramphenicol, neomycin and methicillin resistance (16), this finding is in contrast with numerous *in vitro* studies which have failed to show that increasing antibiotic resistance is associated with decreasing virulence (17, 18). The data of the present study did not reveal significant difference between antibiotic resistance phenotypes of the nasal strains from healthy volunteers and out-patients' nasal strains. The latter undoubtedly possess virulence because of their identity with strains from the site of infection. Seems the antibiotic resistance phenotype is probably not significant feature concerning the virulence and colonising ability of the strains.

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CLOSTRIDIA IN ANAEROBIC INFECTIONS: A 16-YEAR STUDY IN BULGARIA

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SUMMARY

The purpose of the study was to determine the isolation of *Clostridium spp.* from 1054 specimens with anaerobic flora sent to the referent for the country anaerobic laboratory in the period 1983-1998. The study revealed 321 clostridial isolates from 367 specimens (35% of all specimens) from 280 patients with anaerobic infections mainly in surgery. Most of them were from intra-abdominal infections (134 patients), soft-tissue infections (64), bone and joint infections (28), gas gangrene (14) and bacteraemia (9) as well as from genital and pulmonary infections, infections of the eyes, of the CNS, in otorhinolaryngology and from dental infections. The main clostridial isolates were 152 strains *C. perfringens*, 46 unidentified *Clostridium spp.*, 45 *C. clostridioforme* and 32 *C. ramosum* which is 85% of all, followed by *C. sporogenes*, *C. tertium*, *C. bifermentans*, *C. sordellii*, *C. septicum*, *C. innocuum*, *C. sphenoides*, *C. oedematiens*, *C. histolyticum*, *C. butyricum* and *C. paraputrificum*, etc. in lower numbers. The clostridia were the only bacterial isolates in 44 (16% of the patients); 236 (84%) cases had mixed infection. The species most commonly isolated with clostridia were *B. fragilis* group, anaerobic cocci, *Escherichia coli* and *Enterococcus sp.* as half of the specimens were from intra-abdominal infections. Our study illustrates the incidence and the variety of clostridia in anaerobic infections in Bulgaria.

Key words: *clostridia, anaerobic bacteria*

Anaerobic bacteria are known to be important, usually as part of a mixed flora, in various types of infections (10, 20, 21). However, there are only a few review articles or separate case reports on the role of clostridia in those infections (4, 5, 11, 15-18). A prospective study of specimens from anaerobic infections sent to the referent for the country National Anaerobic Laboratory between 1983 and 1998 was performed. The purpose of the study was to determine the isolation of Clostridium spp. from such specimens in this period of 16 years as there are no such data before in Bulgaria.

MATERIALS AND METHODS

Specimens

1054 specimens from the patients with anaerobic flora sent to the National Anaerobic Laboratory, National Center of Infectious and Parasitic Deseases, from different hospitals in Bulgaria were included in the study in a period of 16 years (1983-1998).

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ABBREVIATIONS USED IN THIS PAPER:
CNS - Central Nervous System

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Specimens were collected by avoiding contamination with normal flora and the sources were: body fluids, pus, different tissues (necrotic, etc.), swabs. They were transported to the anaerobic laboratory in: Stuart's transport medium (Port-a-Cul, BBL), syringes and blood culture bottles.

Microbiology

Isolation and identification of anaerobic isolates was carried out according the Wadsworth Anaerobic Bacteriology Manual, 4th and 5th edit. (20, 21) and the VPI Anaerobe Laboratory Manual, 4th edit., 1977 (with the update material, 1991) (12).

RESULTS

The study revealed 321 clostridial isolates from 367 specimens (35% of all specimens) from 280 patients with anaerobic infections mainly in surgery (Patients with botulinus, tetanus and *C. difficile* infections were not included in the study). Most of them were from intra-abdominal infections (134 patients), soft-tissue infections (64), bone and joint infections (28), gas gangrene (14) and bacteraemia (9). Clostrida were recovered also from genital (7) and pulmonary infections (6), infections of the eyes (6), of the CNS (4), in otorhinolaryngology (5) and from dental infections (3) (Table 1).

Table 1. Types of infections with clostridial isolates

Type of infection	Diagnosis	No of cases (%)
Abdoninal infections		134 (47.9%)
	Appendicitis w/o absces and peritonitis	36
	Infections in bowel surgery after perforation or carcinoma	27
	Infections of the gallbladder and the biliary duct	23
	Phlegmone of the abdominal wall	13
	Peritonitis after perforation of ulcer or carcinoma of the stomach and duodenum	8
	Other (ileus, trauma, pancreatic infections, etc.)	27
Soft-tissue infections		64 (22.9%)
	Necrotic fasciitis and phlegmones	28
	Paraproctitis and other perirectal infections	22
	Other (bite wound infections, diabetic gangrene, trauma, gangrene Fournier)	14
Bone and joint infections		28 (10%)
	Osteomyelitis and coxarthrosis	3
	After operations and amputation	15
	Trauma and fractures	10
Gas gangrene		14 (5%)
Bacteraemia		9 (3.2%)
Genital infections	Postabortal, puerperal, after hysterectomy, abscessus cavum Douglasi, tumor ovarii	7 (2.5%)
Pleuropulmonary infections	Empyema, pleuropneumonia, after resection of pulmo and thoracotomia	6 (2.1%)
Eye infections		6 (2.1%)
	Orbital phlegmones	2
	Endophthalmitis	4
CNS infections	Brain abscess, after operations	4 (1.4%)
Ear, nose and throat	Peritonsillar abscess, sinusitis, mastoiditis, after laryngectomia	5 (1.8%)
Dental infections	Dental gangrene, phlegmone of the oral cavity	3 (1.1%)
Total		**280 (100%)**

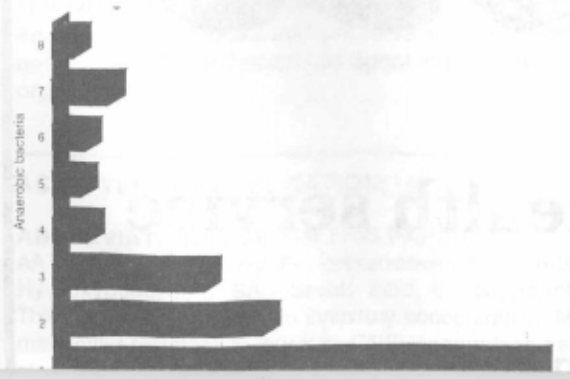
SSD was noted between the susceptibility of MRSA and the susceptibility of Enterococcus strains ($p < 0.001$) to B. SSD was observed between the susceptibility of CNS and the enterococci to B ($p < 0.001$).
MICs of Th against MRSA were: 0.1 mg/l (59 %) and 1 mg/l 2. BHB showed identic antibacterial activity against MRSA, CNS and Enterococcus strains. The enterococci were more resistant than the staphylococci to SA (99.2 % of all enterococci had MIC > 1000 mg/l, while 52 % of MRSA and 25 % of CNS were inhibited by 1000 mg/l of Sorbic

The main clostridial isolates were 152 strains *C. perfringens* (47.4%), 46 unidentified *Clostridium spp.* (14.3%), 45 *C. clostridioforme* (14%) and 32 *C. ramosum* (10%) which is 85% of all, followed by *C. sporogenes*, *C. tertium*, *C. bifermentans*, *C. sordellii*, *C. septicum*, *C. innocuum*, *C. sphenoides*, *C. oedematiens*, *C. histolyticum*, *C. butyricum* and *C. paraputrificum*, etc. in lower numbers (Table 2).

Table 2. Relative incidence of recovery of Clostridium spp. in various infections

Type of infections/Number of isolates Organisms	Intra-abdomina l	Soft-tissue	Bone and joint	Gas gangrene	Bacteremia	Genital	Pulmonary	Eye
C. perfringens	66(4)*	34	20(5)*	12(4)*	6(3)*	3(1)*	3(2)*	4(2)
C. clostridioforme	24(1)*	15	1		1(1)*	1		1
C. ramosum	23(1)*	7	1(1)*					1(1)
C. sporogenes	5		1		1(1)*	1		1
C. tertium	4(2)*						1	1
C. bifermentans	3							1
C. sordellii	1	1(1)*	3(3)*					
C. septicum		1(1)*		2(2)*	1			
C. innocuum	1	1				1	1	
C. sphenoides			2(1)*					
C. novyi		1(1)*		1(1)*				
C. histolyticum			1(1)*					
C. butyricum	1							
C. paraputrificum	1							
C. glycolicum	1							
C. fallax		1(1)*						
C. ghoni								1
Clostridium sp.	28(3)*	3	4(1)*		2	2(1)*	1	1
Total	**158 (11)***	**64 (4)***	**33 (12)***	**15 (7)***	**11 (5)***	**8 (2)***	**6 (2)***	**11 (3)***

Clostridia were the only bacterial isolates in 44 (16% of the patients); 236 (84%) cases had mixed infection. There were 709 other isolated mixed with *Clostridium*. 356 strains (50.2%) were anaerobes and 353 (49.8%) - aerobes and facultative anaerobes or the ratio anaerobes (including clostridia) to aerobes was 1.9:1. The species most commonly isolated with clostridia were *B. fragilis* group (140 strains), anaerobic cocci (101) (Fig. 1), as well as *Escherichia coli* (98) and *Enterococcus sp.* (85) probably because half of the specimens were from intra-abdominal infections. The newly discovered organism *Bilophila wadsworthia* was also isolated in 7 cases. The other aerobic strains were *Proteus sp.* (35), *P. aeruginosa* (13) and other Gram-negative rods (51), *Staphylococcus sp.* (34), *Streptococcus sp.* (29), *Candida* (5), etc.



DISCUSSION

Clostridia could be cultured from 10-30% of wounds in civilians and up to 80% of wartime wounds (after 11). Although the antibiotic therapy has influenced the possibility to recover these organisms in our days, clostridia are commonly isolated from various infections - 35% of all specimens in our study and about half (47.4%) of all anaerobic isolates in those infections. They were also the only bacterial isolates in 16% of the cases which data are comparable to those of Brook (5) with 15% isolation of *Clostridium sp.* in culture and where clostridia were found in 7% of all specimens from children.
Clostridia were especially prevalent in intra-abdominal and soft-tissue infections. Their distribution in these infections corresponds to that as normal flora in the gastrointestinal tract and more rarely - on the skin from where they may originate causing rather more endogenous than exogenous infections (11, 16).
Clostridium was also recovered in other types of infections as gas gangrene, postabortal and postpartum infections, endophtalmitis, empyema, brain abscess, dental gangrene, mastoiditis, sinusitis, etc. which corresponds to the findings of other authors (2, 4, 5, 7, 11, 19).
Although our clostridial isolates included 17 recognized species and 46 strains that could not be further classified, the clostridia that we found most frequently were *C. perfringens* (47.4%), *C. clostridioforme* (14%) and *C. ramosum* (10%) which is 71.4% of all clostridia in our clinical cases. *C. perfringens* accounted also for 48% of all clostridial isolates in a survey of Brook et al. (4) in 2 military hospitals. It was the most common isolate according to Marinella et al. (13) or in cases of clostridial bacteraemia where there was a poor clinical correlation as Gorbach et al. (11) state in their review article. And it is the easiest species among the clostridia to isolate and to identify. The virulence of *Clostridium sp.* is well documented in animal studies and in clinical infections but clostridial infections are more often mixed infections (11) - as in 84% of the cases in our study. Clostridia can act synergically with other anaerobic or aerobic bacteria (4, 5). As a result polymicrobial infections with clostridia may be more destructive and more difficult to eradicate, causing a devastating illness with high mortality as described clinically in our former study (9).
Their management comprised a combination of surgical intervention and antimicrobial therapy. Clostridia are generally susceptible to most antibiotics used in the treatment of anaerobic infections but there are reports of increased resistance to clindamycin, penicillin, cefoxitin, etc. and of production of beta-lactamases (1, 3, 6, 8, 14).
The results of this 16-year retrospective study illustrate the incidence, the variety and the importance of clostridia in anaerobic infections in Bulgaria. With the growing evidence of their resistance to antimicrobial agents, knowledge of the distribution of these organisms and the constant monitoring of their susceptibility may help in the selection of empiric treatment of clostridial infections.

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CLOSTRIDIA IN ANAEROBIC INFECTIONS: A 16-YEAR STUDY IN BULGARIA

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Specimens were collected by avoiding contamination with normal flora and the sources were: body fluids, pus, different tissues (necrotic, etc.), swabs. They were transported to the anaerobic laboratory in: Stuart's transport medium (Port-a-Cul, BBL), syringes and blood culture bottles.

B (2-bromo-2-nitropropan-1,3-diol) (fig. 3) is an aliphatic halogenonitro compound which has a broad antibacterial spectrum including P. aeruginosa but is not sporicidal (4, 6). B has been widely used as a preservative in pharmaceutical and cosmetic preparations at concentrations of 0.01-0.02 % (6). It possesses high aqueous solubility and is effective over a wide pH range (6). Its activity is reduced in the presence of 10 % serum and especially by sulphydryl compounds (4). B inhibits enzymes, which contain thiol (-SH) groups. B oxidized thiol groups to disulphides, an action that is reversed by sulphydryl compounds (2, 4).

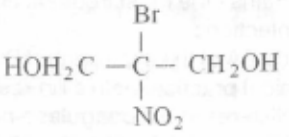


Figure 3. Bronopol - chemical structure

Th (sodium ethylmercurithiosalicylate) (fig. 4) is mercury compound that is widely used as preservative in immunolo-gical, pharmaceutical and cosmetic products (1, 4, 6). It has a broad spectrum including bacteria, M. tuberculosis, spores, fungi and viruses. Th belongs to the antibacterial agents interacting with thiol groups found in enzymic and structural protiens (2, 4). Thiol groups derived from cysteine residues are vital for the activity of many enzymes. Reaction with, or oxidation of, these essential groups produces cell inhibition or cell inactivation, but it is possible to reverse this by adding a thiol-containtig compound, such as thioglycolic acid or cystein (2, 4). Interaction of a mercury compound with enzyme or protien-thiol groups and its reversal by means of sulphydryl compound are depicted in fig. 5.

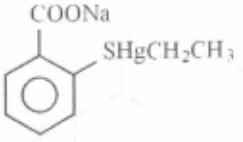


Figure 4. Thiomersal - chemical structure

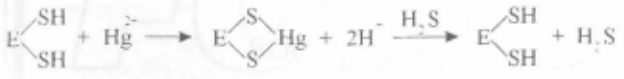


Figure 5. Effect of mercury compound on enzyme (E) - SH groups and reversal by thiol-containing compounds.

Microorganisms

47 clinical isolates of staphylococci (27 MRSA and 20 CNS, mainly methicillin-resistant). 79 Enterococcus strains (65 - from human clinical specimens, and 14 - from veterinary sources). Among the enterococci problematic were 27 strains that were resistant to high level amonoglycosides (HLAm) and/or to β-lactams:

HLStr - 9; HLGen - 4; HLStr + HLGen - 3; HLKan + HLGen + HLStr - 2; HLStr + b-lactams-resistant (BLR) -1; HLGen + HLStr + BLR - 6; HLGen + BLR - 1; and BLR - 1.

Method

The antibacterial activity of the investigated preservatives against Staphylococcus and Enterococcus strains was studied by their minimum ingibitory concentrations (MICs) (3, 4, 7). MICs were determined by serial agar dilution method.

RESULTS AND DISCUSSION

BHB in concentration of 1000 mg/l inhibited all Staphylococcus and Enterococcus strains (fig. 6).

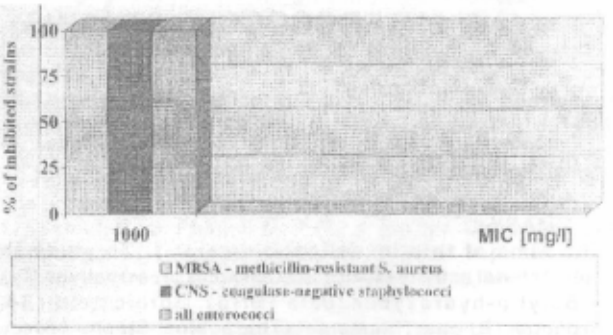


Figure 6. Comparative susceptibility of the tested strains (MRSA, CNS and enterococci) to n-Butyl p-hydroxybenzoate.

52 % of MRSA-strains; 25 % of CNS; 0.8 % of all Enterococcus strains and 7.4 % of the problematic enterococci were inhibited by 1000 mg/l of SA, the rest of strains had MICs > 1000 mg/l (fig. 7).

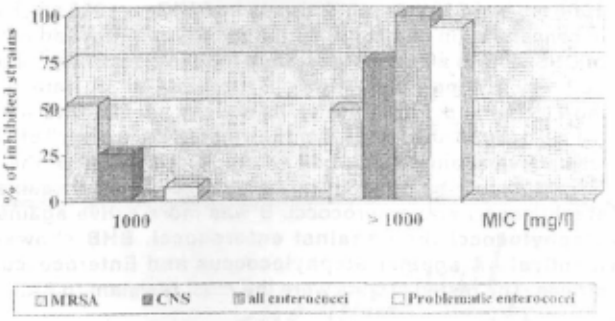
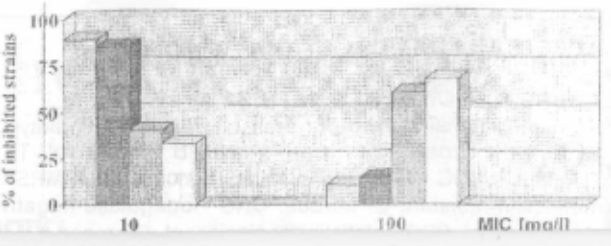


Figure 7. Comparative susceptibility of the tested strains (MRSA, CNS, all enterococci and problematic enterococci) to Sorbic acid.

There was statistically significant difference (SSD) between the susceptibility of MRSA and the susceptibility of CNS to SA ($p < 0.05$). SSD was established between the susceptibility of MRSA and Enterococcus strains to SA ($p < 0.001$). In addition, there was SSD between the susceptibility of CNS and the susceptibility of enterococcal strains towards SA ($p < 0.02$).

B in concentration of 10 mg/l inhibited 89 % of MRSA; 85 % of CNS; 40 % of all Enterococcus strains and 33 % of the problematic enterococcal strains. MIC of 100 mg/l of B inhibited 11 % of MRSA; 15% of CNS; 60 % of the problematic enterococci, tested in this investigation (fig. 8).



SSD was noted between the susceptibility of MRSA and the susceptibility of Enterococcus strains ($p < 0.001$) to B. SSD was observed between the susceptibility of CNS and the enterococci to B ($p < 0.001$).
MICs of Th against MRSA were: 0.1 mg/l (59 %) and 1 mg/l (41 %). Its activity against CNS was: 0.1 mg/l for 50 % of the strains and 1 mg/l - for the other CNS. 32 % of all Enterococcus strains and 26 % of the problematic anterococci were inhibited by 0.1 mg/l of Th, the rest of Enterococcus strains - by 1 mg/l (fig. 9).

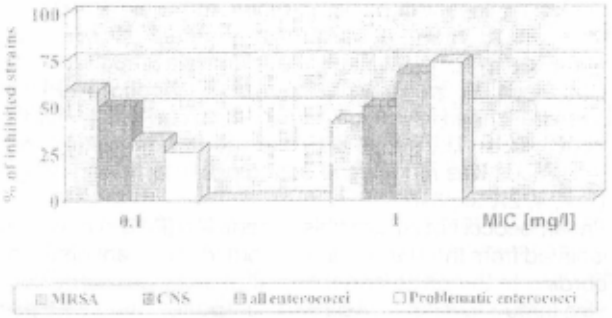


Figure 9. Comparative susceptibility of the tested strains (MRSA, CNS, all enterococci and problematic enterococci) to Thiomersal.

There was SSD between the susceptibility of MRSA and the susceptibility of Enterococcus strains towards Th ($p < 0.02$). The comparative presentation of the antibacterial activity of the considered preservatives (determined by their MICs against Staphylococcus and Enterococcus strains) is shown in fig. 10.

AA	Chemical agents	MICs against MRSA, CNS and Enterococcus strains [mg/l]
↓	Thiomersal	0,1 – 1,0
	Bronopol	10 – 100
	n-Butyl p-hydroxybenzoate	1000
	Sorbic acid	> 1000

Figure 10. The antibacterial activity (AA) of the investigated chemical agents decreases from Thiomersal to Sorbic acid.

CONCLUSIONS

1. The investigated strains showed a different susceptibility to the 4 preservatives. The range of the AA of the studied compounds was as follows: $AA_{Th} > AA_{B} > AA_{BHB} > AA_{SA}$;
2. BHB showed identic antibacterial activity against MRSA, CNS and Enterococcus strains. The enterococci were more resistant than the staphylococci to SA (99.2 % of all enterococci had MIC > 1000 mg/l, while 52 % of MRSA and 25 % of CNS were inhibited by 1000 mg/l of Sorbic acid). B was more active against staphylococci - 89 % of MRSA and 85 % of CNS were inhibited by 10 mg/l, but only 40 % of all Enterococcus strains and 32 % of the problematic enterococci were inhibited by the same concentration of B. Th showed excellent AA. MRSA were more susceptible than enterococci to Th.
3. SSD was established between the susceptibility of MRSA and the susceptibility of CNC to SA ($p < 0.05$). SSD was observed between the susceptibility of MRSA and the susceptibility of the enterococci to SA ($p < 0.001$), to B ($p < 0.001$) and to Th ($p < 0.02$). There was SSD between the susceptibility of CNC and Enterococcus strains towards SA ($p < 0.02$) and to B ($p < 0.001$).
There was not SSD between the susceptibility of all enterococci and the problematic enterococci ($p > 0.1$). There was not difference of the susceptibility of the enterococci from human and veterinary sources to the studied preservatives.

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SUSCEPTIBILITY OF CLINICAL STRAINS *STREPTOCOCCUS PNEUMONIAE* ISOLATED FROM ADULTS WITH LOWER RESPIRATORY TRACT INFECTIONS AND REVIEW OF THE LITERATURE

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SUMMARY

Clinical isolates of *Streptococcus pneumoniae* with decreased susceptibility to penicillin and, in many cases, multi-antibiotic resistant have been reported worldwide. One hundred and eighty three pneumococcal isolates were collected from sputum, pleural fluid and transtracheal aspirate from January 1995 to May 1997. The strains were from adults (over 14 years) with lower respiratory tract infections admitted to the Department of Pulmonary diseases of the Emergency Medical Institute „Pirogov", Sofia for treatment. The age of the patients ranged from 16 to 81 years; the male-to-female ratio was 1.5. The MICs of penicillin, ampicillin, amoxicillin, amoxicillin/clavulanate, cefaclor, cerufoxime, cefotaxime, erythromycin, chloramphenicol, trimethoprim/sulfamothoxazole (TMP-SMX) and vancomycin were determined by agar dilution method. Fifty-eight (31,6%) strains showed reduced penicillin susceptibility with high level resistance (MIC2mg/L) in 49 (26,7%) strains. Intermediate resistance (MIC, 0,12 -1mg/L) was found in 9 (4,9%) of the isolates. Most of the penicillin resistant strains were isolated from patients over 50 years. Penicillin-resistant strains were more frequently resistant to other 8-lactam antibiotics than were penicillin- susceptible strains. One hundred and sixty (87,4%) of the isolates were susceptible to cefotaxime. All cefotaxime-resistant strains were highly penicillin-resistant. Cefaclor showed poor activity against PSP, PIP and PRP and was less active than the other cephalosporins tested. The sensitivity to erythromycin was - 96,7%, to Chloramphenicol - 94,0% and to TMP-SMX - 73,2%. Among 58 PNSP - 37 (63,8%) were defined as multiresistant.

Key words: *S. pneumoniae, penicillin-resistant pneumococci, antimicrobial susceptibility, lower respiratory tract infections*

INTRODUCTION

During the past two decades, significant antibiotic resistance has emerged among the bacteria responsible for a variety of lower respiratory tract infections (7). *S. pneumoniae* is the most commonly identified bacterial cause of pneumonia in adults in most parts of the world (11, 27). In the United States, pneumococci cause more than 500 000 cases of pneumonia annually (19). The majority (50%-90%) of the cases of pyogenic pneumonia with acute onset in middle aged or older adults are due to this microorganism. The morbidity and mortality rates associated with pneumococcal pneumonia are enhanced in patients with underlying diseases or immunological abnormalities (17).

In the past, *S. pneumoniae* was uniformly susceptible to penicillin, allowing most physicians to treat persons who had such infections with penicillin alone and without testing for resistance. The first case of a clinically significant *S. pneumoniae isolate* resistant to penicillin was reported in 1967 (10). Since then penicillin resistant pneumococci (PRP) have been described in various parts of the world with increasing frequency and by the late 1980s the penicillin-resistant pneumococcus was recognized as a globally spread pathogen (1, 2, 4, 27, 33, 39).

Pneumococci not susceptible to penicillin (PNSP) have been isolated from the five continents, but the per cent differs according to the place and the year of isolation. Many countries throughout Africa, Asia and Europe like Hungary, Spain, Korea, South Africa and Iceland have been notorious for harboring penicillin resistant pneumococci with very high MICs to beta-lactams (8, 16, 18, 20, 21, 22, 23). The links between penicillin resistance in pneumococci and high levels of antibiotic consumption are also clear in this countries (19). In Bulgaria a high per cent of isolates with resistance to penicillin has been found during the investigations of clinical isolates and of nasopharyngyal carriers among children under 5 years of age (34, 35, 36). In the same time in northern European countries - Finland, Northern Ireland, Sweden, the United Kingdom, and in countries such as the Netherlands, Switzerland, Belgium, Germany, in Australia, there is still little or no problem with penicillin resistant isolates of *S. pneumoniae (5,* 11, 15, 30, 32, 40).

Increasing resistance to penicillin is only part of the problem. Penicillin nonsusceptibility in *S. pneumoniae* is associated with high rates of resistance to other antimicrobial agents: cephalosporins, macrolides, chloramphenicol, trimethoprim-sulfamethoxazole (26, 27). The emergence of such strains has made the selection of antibiotics for treatment of pneumococcal infections more difficult (4). There were many reports of treatment failure in patients with pneumonia and other infections caused by drug-resistant pneumococci (DRSP) (26).

The spread of penicillin resistant and multiresistant pneumococci is associated with the circulation of certain serogroups. Serotyping and the analyses of other epidemiological markers reveal that a specific resistant strain may spread from country to country and even from one continent to another with increased mobility of the human population (1, 32). Because of the problem of resistance and spread, drug-resistant pneumococci have been said to be „among the most dangerous agents of human disease" (38). The data on antibiotic susceptibility, serotypes and the prevalence of penicilin-resistant and multiresistant pneumococci vary according to several parameters: geographical area, period of analysis, age and sex of population, etc. (1). Since the information about the Balkans is very scarce, our humble efforts in this study are to reveal the present situation with S.pneumoniae in one of the biggest hospitals in Sofia, Bulgaria (Emergency Medical Institute „Pirogov") and to compare our results with those of other investigators.

The aim of this study was to determine the rate of resistance to penicillin and to other antimicrobial agents of pneumococci isolated from adult patients with lower respiratory tract infections in our hospital.

ACCEPTED FOR PUBLICATION:

ABBREVIATIONS USED IN THIS PAPER:

PRP - penicillin resistant pneumococci, DRSP - drug-resistant pneumococci, PIP - penicillin - intermediately resistant pneumococci, TTA - transtracheal aspirate, MIC - minimal inhibitory concentration

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MATERIALS AND METHODS

Bacterial strains : The study was performed at the Departments of Pulmonary Diseases and Clinical Microbiology in

the Emergency Medical Institute „Pirogov". From January 1995 to May 1997 all isolates of S. pneumoniae from sputum, pleural fluid and transtracheal aspirate (TTA) were subjected to uniform microbiological and clinical study. Only one isolate from each patient was included. Blood cultures from the patients were tested.

Patients : All patients included in the study were over 14 years. The relevant patient history and all clinical findings were recorded on structured case record forms. The information on the use of antimicrobials during the previous 3 months, immunosuppression and hospitalization within the previous 3 months was based on interviews with the patients.

Quantitative examination of sputum: Samples with more than 25 polymorphonuclear leukocytes and fewer than 10 epithelial cells per low power field were classified as purulent.

Idintilication : The strains were identified by optochin susceptibility, bile solubility test and by API Striped test (Bio-Merieux). After identification, some were lyophilized and some were kept frozen at -70° C in BHI broth (Difeo) containing 10% (vol./vol.) glycerol for further examination.

Antibacterials: The antimicrobial agents used in this study were: penicillin G potassium (P), ampicillin (A), amoxycillin (AMX), amoxycillin/clavulanate/2:1/ (AMC), cefaclor (CEC), cefuroxime (CW), cefotaxime (CTX), chloramphenicol (C), erythromycin (E), trimethoprim-sufamethoxazole (TMP-SMX) and vancomycin (V). The agents were supplied as laboratory powders of known potency, and stock solutions were, made according to the recommendations of the manufacturer.

Susceptibility tests: All pneumococci were screened for susceptibility to penicillin by the 1-μg oxacillin disk (BBL) method as recommended by the National Committee for Clinical Laboratory Standards /NCCLS/ (28). The minimal inhibitory concentrations (MICs) were determined by the standard agar dilution method (NCCLS) (28). The final inoculum of 10^4 organisms per spot (2 mL) was delivered by using a Steers replicator onto the surface of Mueller-Hinton agar (Difco) plates enriched with 5% sheep blood (for TMP-SMX, the media was supplemented with 5% lysed horse blood). Plates were incubated at 37°C for 20 to 24 h in a CO_2 - enriched atmosphere. Standard control strains *(S.pneumoniae* ATCC 49619, *Speumoniae* R 6, *S. aureus NCTC* 11 561) were included in each run.

The MIC was defined as the lowest concentration of an antimicrobial agent that completely inhibited growth (28). The criteria of NCCLS (1995) were used for the definition of sensitivity or resistance to the antimicrobial agents studied (29). The pneumococcal strains were classified for penicillin susceptibility as follows: susceptible (PSP) - MIC ≤ 0.06 mg/L, intermediately resistant (PIP) - MIC 0.1-1 mg/L and highly resistant (PRP) - MIC ≥ 2 mg/L (29). The isolates were defined as multiply resistant to antimicrobial agents if they showed moderate or full resistance to three or more different groups of antibiotics. To clarify the terminology used in the discussion of clinical results, we grouped all PIP and PRP strains as nonsensitive (PNSP).

Serotyping : Serotyping was performed by the capsular swelling method (Quellung reaction) with antisera available from the Statens Seruin Institute (Copenhagen).

RESULTS

Patients and samples available: All 183 patients with lower respiratory tract infections included in this study were admitted to the hospital for treatment. A definite infiltrate on the X ray was seen in 87 (47.5%) of the patients. In the remaining cases, the diagnosis of lower respiratory involvement was based on clinical signs or symptoms. In all samples included in the research gram-positive diplococci were seen as the predominant finding in Gram-stained smears.

Of all 183 patients, 171 had medical records available for review: 135 (79%) had received one or more courses of oral antimicrobial therapy in the previous 3 months before hospitalization. The antibiotics used were: penicillin, cephalosporin, doxycycline, TW-SNIX or quinolons in 22%, 19%, 18%, 13%, 7% respectively. None of these patients had received cefotaxime, erythromycin or chloramphenicol. Twenty one (12,3%) were imrnunosuppressed and 37 (21,6%) had been hospitalized within the preceding 3 months.

Seventy three (42,7%) of the patients gave a history of the following predisposing factors: chronic obstructive pulmonary disease, chronic bronchitis, asthma, viral respiratory syndromes.

Of 183 pneumococcal strains collected during the studied period, 174 (95%) strains were isolated from sputum. 6 (3,3%) from pleural fluid and 3 (1,7%) from TTA. Pneumococci from blood cultures of patients were isolated only in 14 cases.

The relationship between the specimen types and the sex of the patients is shown in table 1 (male-to-female ratio was 1,50 with the predominance of males). The youngest patient was 16 years and the oldest was 8 1 years of age.

Antibacterial susceptibility of S.pneumoniae strains:

The oxacillin disk screening test used to detect reduced susceptibility to penicillin predicted that 65 (35,5%) of 183 pneumococci tested had some resistance to penicillin. When the agar dilution method was applied to determine the MICs of penicillin to all isolates, 49 (26,7%) were confirmed as resistant, 9 (4,9%) were relatively resistant and other 7 were susceptible (Fig.1). The strains for which there were discrepancies between the results of oxacillin disk test and the MIC technique had zones of inhibition ranging from 16-19mm and there MICs were 0,06mg/L. The distribution of all pneumococcal isolates (183) and penicillin resistant strains (58) according to the age of the patients is shown in fig. 2. A higher frequency of isolation of all pneumococci - 138 (75,4%) and of PNSP strains - 50 (86,2%) was observed in patients over 50 years.

Susceptibility test results and MIC ranges of 11 antimicrobials are presented in table 2: 31,6% of the 183 isolates displayed reduced susceptibility to penicillin - intermediate resistance (MIC 0,1 - 1mg/L) in 4,9% of the cases and high level resistance (MIC ≥ 2mg/L) in 26,7% of the isolates. All isolates from TTA were penicillin resistant. A slightly higher was the resistance to ampicillin (39,9%, including the intermediate strains) with MIC values for resistant organisms reaching 8mg/L (8 strains). MICs for amoxicillin and amoxicillinle-lavulanate were similar and their WC ranges were the same as for penicillin. We found these two drugs a little bit more active in vitro against our isolates than penicillin.

The resistance to cephalosporins varied from 47,5% for cefaclor and 24,1% for cefuroxime to 12,5% for cefotaxime. The MIC range of cefotaxime was identical with that of penicillin. The MIC values of cefaclor were the highest among all β-lactams (>32 mg/L). A total of 177 strains (96,7%) were susceptible to erythromycin with MICs that ranged from 0,03-0,5 mg/L. Chloramphenicol was also very active against our strains as only 11 of them (6%) were resistant to it with growth inhibited at MICs from 8 to 32 mg/L. The MIC range of susceptible strains varied from 0,125-4 mg/L. Forty nine (26,8%) of the pneumococci included in this study were resistant or intermediately resistant to TMP-SMX. All isolates were susceptible to vancomycin.

Susceptibility of penicillin resistant strains to other antimicrobial agents: The distribution of MICs of 10 antimicrobials against penicillin - intermediately resistant (PIP=9) and high level penicillin resistant (PRP=49) isolates of *S.pneumoniae* are depicted in tables 3 and 4 respectively. Most of PIP (MIC = 0,1-1 mg/L) were sensitive to arnoxicillin and amoxicillin/clavulanate. All nine strains were susceptible to cefotaxime and erythromycin and 8 of them showed chloramphenicol and TMP-SMZ sensitivity (table 4).

However penicillin resistant isolates (MIC ≥ 2mg/L) demonstrated higher MIC levels to the antimicrobial substances tested (table 4). All strains were resistant to amoxicillin (30)

and amoxicillin/clavulanate (26) and most of the intermediate resistant pneumococci (except 2 isolates for each drug) belonged to the group of PRP. Almost every penicillin resistant strain (45 of 49) was also resistant to cefaclor. Isolates resistant and moderatly resistant to cefotaxime were all PRP. Thirty of 32 cefuroxime resistant strains also displayed resistance to penicillin and the remained 2 strains belonged to PIP. The observed erythromycin and chloramphenicol resistance in S.pneumoniae was found only in penicillin resistant strains and 3/4 of TMP-SMX resistance occurred among PRP.

Antibiotic resistance patterns and multiple resistance: The penicillin resistance in the isolates tested was associated with resistance to at least one antimicrobial other than penicillin (in 57 from 58 strains) - table 5. There were 15 resistance patterns and three of them were most frequently encountered (50%): P, AMX, AXC, A, CEC, CXM, TMP- SMX (in 11 PRP); P, AMX, AXC, A, CEC, CXM, CTX, TMP-SMX (in 10 PRP) and P, AMX, A, CEC (in 8 PRP). The rate of isolation of the next two patterns was about 1/2 of the above mentioned.

Multiple resistance, defined as a resistance to three or more antibiotic groups, was a common phenomenon among our penicillin resistant pneumococci - 37 (63,8%). The predominant multiresistance patterns comprising about half of the strains (24) included resistance to at least two penicillins, 2 cephalosporins and TMP-SMX (Nr. 1, 2 and 8). Second in frequency of isolation was a multiresistance patterns (7 strains) that had added chloramphenicol to the previous drugs (Nr. 7, 12 and 13). Multiresistance to all five groups of antibacterials tested (penicillins, cephalosporins, TMP-SMX, chloramphenicol and erythromycin) was observed in 4 strains (Nr. 6 and 14).

Two different serotypes groups were identified among 10 highly penicillin-resistant strains in our study: 8 strains were serotype 23F and 2 strains were 19F.

DISCUSSION

The increasing number of penicillin-resistant S.pneumoniae isolates has become a worrisome problem worldwide (17). The results of the current investigation showed high level resistance to penicillin in 26,7% of the strains and intermediate resistance in 4,9%. The same prevalence of high-level resistance to penicillin, 19,6% with 9,3% intermediate resistance was found by Kam et al (16) in pneumococci isolated from patients with respiratory infections in Hong Kong.

It has been established that penicillin resistance in pneumococci often may not be detected by the routine disk diffusion method using penicillin disks. Resistance is mediated by alternation in penicillin binding proteins and not by β-lactamase production rendering detection of resistance more difficult. To overcome this problem the NCCLS method recommended that a 1 μg oxacillin disk to be used (28). Our results showed that not all strains (89,2%) determined as resistant by this screening method were confirmed as such by the agar dilution technique. Some of them turned penicillin susceptible with MICs ≤ 0,06 mg/L which are just one two-fold dilution below the breakpoint for resistance. Almost the same agreement (≈ 90%) between the two methods has been reported by other investigators (5, 24, 35) so oxacillin disk test is found to be reliable for predicting susceptibility to penicillin. However this technique can not distinguish strains that are penicillin resistant (MIC ≥ 2 mg/L) from those that demonstrate relative resistance (MIC = 0,1-1 mg/L). Therefore all pneumococcal isolates recorded as resistant by the screening method, must overdo MIC determination for more precise appraisal of their resistance.

Infection with drug-resistant pneumococci have most commonly been associated with extremes of age, hospitalization, the presence of underlying diseases, the presence of immunosuppression, prior episodes of pneumonia and prior treatment with antibiotics, especially beta-lactam drugs (4, 19, 20, 21, 24, 26, 27, 32). One of the predisposing factors to pneumococcal pneumonia is bronchopulmonary disease (chronic obstructive pulmonary disease, chronic bronchitis, asthma, and viral respiratory syndromes) (27).

Our data indicate a higher frequency of isolation of pneumococcal strains - 138 (75,4%) and PNSP - 50 (86,2%) from patients over 50 years, which is in agreement with the ascertainment that the incidence of pneumococcal infections occurs more often (about 3 times) in patients over the age of 50 than in patients between 20 and 50 years (32).

We encountered more often the isolation of pneumococci from males with respiratory infections. The data of Hong Kong showing male-to-female ratio 2.8 with a predominance of males are in collaboration with our results (16). In a hospital in Atlanta the male: female ratio was 1,25, 3,43 and 1,94 for patients with pneumococcal bacteraemia and PSP, PIP and PRP isolates respectively (24). A predominance of male-patients with serious pneumococcal infections (63%-males and 37% females) was also reported in the University Hospital in Korea (20). Jacobs and co-workers (13) found male-to female ratio of 2:1.

The data from the USA (Centers for Disease Control and Prevention) including 12 hospitals (1993-1994) showed that 53,8% of such patients were male (4). An investigation in Spain documented that of 139 patients studied (89 with pneumococcal pneumonia), 88 were male and 51 were female (8).

In the present investigation, 135 (79%) of the patients had received one or more courses of oral antimicrobial therapy in the preceding 3 months before hospitalization. Klugman (38) reported about 65% incidence of invasive pneumococcal infections among patients previously treated with beta-lactams, compared to 17% incidence among untreated patients.

We found pneumococcal bacteremia in 14 (7,65%) patients. Authors from Spain found a high rate (67,4%) of concomitant bacteremia among patients with pneumococcal pneumonia and associated this with the routine performance of early blood cultures in all cases of pneumonia (8). According to Musher (27) blood cultures were positive in 15% - 30% of the cases with pneumococcal pneumonia if antibiotics have not been administered. Other rates reported in the literature range from 7% to 25% (8).

Penicillin has been used as a standard drug for treating pneumococcal infections for nearly half a century but recently resistance among S.pneumoniae strains has increased worldwide (13, 27). Whereas in some countries like Northern Ireland (0,8%), Sweden (1,5%), Belgium (1,5%), the United Kingdom (1,5%), Finland (1,7% PIP), Australia (1.7% PEP), Germany (1,8%), Alaska (3,8% PIP) and Switzerland (7%) penicillin resistance is still relatively low; in France it is twice higher (12,5%) (1, 9, 15, 20, 30, 31, 32, 40). The rates of resistance in the United States differed from 6,6%, 8% in 1981-1983 to 19% (1993) and 22%(1994) (4). Much more worrisome is the situation in Romania, Poland, Japan, Israel, Hong Kong, Greece where the rate of isolation of strains with reduced susceptibility to penicillin is 25,1%; 26,7%; 27,0%; 28,4%, 28,9% and 29% respectively (1, 16, 25, 37). Most alarming reports about a dramatically increase in penicillin resistance of S.pneumoniae come from Spain (42,5%), South Africa (48,4%), Hungary (57,8%) (1, 8, 18, 21, 22, 23). In the Southeast Asian Region, in recent studies on penicillin resistance, the rate of 70% in Korea was one of the highest reported data, while in Hong Kong, only 28.9% of the investigated strains showed reduced penicillin susceptibility and the first isolation was in 1993 (16, 20). In some Spanish hospitals the percentage of S.pneumoniae strains not susceptible to penicillin ranged from 37,8% to 52,8% (8). In Portugal penicillin resistance was found to increase between 1989 and 1993 from 4,6% to 17%(39).

Our data, with about 31,6% (intermediate plus high level) penicillin resistance in the isolates tested, are similar to those reported from the Balkan countries - Greece (29%) and Romania (25,1%) (25, 37).

Table 3. The therapeutic effect of iridazole, Metrifonate and Praziquantel for treatment of genitourinary schistosomiasis

Therapeutic	Dose scheme	treated/recovered	therapetic

Table 4. The therapeutic effect of Niridazole, Oxamniquine and Praziquantel for the treatment of intestinal schistosomiasis

Therapeutic	Dose scheme	treated/recovered	therapetic

There are a few publication on the penicillin susceptibility of S. pneumoniae in Bulgaria: in one concerning the Hospital for Infection Diseases, the rate of isolation of PRP from sputum was 30,2% (1991-1995) (35); in others dealing with nasopharyngeal carriage in children (1991-1994), the incidences of resistance were even higher - 40% and 41,6% (34, 36).

The success in the therapy of pneumococcal diseases may depend on the type of infection and the degree of penicillin resistance. The experience of Linares et al (2 1) has shown that patients with pneumococcal pneumonia due to penicillin-resistant strains with MICs between 0, 12 and 2 mg/L who were not initially in critical condition may be successfully treated with a high dose of i.v. penicillin. The opinion of Musher (9) is that infections due to PIR pneumococci are readily treatable with moderately increased doses of penicillin.

Inappropriate antibiotic use, including an excessive length of therapy, use of broad- spectrum agents, and unjustified administration of antibiotics, is common in clinical practice and provides conditions favorable for selection of drug-resistant bacteria (4, 9, 22, 23, 26). This is supported by the observed positive correlation between the amount of antibiotic consumption and the frequency of antibiotic-resistant pneumococci (2, 9, 21). The effectiveness of limiting antibiotic use on preventing continued emergence of penicillin-resistant pneumococci is unknown: however, preliminary data from Iceland suggest that the prevalence of pneumococci with reduced susceptibility to penicillin has fallen as penicillin use has decreased (4). The relatively restrictive use of antibiotics in Germany, Finland, Belgium and the United Kingdom may be one of the major factors underlying the favorable situation with regard to antibiotic resistance in pneumococci (1 *5,* 30, 32).

In addition, the uncontrolled, injudicious and frequent administration of penicillin and/or its derivatives are most probably the base of the process of selection of penicillin resistant S.pneumoniae in Bulgaria.

Reduced penicillin susceptibility is associated with certain serogroups (1, 31, 32). The results of serotyping indicated the presence of serotypes l9F and 23F in some of our penicillin resistant strains. Although various proportions of different serotypes predominate in each country, some of the serogroups are common for all: in Hungary - serotypes 19, 6, 23 (22, 23); in France, have been found serotypes 23,19, 6,14, 9 (9); in Spain - serotypes 23, 6, 9, 19 2 1); in Germany - 6, 9 19, 23 (32); in Switzerland - 6, 19, 14, 23 (40); in Korea - 19, 23, 14, 6, 9 (20); in Brazil - 6, 23, 14, 19 (33); in South Africa - serotypes 6, 14, 19, 23 (18); in Finland - 23, 6, 19 (30); in Portugal - serotypes 23, 19, 3, 6 (39). In a study from Canada, most of the highly resistant to penicillin strains (76,5%) belonged to serotype 23F (14).

MIC values of amoxicillin and amoxicillin clavulanate were almost identical. However these agents were slightly more effective *in vitro* than penicillin and ampicillin as found by other investigators (27, 35). Amoxicillin provides a substantially broader spectrum of coverage than is necessary for the treatment of pneumococcal infections and the absorption is much more reliable after oral administration (27). The half life is substantially longer than that of penicillin, thus making it a good choice for an orally administered drug in the treatment of some cases of pneumococcal pneumonia (27).

In recent years, attention has turned from penicillin to other β-lactams such as cephalosporins. In our study the less effective against PSP, PIP and PRP strains was cefaclor. Its

Most active against the pneumococcal isolates (87,4% sensitivity) was cefotaxime. In 1994-1995 surveillance study in USA found only 3,4% high level cefotaxime resistance (4). Very interesting was the fact documented by Garcia-Leoni and colleagues (8) in a hospital in Madrid, where 42,5% of all strains. were PNSP but none of them was resistant to cefotaxime, imipenem, vancomycin or ciprofloxacin. A recent report from Atlanta emphasized that 25% of the isolates from patients with invasive pneumococcal disease were no longer susceptible to penicillin and only 9% were considered not susceptible to cefotaxime (12). Only one (0,1%) strain was cefotaxime-intermediate resistant in Germany (32). The lowest MIC values demonstrated by this third generation cephalosporin together with the high serum levels achievable outlined it as one of the best antibiotics for treatment of serious pneumococcal infections (3, 8, 13, 30). Clinical data indicate that most penicillin- intermediate resistant strains can be treated with cefotaxime monotherapy (3, 13, 21). All cefotaxime resistant strains were also resistant to penicillin and other antimicrobials (14).

Since the first description of an erythromycin-resistant pneumococcus in 1967 (6), several reports have documented variable rates of erythromycin-resistant S.pneumoniae on all continents. In the current study, nonsensitive to erythromycin were only 3,3% of pneumococcal isolates. A similar the low resistance has been reported from Finland - 0,6% (30), Sweden - 1,7% (11), Alaska - 1,9%(3 1), Germany - 3,8% (32), South Africa - 2,3% (1 8), Switzerland - 6,3%(40), Brazil - 6,1% (33) and Spain - 9,4%, 10,8% (8, 2 1). Moreno and co-workers (26) found that resistance to erythromycin among clinically significant pneumococci had increased from 7,6% (1988) to 15,2% (1992) in their hospital. Much higher rates of erythromycin resistance were seen in Greece - 20% (17), France - 29% (9), Hong Kong - 39,2% (16), Hungary - 48,5% (22, 23) and Korea - 52% (20). The high level of resistance to macrolides was linked to its frequent use in some countries, particularly for the treatment of upper respiratory tract infections in children and acute lower respiratory tract infections in adults (9, 20). The uncommon incidence of resistance to erythromycin among our strains and their low MIC values are most probably a result of the restricted use of this agent for upper and lower respiratory tract infections during the last 1 5 years in Bulgaria.

Chloramphenicol still appears to be effective in vitro against pneumococci (94% sensitivity) included in this investigation. These data are comparable with the results from other countries, where the rate of resistance was: Brazil - 0,5% (33), Greece - 1%(clinical isolates from adults with pneumonia)(17), Germany - 1,9% (32), Sweden - 2,1% (11), South Africa - 5,5% (18), Switzerland - 7,7% (40) and France - 9% (9)) and they differ from the observed percents of resistance in Spain - 23% (8) and 36,6% (21), Greece - 27%(carriers) (37), Hungary - 29% (22,23) and Hong Kong - 37,7% (16).

Higher values of erythromycin - 15,2%(PSP) and 31,8%(PRP) and Chloramphenicol resistance - 21,2%(PSP) and 31,8(PRP) were reported by Setchanova for clinical pneumococcal isolates in the Hospital for Infectious Diseases, Sofia, using disk diffusion method (34).

The data of a study of 1,527 clinically significant S.pneumoniae isolates from lower respiratory tract infections collected from 30 U.S. centres (1994-1995) were similar to ours: the overall rate of penicillin resistance was 23,6%, but resistance to chloramphenicol and erythromycin was only

and amoxicillin/clavulanate (26) and most of the intermediate resistant pneumococci (except 2 isolates for each drug) belonged to the group of PRP. Almost every penicillin resistant strain (45 of 49) was also resistant to cefaclor. Isolates resistant and moderatly resistant to cefotaxime were all PRP. Thirty

pneumococcal pneumonia is bronchopulmonary disease (chronic obstructive pulmonary disease, chronic bronchitis, asthma, and viral respiratory syndromes) (27).
Our data indicate a higher frequency of isolation of pneumococcal strains - 138 (75,4%) and PNSP - 50 (86,2%) from

RESULTS AND DISCUSSION

The data shown in table 1 represent that the population in Angola is greatly infected with S. haematobium - 57.39%. The extent of the infection is great among children younger than 14 years (63.14%) while among adults it is 52.18%. Women are more affected by schistosomiasis comparing to men (52.54%). S. mansoni has been found only in one settlement
in the northern part of the country that appeared to be an endemic area of intestinal schistosomiasis-table 2. The total percent of infected people was 5.63%. 6.44% were children younger than 14 years and 4% were adults.
We have recognized two clinical cases of S. intercalatum infection in emigrants who had returned from Zaire. According to the data published by C. Wright (1963) the main intermediate hosts of S. haematobium in Angola are: Bulinus angolensis widely distributed throughout the whole country, B. globosum and B. truncatus - in the northern part, B. africanus - in the southern. The intermediate host of S. mansoni is B. pleiferi that could be found everywhere in Angola (6). Our data as well as data received by other authors confirm that S. haematobium is a widespread parasit while S. mansoni is a rare one (6).
The great extent of the genitourinary schistosomiasis and the presence of endemic areas of the intestinal form of the disease result from the favourable climat and fauna as well as from special social, economical features and the living standard. The permanently occuring reinfections increase the worm load and complicate the course of the disease.
According to the expert recommendations of the WHO courses of individual and mass treatment must be performed periodically for people at risk in the infected settlements and areas (10, 13, 14).
We have studied the therapeutic efficacy of some most commonly used medicines against schistosoma: Niridazole and Praziquantel for treatment of the genitourinary and intestinal schistosomiasis; Metrifonate - for the genitourinary form and Oxamniquine-for the intestinal form (4, 5, 12).
A total of 195 patients have been treated for genitourinary schistosomiasis. 118 of them have been cured with Niridazole and some took also tablets of Phenobarbital tab. 0.1, 2 mg/kg once a day in order to minimize the side effects of Niridazole ,which appeared to be predominantly neurologic (4, 6). 18 patients were treated in a hospital while the rest - in their homes. 102 people had a negative result from the control microscopic examination of their urine samples with an estimated therapeutic effect of 86.44%.
Some patients who did not take their medicines regularly,in particular Phenobarbital, revealed side effects, such as a headache, nausea, anxiety, sleeplessness. The side effects usually passed after the treatment course had been finished.
Metrifonate was the medicine that had been taken by 42 patients. The control examinations revealed 32 completely recovered people, so the therapeutic effect was estimated to be 76.19%. We did not observe any side effects when Metrifonate had been taken.
The treatment of 36 individuals with Praziquantel was very successful with estimated efficacy of 94.28%. No side effects have been observed. Paziquantel revealed the best therapeutic efficacy for the treatment of the genitourinary schistosomiasis. This medicine is suitable both for individual and mass therapeutic course (3).
The treatment with Metrifonate has proved to be also highly effective, but because of some reported cases of resistance its practical application is limited (13). Despite of its possible satisfactory therapeutic result,Niridazole should be avoided,especially for mass therapy for reasons of its side effects. Another negative point is that the prolonged treatment course makes some patients stop their therapy prematurely, when the macroscopic haematuria dissapears and the urine looks like normal.
We have chosen Niridazole to cure 110 patients with intestinal schistosomiasis. 46 people took Niridazole in a hospital. The control analyses showed that 30 patients had no schistosomal eggs in their stool samples,thus confirming 65.21% therapeutic efficacy. In order to avoid side effects,a daily dose of 2 mg/kg Phenobarbital has been given.
40 patients have been treated with Oxamniquine. The control analyses determined 35 completely recovered individuals with negative results, so the achieved therapeutic effect was 87.5%. Four patients had slightly expressed side effects such as a headache, nausea,vertigo,which passed within two days after the treatment had begun.
22 individuals of 24 cured with Praziquantel have completely recovered and the therapeutic efficacy associated with that group of people was 91.66%.
We have observed one case of eyelid·s oedema on the second day of treatment with Praziquantel,but it passed afer

Table 1. Structure of genitourinary schistosomiasis in Angola.

Age groups	men			women			Total		
	number of examined	total of infected number	%	number of examined	total of infected number	%	number of examined	total of infected number	%
0-14	443	268	60.49	493	323	55.51	936	591	63.14
more than 15	464	209	45.04	567	32	58.02	1031	538	52.18
Total	**907**	**477**	**52.59**	**1060**	**652**	**61.50**	**1967**	**1129**	**57.39**

Table 2. Structure of intestinal schistosomiasis in an endemic settlement.

Age groups	men		women		Total	
	number of	total of	number of	total of	number of	total of

Table 3. The therapeutic effect of iridazole, Metrifonate and Praziquantel for treatment of genitourinary schistosomiasis

Therapeutic agent	Dose, scheme	treated/recovered	therapetic effect, %
Niridazole tablets 0.5 and 0.1	25 mg/kg for 7 days	118/102	86.44
Metrifonate tablets 0.1	10 mg/kg single dose once	42/32	76.19
Praziquantel tablets 0.6	40 mg/kg single dose once	35/33	94.28

Table 4. The therapeutic effect of Niridazole, Oxamniquine and Praziquantel for the treatment of intestinal schistosomiasis

Therapeutic agent	Dose, scheme	treated/recovered	therapetic effect, %
Niridazole tablets 0.5 and 0.1	25 mg/kg for 7 days	46/30	65.21
Oxamniuquine caps 0.25	15 mg/kg single dose once	40/35	87.50
Praziquantel tablets 0.6	40 mg/kg single dose once	24/22	91.66

Allergosan (dragee three times a day for 3 days) had been applied. Praziquantel has proved to be the most effective antiparasitic agent for the management of intestinal schistosomiasis. It appeared to be the most suitable medicine both for individual and mass therapy, as well (9).

Oxamniqune is easily applicable. It is possible to achieve satisfactory therapeutic results when using it. However,it should be avoided for mass therapy,despite of being useful for individual treatment, because of epileptiform attacks that could rarely appear (7).

Niridazole has a good therapeutic effect,but it schould be replaced with one of the other drugs mentioned (5).

In conclusion, Praziquantel is a highly effective therapeutic agent that is currently available for ambulatory and hospital treatment of the genitourinary and intestinal shistosomiasis. Its major advantages are: the onefold application, the exellent therapeutic result,the lack of side effects and the low toxicity. These features make Praziquantel a drug of choice for individual and mass therapy of schistosomiasis. Some important special features to mention in order to achieve good therapeutic results are (8): after the effective treatment the exretion of schistosomal eggs decreases and stops for 1-2 weeks, but single viable eggs might remain and be present for one month. Eggs that are not viable could be excreted for several months or even for one year. If the parasites are paralyzed,they stop disposing eggs for 2-3 months. In case that viable eggs appear again 4 months or later after conducted treatment course in an endemic area, it is regarded as a reinfection, not as a relapse.

The therapeutic results should be monitored by microscopic examination for presence of ova or larvae. The immunologic assays could also be helpful in determination of therapeutic results. They usually become negative 3 months after the conducted treatment (6).

The genitourinary and intestinal schistosomiasis are imported tropical parasitic infections that do not have autochtone distribution in Bulgaria. Schistosomiasis is predominantly a clinical problem that concerns mainly the diagnosis and therapy. The treatment of patients and their convalescent period are strictly followed up, by dispensary control.

CONGENITAL TOXOPLASMOSIS, CLINICAL MANIFESTATIONS AND PATHOMORPHOLOGICAL CHANGES IN CNS

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SUMMARY

Samples taken from 14 dead children and directed for specialized examination of CNS in the Laboratory of neuropathology at the University Hospital „Aleksandrovska" - Sofia for the period 1976-1998 have been studied. 13 of them were described as cases with congenital toxoplasmosis. It is predominant the clinical symptomatic of manifested meningoencephalitis, spastic quadriparesis or bulbar paralysis, epilepsy, hydrocephalus, retinochoroidites or atrophy of the ophthalmic nerve. The authors described the pathomorphological changes in the brain into three forms - first form (subacute inflammation at infants died in the period of new born), second form (acute inflammation at children died between first and eight months after delivery) and third form (chronical-destructive inflammation with initial damages in the early gestation age in the period between 9th and 28th week of pregnancy). The described changes of the brain would be of favour to specialists who investigate neuropathology of central nervous system (CNS) at immunodeficiency when the reactivated toxoplasmosis is one of the reasons for the dead of these individuals.

Key words: *congenital toxoplasmosis, Central nervous system (CNS), pathomorphological changes*

Toxoplasmosis is a parasitic disease caused by the protozoan parasite T.gondii. The first records described a possible pathogenicity in humans were in 1913-1914, but 10 years later in 1922 the Czech ophthalmologist J. Janku discovered toxoplasms in the eyes of 11 years old child died from seriously congenital disease and described what was probably for the first time the clinical picture and pathoanatomical changes caused by congenital toxoplasmosis (2).

In the course of years the interest to the toxoplasmosis more and more has been increased. Now a days, as medical problem, the infection is associated mainly with the three possible clinical manifestations in humans: 1. Acquired; 2. Congenital; 3. Infection connected with immunodeficiency. Each one of them has its own place in the human pathology (9).

Generally the congenital toxoplasmosis is a result of acquired for the first time toxoplasmic infection during the pregnancy (2,6,9,11,14,16,20). There are involved many factors in the pathogenesis - the immune state of the pregnant women, the gestation period in which the fetus was infected, the virulence of the strain T.gondii, the functional condition of the chorion s trophopblast layer and later on - the state of the placenta, etc. All these factors influence the frequency of congenital toxoplasmosis which is not so high. According the WHO data a fetus could be infected in 30 to 50% from pregnant women with infection acquired recently and only in 13% of them was occurred the clinical picture of congenital toxoplasmosis (8,12).

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ABBREVIATIONS USED IN THIS PAPER:
CNS - Central nervous system

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Mainly the diagnosis of infection in pregnant women, respectivelly in fetus, is generally based on determination of the specific toxoplasmic antibodies - immunoglobulins class M, A, G (1,5,6,21) having in mind that the heaviness of clinically manifested toxoplasmic infection has not been connected with the degree of immune response (15). The congenital toxoplasmosis is confirmed not only serologically but also by determine trophosoits, in the amnionic fluid or liquor of the neonates or histologically - pseudocysts or real cysts in the tissues of organs and systems affected by the endometrial infection (5,13,15,19).

The congenital toxoplasmosis varies clinically from asymptomatic (only serologically confirmed) to subclinically and clinically manifested, sometimes fulminant incompatible with the life of child. The damages of congenital toxoplasmosis are mainly at the CNS but could be extended with appearance of retinochoroidites, myocarditis and other organic lesions. Pathoanatomically the brain lesions are manifested as meningoencephalitis, sections of necrosis and intracranial calcifications with hydrocephaly or microcephaly, atrophycally-destructive changes in the cerebral cortex, etc. (4,7,10,15,18).

The aim of the present publication is to introduce to the medical specialists our results concerning the brain changes in deceased and direcred for specialized examination of CNS children. This study has been undertaken to stress the attention to the problems of congenital toxoplasmosis.

MATERIAL

Samples taken from 14 dead children have been examined for the period from 1976 to 1998 in the Laboratory of neuropathology at the University Hospital „Aleksandrovska" - Sofia and 13 of them were described as cases with congenital toxoplasmosis. The children were born without visible malformations or monstrosities but days or months later developed symptomatic that reduce to the status of exitus letalis. The clinical data obtained for the dead children are given in Table 1.

RESULTS AND DISCUSSION

The clinical diagnosis accompanied all 13 cases indicated affection of the CNS. The diagnosis mostly was „Meningo-encephalitis" - in six of them determined as suppurative, in four was accepted that the cerebral infection is with virus etiology and in two cases the suppurative inflammation was with presence of sporadic or multiple abscesses. The preliminary orientation about congenital infection was available only in two of the cases - an infant No 12 without specifying the reasons for that and an infant No 7 determined as „Toxoplasmosis" because of trophozoites of T.gondii found in the liquor that has been examined. Four of the deceased children (No 1, 3, 6 and 13) survived the initial manifestation of meningoencephalitis but No 1, 6 and 13 later on developed spastic quadriparesis which one is the clinical evidence that the pyramidal and extrapyramidal pathways were affected. The bulbar paralysis was found in No 3. In two of the cases (No 1 and No 6) the clinicians were considered for degenerative affection of the brain. In six cases (No 2, 5, 7, 8, 9 and 13) became apparent the presence of hydrocephaly with the typical elargement of the head, moving apart of the cranial satures and specific symptom of „sunset" into the glance.

Symptoms of epilepsy have been rather often described in the clinical syndrom. The first appearance of epilepsy is either Jacksonian motional epilepsy with isolated clonic or combined clonic-tonic convulsions in a single limb or symptoms of hemi-Jacksonian disease which in some of the cases pass into generalized epilepsy (cases No 3, 6, 8 and 10). It was described a disease that started straight with the clinical picture of grand mal but some of the children fell into the state of epileptic status (No 1, 11 and 12). It is well known from the literature that different somatic diseases could be observed at congenital infections and some of them became a direct reason for the patient s dead. In five of our cases (No 3, 4, 5, 12 and 13) a severe enterocolitis with different genesis was developed and in case No 10 in the course of septic

Table 1. Dead children directed to the laboratory of neuropathology

No	Name	Age	Sex	Date of dead Autopsy No	Term for appear of the clinical manifestations after delivery	Clinical diagnosis	Mode of delivery	Mode of pregnancy
1.	MDA	11 months	M	28.06.76 614	9 months	Desseminated cerebral paralysis. Spastic quadriparesis. Oligophrenia. Epist. Hyperthermia	Normal	Normal
2.	MDD	1 month	F	18.12.76 1171	No records	Purulent meningitis. Cerebral abscess. Hydrocephalus.	No records	No records
3.	MSS	2 months	F	10.05.78 471	15 days	Purulent leptomeningitis. Bulbar paralysis. Epilepsy (Jack. and grand mal). Enterocolitis	Normal	Pathologic
4.	BMD	8 months	M	25.09.80 852	3 months	Status after meningoencephalitis. Idiopathy. Atrophy. Dysentery (Shigella flexneri and E.coli O_{114}	Normal	No records
5.	HZU	6 months	M	29.09.80 873	9 days	Status after purulent meningitis. Colienteritis O_{25}. Hypotrophy. Hydrocephalus. Liquor-Enterolacter, Klebsiella Asphyxia High bilirubinuria	Normal	
6.	MVD	10 months	M	04.12.83 1284	24 days	Leukodystrophy. Spastic quadriparesis. Hypotrophy. Atrophy of the ophthalmic nerves.	Asphyxia	Pathologic
7.	DBA	1 m. 22 d.	F	10.11.84 1115	30 days	Toxoplasmosis liquor trophozoites). Purulent meningoencephalitis. Hypotrophy II-III. Hydrocephalus. temperature 2 weeks before delivery	Premature	Elevated
8.	ABA	1 m 13 d	F	19.02.85 215	17 days	Meningoencephalitis (influenza type A), Herpes simplex. Hydrocephalus.	Normal	Normal
9.	JGT	1 m 23 d	F	03.10.87 1127	10 days	Sepsis of the new born infant. Asphyxia. Meningoencephalitis. High bilirubinuria. Hydrocephalus.	Premature,	No records
10.	NGC	3 months	M	03.06.91 341	17 days	Meningoencephalitis (Herpes simpl.). Postencephalitic multicyst encephalomalacia. Status after double pneumonia and sepsis.	Asphyxia pressure	High blood
11.	PVL	1 m 25 d	F	11.02.92 103	14 days	Acute meningoencephalitis. Complicated cardiac failure. Edema cerebri.Pneumonia bilateral. High titre of antibodies □ and □	Normal infection 10 days before delivery	Herpes
12.	IMI	1 m 10 d	M	05.07.93 31	No records	Congenital infection. Double pneumonia. Acute enterocolitis. Multicysts encephalopaty.	No records	No records
13.	LMA	2 y 11 m	F	10.04.98 169	2 months	Status after viral meningoence phalitis. Acute enterocolitis Double pneumonia. Acute bronchiolit. Hydrocephalus.	Normal	Normal

condition was isolated enterococcus from the hemoculture and liquor. It is possible to indicate some of the symptoms typical for visceral pathology as follows: pneumonia - twelve of the children, myocarditis - two, nephritis - one but suppose to have in mind that myocarditis and especially enterites are diseases later on accompanied the congenital toxoplasmosis (usually 4-6 months after delivery). Lowered body temperature, asphyxia and cyanosis, edemas, anaemia, thrombocytopenia, skin eruption, petechial haemorrhagies, extramedullar hematopoiesis, jaundice which are rather well exhibited at the premature born infants are referred to the common clinical symptoms (7,18). These symptoms were observed more or less in the clinical description of our cases with exception of the jaundice that was not found in all of the cases. The eye injuries as retinochoroidites and atrophy of the optic nerve were occurred only in cases No 6 and No 12. Results reported up to here confirmed the position of the leading authors that the clinical symptoms characterized congenital toxoplasmosis could be divided into four large groups: (1) common; (2) visceral; (3) brain lesions and (4) eye injures, but in the case of dead the pathomorphological changes are of significant importance for the final etiologic diagnosis. The most typical changes were observed in CNS, respectively in the encephalon. According our investigation we qualified the brain changes into three forms:

First form (an acute inflammation described at cases No 2 and No 7).It is characterized with comparatively preserved macroscopic structure of CNS. There is a some kind of brain atrophy (common) and purulent inflammation of the meninges as a result of the supplementary infection.

The acute inflammation with large inflammatory infiltrations and great number of phagocytes in some of which is possible to be seen toxoplasms forming the so-called pseudocysts was established at microscopic investigation of diferent levels of the cerebral hemispheres, brain stem, cerebellum and spinal cord. The parasites could be also seen in other cells such as astroglia and even neurones. The ventricular system is full of similar to jelly substance as in the case of an infant No 7 the substance fill also the space of large cysts in Centrum semiovale formed in result of unification of the multiple small softening sections. The sections of necrosis accompanied the large spaces of inflammatory infiltrations. The dead occurred in the period of newborn infant approximately one month old.

Second form (a subacute inflammation described at cases No 1, 4, 5, 10 and 11). Usually the microscopic picture was a combination between the acute and chronico-destructive (the third one) form of inflammation and it does not depend on continuation of the disease. For instance, nevertheless that in the cases No 1 and No 4 the suffering was continued 11 and 8 months respectivelly, the common structure of the cerebral hemispheres was comparatively well preserved while there have been already seen destructive changes in the pallium concerning cases No 5, 10 and 11.

The microscopic investigation presented a wide variety of different inflammation forms. In the three of the cases (No 1, 4 and 5) were established leptomeningitis with different heaviness but usually moderately expressed and the meninges were in less or more degree thicken with predominant lymphonuclears. There were seen multiple calcifications among the brain substance in almost all levels of the brain with exception of the spinal cord. They were the most abundant in the zone of Centrum semilunare and usually closely to necrosis and engaged vascular walls. The calcifications were small, fine and sometimes accumulated in a heap. They resembled blackberry and represented calcinated tissues of toxoplasmic cysts. It could be notice pseudocysts (phagocite with parasite) near-by the zones of necrosis. Progressive gliosclerosis

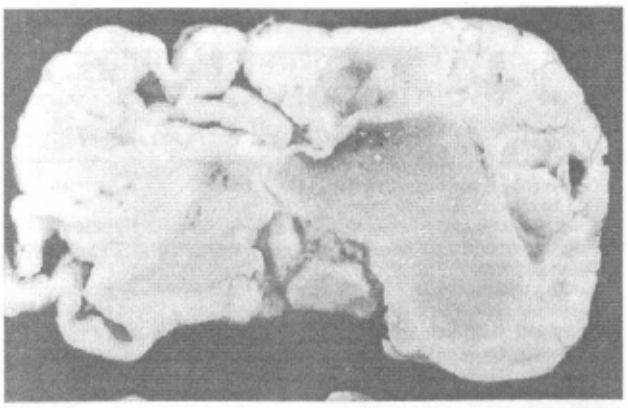

Figure 1. Acute forme of inflammation in case of congenital toxoplasmosis. Piohaemocefalia and enlargement of brain ventricles as well as paraventicles necrosis.

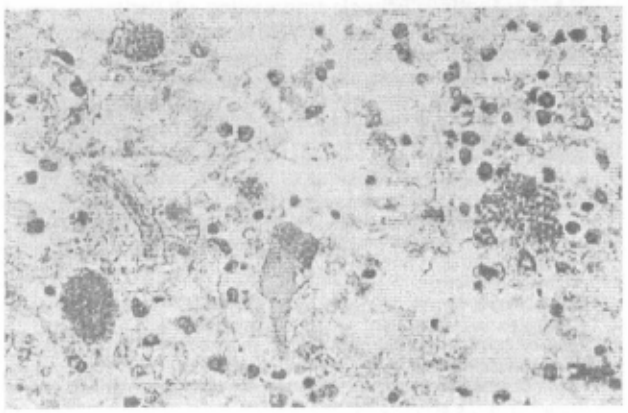

Figure 2. Acute forme of inflammation in case of congenital toxoplasmosis. Pseudocysts of T. gondii in brain.

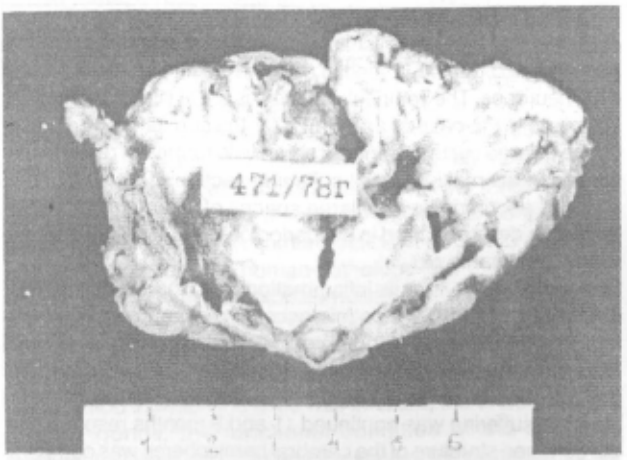

Figure 3. Chronical atrophically - destructive inflammation in case of congenital toxoplasmosis. Atrophy and deformation of the enchephalo-hemispheres. The thalamus is not destroyed. The third ventricle is not enlarged.

was accompanied necrosis and calcifications in subcutaneous inflammation in contrast with the acute one.

In the cases No 1, 4 and 5 granulomatous inflammation was observed while at No 5 the inflammation process was with presence of the so-called „giant cells". The second form was described at children that acquired the disease more than one month after delivery but rearly goes beyond 6-8 months.

Third form (a chronical atrophically-destructive inflammation described at cases No 3, 6, 8, 9, 12 and 13).

Macroscopically the brain was with significant deformities, heavy atrophy of the sulcuses and generally of the cerebral hemispheres. On the background of these massive changes in the hemispheres the tissues were markedly preserved in the other levels such as thalamus, hypothalamus, mesencephalon, pons, bulbus and cerebellum hemispheres.

The microscopic investigation reveals a typical picture: atrophycally-destructive process was observed in the encephalon hemispheres at which the cerebral cortex was almost replace by glial proliferation forming on some places nodules. The inflammation process was more often poorly presented and sometimes similar to that one described at the second form with respect to the presence of the „giant cells" someone of which polynuclear. The toxoplasms (pseudocysts) were still occurred in the part of the phagocytes and it could be seen a single tissue cysts among the poor inflammatory and necrotic changes sometimes in process of calcification. The third form was described at children where the initial clinical symptoms started at the first 2-3 weeks after delivery (with exception of No 13). This is an evidence that the damages started in the period between the 9th and 28th week of pregnancy (3). The hydrocephaly that was not always marked at the third form presented at the first and second one. It is evident that the congenital toxoplasmosis is in causative-consequence relationship with the problem of toxoplasmosis and pregnancy. The knowledge of this problem is of particular importance on one hand for diagnose in time of the initial toxoplasmic infection in pregnant women and on the other hand for prophylactic of that congenital infection. There are no data for tracing the immunological status of the mothers with dead children regarding specific toxoplasmic antibodies during the pregnancy in order to avoid infection. There was investigated serologically for toxoplasmosis after the delivery only the mother of No 7 because of the reason that toxoplasms were found in infant·s liquor. This approach is insufficient and does not contribute for influence at the frequency of the cases with congenital toxoplasmosis.

It is possible to be cure the initial toxoplasmic infection in pregnant women if would be diagnosed in time. This creates a possibility for prevention or localization of the clinical manifestations of the congenital toxoplasmosis, respectively the lethal damages of the fetus.

In our opinion the described changes in the brain would be of favour to specialists who investigate neuropathology of CNS at immunodeficiency when the reactivated toxoplasmic infection is one of the reasons for the dead of these individuals.

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CLINICAL OBSERVATIONS OF HUMAN MYIASIS

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SUMMARY

Human myiasis are sporadic diseases in our country caused by parasitic larvae from various fly species. There are described the most specific clinical special features of the myiasis and particular cases observed from the authors were included. The clinical symptoms depend on the anatomical site and biological species of the larvae. They are divided into groups according acquired classification such as accident, facultative and obligatory myiasis with cavity and tissue localization. Recommendation are given concerning the basic therapeutical measures and for prophylaxis of myiasis. The publication is directed especially for medical doctors - general practitioners as well as specialists from the specialized health system.

Key words: *arachnoentomology, myiasis, clinic and treatment*

Myiasis are diseases caused by the larvae of the flies that parasitized in human and vertebrates (myiasis, from myia - fly). They are widely spread all over the world and could be found predominantly in the regions with developed stock-breeding farms. Myiasis are sporadic diseases in our country. Cases of imported from tropical countries myiasis where they are often diagnosed in the clinical practice have been observed (1,4,11,12).

The larvae parasitized in human are from synantropic fly species belonging to the order of Diptera that includes the families Muscidae, Calliphoridae, Sarcophagidae, etc. as well as from symbovilic flies (steppe, pasture) - warble and bot flies from the families Hypodermatidae, Oestridae, Gasterophilidae, etc. that parasitized on vertebrates (2,5.8). The dieseases could be divided into incidental, facultative and obligatory myiasis according the special features in the biological cycle of the parasites, respectively the mode of their life. They have also been divided into tissue and cavity (hole) type according the localization of the parazitized larvae (6,8,10).

Cases of local and tropical myiasis that are referred to the above mentioned groups have been often observed during our practical research in the specilized outpatient's department for parasitic and tropical diseases at the National Center for Infectious and Parasitic Diseases as well as in the tropical countries.

The main purpose of the present publication is to summarize the most typical clinical special features of the different types of myiasis and to include also the cases that have been diagnosed, consulted and treated from us.

Incidental myiasis. Intestinal and urinary (cavity myiasis). They are manifested when the larvae get into the human body where they are developed into putrefactive compounds. If this happened by contaminated food and water it would developed intestinal myiasis. The larvae could crawl up to the urethra and urinary bladder and in this case would be manifested urinary myiasis.

Intestinal myiasis are caused very often from the larvae of the common housefly (Musca domestica), small housefly (Fannia canicularis), big housefly (Muscina stabulans), blue and green flesh flies (different flesh flies species from genus Callyphora and Lucilia), common cheese skipper (Piophila casei), and fruit fly - drosophila (Fam. Drosophilidae). In total approximately 50 various larvae of fly species have been determined as a causative agent caused intestinal myiasis. The larvae of these flies entered with food undamaged into the stomach by ingestion and at decreased stomach acidity continued to develop into the bowels and provoked irritation and inflammation of the intestinal mucus. The larvae of some flies developed from eggs and deposited in the perianal sulcuses could be penetrated into the intestinal tract and retrograde through the anus (9).

Clinically the intestinal myiasis in more of the cases run acute with vomiting, abdominal pain, diarrhea stools sometimes mixed with blood, tenesmus and anal pain. Larvae of flies could be found out in the vomited matters as well as in the faeces. The ethiologic diagnostic and differentiation from intestinal helminths was done by their morphological special features by enthomologic stereomicroscope. It is necessary to be excluded outer contamination of the faeces by flies. The similar cases of intestinal myiasis passing with symptoms of acute enterocolitis have been observed by us in children and adults as in the more of the cases have been differenciated the isolated from the faeces larvae of drosophilas (fruit flies) which are developed in rotting and overripe fruits and vegetables. An intestinal myiasis provoked from common cheese skipper (Piophila casei) that could be found all over runs with more seriously expressed symptomatic. The successfully treatment with antinematodic drugs such as pyrantel, decaris and vermox has been conducted. Moreover, for elimination of the larvae non-drastic laxative medications have been administered and also cleasing emena has been described.

The urinary myiasis is caused from the larvae of flies Fannia canicularis, Musca domestica and others that penetrate into urethra. This could happened when was used contaminated with urine underwear and from deposited on it eggs have been developed larvae of the flies. The clinical symptoms in these cases are typical, i.e. mucopurulent secretion from the urethra, frequent and painful urination, pain above symphysis and elimination of the fly larvae with urine which one is the basic diagnostic symptom. Larvae of M.domestica have been found in urine of elderly patient who was with mentioned above disury symptom and painful cysto-urethral complaints. Larvae of F.canicularis has been observed in sample of another patient who was with acute urethritis at native microscopiag ofurethral secretion. It could be revealed a vaginal myiasis in women - autonomically or combined with urethral myiasis. Cystoscopy was seldom necessary to be conducted at the diagnosis of urinary myiasis because the larvae are very often eliminated spontaneouly with urine. The treatment is consists of mechanial elimination of the larvae by urethral douche with antiseptic solutions.

Facultative myiasis. The different fly species predominantly green and blue flesh flies (reproducting on animal's corpses and excrements) as well as other species such as common housefly, small and big housefly, in the case of outer injuries of the body (especially if they

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are suppurated) lays their eggs above and the incubated larvae use the necrotic tissue of the wounds (necrophagia). Although that the healthy tissue is not destroyed the invasion is dangerous because introducing of secondary infection. These types of invasion were observed at a child with diagnosis otitis media supporativa as the larvae of Musca domestica have been repeatedly isolated from the purulent secretion of the auditory canal. Another case - a child with diagnosis acute rhinopharingitis - larvae of Fenia canicularis came out repeatedly and spontaneously with the nasal secretion from the nostrils. In a patient, who was victim of a car accident, after removing of the old contaminated bandage from the wounded lower limb which one was not initially proceeded have beenfound larvae of blue flesh flies (Callyphora erythrocephala) in the wounded place that do not cause special complaints of the victim. When there were not manifested symptoms of introducing additional infection, the wound was not particularly painful but most frequently were the cases with secondary infection with myiasis that significantly complicated the clinical progress of the suffering. The treatment was carried out by mechanical removal of the larvae with tweezers from the place of invasion followed by douche and bandage with antiseptic medications (flavine and others).

Obligatory myiasis. They are cause by larvae of the flies having no alternative way of living except parazitized in human and animals. They are fed both with necrotic and alive tissue which one quickly and systematically destroy. They are divided into malignant and non-malignant according the damages that would caused.

Malignant myiaisis are caused by larvae of flies that are developed significantly fast (for 3 to 10 days). The widespread wohlfahrtia fly (Wohlfahrtia magnifica) and other species (Wohlfahrtia vigil, Collitraga hominivorax, Chrisoma bizziuna, etc.) belong to them. The larvae penetrate through the mucous or injured skin and migrate as by digestive ferments cause necrosis of the subcutaneous tissue and skin. They could quickly destroy large areas from the skin covered the head accompanied with haemorragies, suppuration and sphacelation. There were cases with compete destruction of the eyeball and scalp, osteonecrosis, penetration of larvae into the cranial cavity with followed encephalitis. Clinically these damages are developed quickly and accompanied with acute pains at seriously common state sometimes with lethal exitus. The diagnosis of these myiasis when have in mind of such a disease is not dificult because on the bottom of the injured zones are seen with the naked eye the mobile larvae of parasites. The treatment of these myiasis should be started immediately. The affected zones are washed out with 2% solution of chloroform, after that mechanically with tweezers the larvae are removed. Sometimes a surgical intervention is necessary which purpose is to clean the larvae and complete processing of the affected areas from the skin and subcutaneous layer. The clinical cases of malignant myiasis have not been recorded from us in our country but there were such cases only in the tropical countries, for instance Angola. Malignant myiasis with very heavy passing were observed and described in the countries with moderately climate (6,8).

Non-malignant myiasis are caused by larvae that developed slowly (more than 10 days) and are in small amount. More often these larvae are from different species of warble flies. Their way of parasitizm does not lead to significant destruction of the affected tissue. These types of larvae have different localization (in animals) according wich are divided into diseases of the skin, stomach and cavities.

People in contact with cattle are invaded with eggs and larvae of warble flies from family Hypodermatidae, most frequently with Hypoderma bovis, which one developed on the skin, drived through it and penetrated in the subcutaneous layer. They could caused local inflammatory infiltrations when migrate to the upper part of the body but they are not so strongly painful and could manifested on different places in accordance with the direction of larvae movement. After few days the larva drives through the skin and forms a fistula from which flows a serous liquid. Accidentally the larva could got into the eye (ophthalmomyiasis) as the eye swells accompaned with pain, hemorrhagic and at neglected cases is observed a destructive damage of the eyeball with followed blindness. The treatment was the most successful at incision with extraction of the parasite.

The widely spread in the horses, including our country, abdominal bot flies (Gastrophilus intestinalis) are the reason for appearance of so-called „linear myiasis" in humans. The larvae of horses bot flies migrate in the skin up to two months and remain threadlike, slowly crawling erythemic trace (up to 2-5 cm daily) in which end is located the larva and all this movement is accompanied with heavy local itching and scratching. Similar clinical picture have been observed from as in our country as well as in the tropical countrues so in the view of effective treatment it became necessary a differential diagnostic with skin invasion of larvae from Strongyloides stercoralis, Ancylostoma caninun and other nematodes that also frequently could cause similar itching „linear dermatitis". In such cases suppose to be conducted diagnostic and in thensame time curative incision. The drug mintezol unguentum has been successfully administered for conservative therapy of „linear myiasis" as well as „linear dermatitis" with another parasite ethiology.

The species Rhinoestrus purpureus and Oestrus ovis from the warble flies that localized diseases on humans in cavities of the body are viviparous and instantly eject their larvae in the eye, nostrils, ears flying or at clashing. The larvae in the eye are directed to the lacrimal gland, eyelid or through the conjunctiva in the eyeball. They cause blepharoconjunctivitis with strong pain, edema, delacrimation, suppuration. The larvae that are located in the nose also cause a strong inflammatory reaction with temperature, headache and muco-bloody purulent secretion. The treatment is conducted immediately as is administered a local anesthesia with dicain collyr and mechanically with tweezers or douche are removed the larvae from affected organ. We have been observed a case of acute dacriocystitis in child caused by invasion with larvae from Oestrus ovis where in the course of 1-2 weeks the larvae of the fly were isolated from the orbital cavity. Besides mechanical removal of the larvae we have been applied locally sulfacetamid collyr and nemybacin ung. ophthalmic.

In the tropical countries where myiasis are usually occurred in the clinical practice, the more significant are the obligatory myiasis caused from the larvae of the flies Cordylobia anthropophage (Africa) and Dermatobia hominis (South America). The invasion larvae of these flies unnoticed drive into the skin after contact of the skin with contaminated from the flies underwear or at direct contact. After 12 to 15 days on the place is formed furunculo-similar infiltrat that is burst and the alive larva of the fly crawled out. The invasion could be multiple with different localizations. These myiasis are often a reason for intractable purulent dermal infections. In these cases is necessary to be done differential diagnosis with

dermal furunculosis. The treatment consist of incision or removal of the larva by compression and subsequent antiseptic processing. Cases of myiasis caused by Cordylobia anthropophaga have been often observed in our clinical practice in Angola. Three cases of cordylobiosis with Bulgarian cytizens returned after employment from Guinea, Zambia and Zimbabwe were observed in our country (3).

The ethiologic treatment of myiasis with chemoterapeutic is applied rarely (for instance, at intestinal and linear myiasis). The resultant treatment depending on the type and localization of larvae, as we mentioned above, has been acheved with their mechanical removal by instrumental and surgical methods, parallel and susecuent local application of antibiotics, sulfonamides and antiseptics with the purpose to avoid eventual secondary infection. The new broad-spectrum antiparasitic drug livermectin presented significantly expressed therapeutic activity both against nematodic invasions and larvae of some flies from family Calliphoridae that provoked myiasis. These observations are rather perspective for successful application and systematic treatment of myiasis with ivermectin (7).

Prophylactic of myiasis is orientated in several directions. It is necessary to be protected the injured places of the body from access of the flies and to process and ligature in time. The foodstuffs suppose to be prepared and storage without access of flies to them. The insects would to be drived off from the body using repellants for external application in the form of lotions, aerosols and ointments. The windows and doors of the residential buildings is necessary to be covered with a net during the sommer season when are also used insecticide preparations. In the conditions of tropical climate suppose to be covered with a net even the beds. It is necessary to avoid lying down on the ground (for instance, on the beach) and when work in a stock-breeding region by all means to wear outer garments using sistematically repelents and insecticides.

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INTERFERON-ALPHA TREATMENT OF CHRONIC HEPATITIS B IN CHILDREN

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SUMMARY

Forty-three children with chronic hepatitis B were entered into a randomised controlled trial of recombinant human interferon-alpha (IFN-α). All of them had been with viral replication (HBeAg and HBV-DNA positive) and increased levels of the aminotransferases for at least one year. Twelve children received IFN-α in dosage 3MU/m² three times weekly during three months (Group 1); nineteen children received IFN-α in dosage 3MU/m² daily during 10 days and afterwards twice weekly at least 6 months. (Group II); twelve children served as controls (Group III). During a 12 months follow up 13 of 31(41,9%) treated children and 2 of 12 controls were HBeAg-negative and lost hepatitis B viral DNA from the serum. In 3 of these 13 children with negative HBsAg anti-HBs were tested. A statistically significant decrease of the serum ALT among the treated children in comparison with the control group was observed. In conclusion this findings indicate that IFN-α treatment leads to elimination of the replicative forms of HBV infection in children. Good correlation with serological response and biochemistry was obtained in 41,9% of them.

Key-words: Chronic hepatitis B, IFN-α, HBeAg/anti-HBe, anti-HBs, HBV-DNA, childhood

The effectiveness of interferon-a as treatment of chronic hepatitis B in children has been well documented in several studies. (1,2) The role of interferon in treating children is less clear. A controlled trial of a recombinant IFN-a in Chinese children with chronic - hepatitis B yielded disappointing results because no differences were found between the controls and the treated patients (4). In contrast, Ruiz - Moreno et al. demonstrated a relatively high response rate using two different dosages of a-interferon.(5) In the groups treated with 5 or 10 million units/m² three times a week for six months 50% became HBV DNA - negative and seroconverted to anti-HBe compared with 17% of the control group. The children in this series most likely acquired the infection later in infancy or childhood, as reflected by a more active hepatitis and higher transaminase levels.

The aim of the treatment is to eradicate the infection with hepatitis B virus (HBV) and to improve the liver function. We ought to access the results of applying IFN-α during the course of the treatment.

PATIENTS AND METHODS

Between January 1989 and December 1993 we have treated 43 children with chronic viral hepatitis B ageing from 2 to 14 years, 29 male and 14 female. The diagnostic criteria included persistent evaluation of the serum aminotransferase levels and the presence of markers of HBV replication in the serum (HBeAg and HBV DNA) for at least 1 year. A liver biopsy was performed in 10 children. Six had CAH, 3 had chronic persistent hepatitis and 1 was with cirrhosis.

Vertical transmission appeared to be the source of HBV infection in two children. In 12 children, vertical transmission was not documented, but their mothers had H8sAg. In 10 other children other members of the family had HBsAg. Eleven children had a clinical history of acute hepatitis. In the rest the disease was discovered incidentally on routine screening.

The children were randomly assigned to three groups. Patients in group 1 (n=12) received 3 MU/m² IFN-α (Roferon-A- 2a-ROCHE) three times a week for 12 weeks by intramuscular injection and in 3 children two courses (24 weeks) were performed. Patients in group II (n=19) received IFN-a at a dose of 3 MU/m² every day for 10 days and afterward twice weekly during 6 months.

The patients in control group III (n-12) were treated only with hepatoprotective medications. On table one are given the three groups and the analysed parameters. Patients were tested at 1month intervals during therapy and every 3 months thereafter until the end of the study. At each visit a clinical examination was performed and blood samples were taken for blood cell counts, biochemical liver function tests (ALT, protrombin time, serum albumin, total bilirubin levels, etc.) and HBV markers (HBsAg, HBeAg and HBV DNA).

HBsAg, HBeAg, anti-HBe, anti-HDV were determinate by commercial immunoassays, HBV-DNA in the serum was tested by dot-blot hybridisation. (N.Naumov, Clinic of Gastroenterology - Medical University, Sofia)

RESULTS

In Group I in 3 children after 3 months seroconversion to anti-HBe and undetectable HBV DNA was observed. In 2 children in which the course of treatment was repeated (24 weeks) HBV DNA was undetectable to the end of the sixth month. In 5 children(from 12) HBV DNA was undetectable in the end of the sixth month from the beginning of the treatment.

Towards the end of the sixth month in Group II anti-HBe appeared in 4 children and during the first year in another 4 children HBV-DNA was undetectable. Totally HBV DNA was negative in 8 cases (42,1%).

Only 2 children from the control group were with spontaneous seroconversion to anti-HBe towards the end of the first year (I6,6%).

Three of the treated with IFN-α children were negative for HBsAg with appearance of anti-HBs (1 from group I and 2 from group II).

Comparing the results in both treated groups and the control group there is a tendency to suppression of the HBV-replication in 41,9%.

Transitional mild side effects were observed in 26 children (83,3%) manifested with fever, headache, anorexia and in one child haematuria. There were no differences in the frequency or the severity of the side effects between both of the treated groups.

DISCUSSION

The treatment of the chronic hepatitis B with interferon IFN-α started in the midst of the 70's. After 1980 thanks to the introduction of a recombinant DNA technology and of lymphoblasttoid cell cultures it was possible to produce significant quantities of IFN. Therefore, well

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ABBREVIATIONS USED IN THIS PAPER:
HBV - hepatitis B virus, IFN-α - interferon-alfa

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planned and controlled clinical trials were set into course which promoted better understanding of virus induced liver injury (2,6).

The results of IFN-α treatment in children are still disputable. We have studied the effect of IFN-α treatment in 43 children with chronic hepatitis B. Two different doses of IFN-α (Roferon-A-2a, Roche) were used. HBV-DNA was negative in nearly half of the patients who had received 3 MU/m² IFN-α three times weekly during a 12 - 24 weeks period.

For this time all of the children serving as controls were HBeAg and HBV-DNA positive. At the end of the first year from 19 children (group II) in 8 (42,1%) HBV-DNA was negative and seroconversion to anti-HBe was observed. These results are encouraging, because in only 2 children from the control group spontaneous seroconversion to anti-HBe was observed. In conclusion a positive effect was observed in 13 of 31 treated with IFN-α children (41,9%). This effect was manifested by the ceasing of HBV replication. In three of them anti-HBs appears after the clearance of HBV-DNA and loss of HBsAg. There were no differences in the response rate of both groups. Histological improvement consisted of significant regression of portal inflammation and lobular necroses but there were no changes in fibroses. Thus the natural history of chronic HBV infection appeared to be improved in those treated children who lost HBV DNA. The post treatment values of ALT were as follows: for group I - 24,8±5,4;for group II - 28,7±7,0. Statistically proven decrease ($p<0,05$) of the ALT values was observed in the treated children in comparison with the data before the treatment and the children in the control group. Definitely even in the cases where the HBV infection persisted (these were the majority of the treated children) a reduction of the liver injury demonstrated with decrease of the values of ALT and good clinical condition.

In conclusion IFN-α treatment leads to elimination of the replicative forms of HBV infection in children. Good correlation with serological response and biochemistry was obtained in 41,9% of them.

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EVALUATION OF RECONSTITUTION OF CELL-MEDIATED IMMUNE FUNCTION IN HIV/AIDS BULGARIAN PATIENTS RECEIVING SPECIFIC ANTIRETROVIRAL THERAPY

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SUMMARY

In a prospective, ongoing study, 13 patients in different stages of HIV-1 infection, 4 of them ZDV- experienced, have been followed-up at 3-month's intervals during the application of ZDV/3TC for 9 months and ten months later, during which 11of the patients were free of therapy ZDV/ 3TC/SQV was introduced. CD4+, CD8+ and CD3+ cell counts and the CD4/CD8 ratio have been determined by flow cytometry; lymphoproliferative responses (LPRs) to PHA, PHA+rIL-2 and the ratio of CoLSI/LSI, PPD M. tuberculosis and M. avium, and the HIV-1 specific antigen as well as the short-term (1h) and long-term (72h and 144h) spontaneous lymphoproliferation (SLP) – radiometrically by [^{3}H] thymidine incorporation. The concentration of sIL-2R was measured in supernatants of HIV-1-stimulated PBMCs in ELISA. Three months after the onset of ZDV/3TC treatment, gradual recovery of T-cell function as measured by LPRs to PHA, mycobacterial and HIV-1 Ags, a reduction in the degree of cellular activation (SLP and sIL-2R) and an increase in CD4 cell counts was found in all individuals. This tendency persisted till day 270, mainly in ZDV-naive patients with CD4 cell counts > 200 cells/μl at base line. At the beginning of the triple combination CD4+ T cells have been found decreased together with an impaired T-cell function in all cases. Six months later a slight, insignificant immunologic improvement was found. The results obtained even preliminary and from a small number of patients demonstrate that the degree of reconstitution of T-cell immune function during specific antiretroviral therapy depends on the stage of infection, time of initiation of ART, previous therapy experience and the choice of the antiretroviral drug regimen.

Key words: HIV-1 infection, cell-mediated immunity, CD4+count, lymphoproliferative responses, spontaneous lymphoproliferation, sIL-2R, specific antiretroviral therapy.

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ABBREVIATIONS USED IN THIS PAPER:
HIV - human immunodefficiency virus; SLP - spontaneous lymphocyte proliferation; LPR - lymphoproliferative response; LSI - lymphocyte stimulation index; CoLSI - lymphocyte costimulative index; PHA - phytohemagglutinin; Ag - antigen; sIL-2R - soluble interleukin 2 receptor; ART - antiretroviral therapy; HAART - highly active antiretroviral therapy; PBMC - peripheral blood mononuclear cell; ZDV - Zidovudine; 3TC - Lamivudine; SQV - Saquinavir.

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The early pathogenic events involved in the transmission of HIV-1 and establishment of chronic infection are the results of a complex series of virologic and immunologic mechanisms that favor recruitment of susceptible target cells for infection with HIV, rapid virus spreading in the appropriate anatomic site and development of multiple mechanisms of virus escape from the immune response (1). The recognition of the crucial role played by host factors in determining the outcome of HIV-1 infection has not only allowed a better understanding of HIV pathogenesis but it is also providing new strategies of antiretroviral therapy in order to achieve effective suppression of virus replication.

Current antiretroviral therapy (ART) has greatly modified the poor prognosis of HIV-1 infection. Treatment with combination drugs that block two critical steps of the virus's life cycle, reverse transcriptase and protein cleavage, is referred to as highly active ART (HAART). This powerful treatment can reduce viral load in the circulation, and in lymphoid tissue, although virus persists in reservoirs (2,3), and increases circulating CD4+ T cells (4,5,6). It is of great clinical importance to establish whether the available regimens of HAART achieve functional immune recovery since improvement of T cell immunity might be an additional factor involved in the control of HIV-1 during therapy. Initial interpretations of the transient increase in CD4+ T cell number observed during monotherapy with a protease inhibitor suggested that the natural T cell homeostasis was reestablished and that lymphocytes were driven to proliferate toward normal levels (7,8). Transient functional improvement was also detected in lymphocyte responses to mitogens and recall antigens (Ags) (9). Preliminary observations suggested that, nonetheless, memory T cell function, measured as in vitro lymphoproliferative responses (LPRs) to mycobacterial and CMV Ags, barely detectable before therapy, was at least partially restored (10).

Current interest in immunologic reconstitution following the administration of potent antiretroviral therapy has focused mainly on the return of CD4 lymphocyte numbers, both naive and memory subsets, and on the return of cellular immune function to Ags of opportunistic infection pathogens. At the same time a more demanding definition of immunologic reconstitution should include a return of a complete repertoire of effective immunologic responses to specific HIV-1 Ags because lymphocytes from the majority of HIV-infected individuals do not respond by proliferation when stimulated in vitro by these Ags (11), even at the early stages of HIV-1 infection.

The present paper includes data from an ongoing prospective study evaluating the immunologic efficacy of different regimens of specific antiretroviral therapy, applied in HIV/AIDS Bulgarian patients since 1997. Here only data demonstrating both lymphoproliferative responses of T cells to the polyclonal mitogen PHA, costimulation with PHA+rIL-2, the two mycobacterial and the specific HIV-1 antigen, cellular activation as measured by the spontaneous lymphoproliferation and the concentration of sIL-2R and the absolute counts of CD4+, CD8+, CD3+ T cells together with the CD4/CD8 ratio are presented.

MATERIALS AND METHODS

Patients and treatment

13 HIV-1 infected Bulgarians in different stages of infection, 4 of them ZDV-experienced received dual combination therapy with ZDV/3TC for a period of nine months. Ten months later, during which 11 patients were free of therapy, the triple combination with ZDV/3TC/SQV(Invirase) was introduced. During double combination therapy all patients received 600 mg (3x200 mg/day) of ZDV and 300 mg (2x150

mg/day) of 3TC and during the application of the triple combination - the same doses of ZDV/3TC + 1800 mg (3x600 mg/day) of SQV(Invirase). Three groups of patients were set up according to their baseline CD4 counts. The first one (CD4 ≤100 cells/μl) included 7 persons, 6 men and a woman, aged 27-53 years, 2 of them ZDV-experienced, the second one (CD4+ 101-400cells/μl) - 4 individuals, 3 men and a women, aged 26-58 years, 2 of them ZDV-experienced, and the third (CD4+ > 400cells/μl) - two persons, a woman 25 years old and a man 50 years old, ZDV-naive, who entered triple therapy recently. The first patient from this group was followed-up two months after the onset of therapy and the second one - only before it's start. One patient from the first group and one from the second did not stop therapy during the whole observation period. To the therapy of the patient from the second group, who did not stop treatment througtout the whole investigation, at the end of month four after the beginning of dual combination, a protease inhibitor Indinavir (Crixivan) was introduced; at the time when triple therapy was started for all other patients Indinavir was replaced by SQV (Invirase).

Sample collection and isolation of PBMCs

Whole blood (5 ml) was collected by heparinised vacutainers (B-D). Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Ficoll-Paque (Pharmacia, Uppsala), washed twice in HBSS (Sigma), counted and adjusted to 10^6 cells/ml. Cells were resuspended in RPMI 1640 medium (Sigma), supplemented with 10% foetal bovin serum (Sigma), 2mM L-glutamine (Sigma) and 100 U/ml penicillin and 100 mg/ml streptomycin.

Lymphocyte proliferation assay

The proliferative responses of isolated PBMCs were measured by incubating 10^5 cells/well in a total volume of 200 ml using 96-well, flat-bottom microtiter plates (Falcon). Cells were cultured in triplicate with PHA (5 μg/ml), PHA +rIL-2 (10 U/ml), PPD M. tuberculosis (25 μg/ml) and M. avium (25 μg/ml), and HIV-1 Ag (10 ng/ml) for 3 and 6 days respectively. 18h before the end of cultivation cells were pulsed with 37 Bq ^{3}H-thymidine (Amersham). After cell harvesting the amount of ^{3}H-thymidine incorporation was determined as counts per minute (cpm) in a β-counter (Beckman). Results were expressed as lymphocyte stimulation index (LSI), calculated by dividing the mean cpm of 3 replicate-stimulated wells by the mean cpm of the unstimulated ones. In healthy individuals the value of the costimulation index (CoLSI) to PHA+rIL-2 has to be always above that of LSI to PHA only, and in this sense it is indicative and predictive for the degree of recovery of T-cell function. The spontaneous lymphocyte proliferation has been measured in triplicate in the absence of stimulators for one, 72 and 144 h (SLP1h, SLP72h, SLP144h) and results were expressed as mean cpm.

Measurement of sIL-2R

The concentration of sIL-2R in supernatants of HIV-1-stimulated PBMCs has been measured in ELISA (Endogen) according to the manufacturer's instructions. Briefly, ready to use ELISA microplates were coated with supernatants from stimulated and unstimulated lymphocyte cultures together with the conjugate. After an incubation for three hours at room temperature plates were washed and Straptavidin-HPR was added for 30 minutes. The chromogen TMB was added after washing and the colored reaction was read at 450 and 550 nm. Results were calculated using a standard curve and the concentration of sIL-2R was determined as pg/ml.

Immunophenotyping: Blood samples were taken using Sodium heparin as anticoagulant. The collection and analysis of blood samples was performed according to the requirements of NCCLS (H-42A,1998). Staining of lymphocytes was carried out by direct immunofluorescence procedure. The following combinations of Mabs were used: ah4F/ahCD14PY/ahCD45PYCy5; ahCD3F/Leu-12(CD19)PE/ahCD45PYCy5; ahCD3F/CD56PE/ahCD45PYCy5 from NCPID and the TriTest CD4F/CD8PE/CD3PerCP with TrueCount tube purchased from B-D. 20 ml of each combination was incubated with 100 ml blood for 15 minutes at room temperature. The samples were lysed by 0.5 ml Lysing solution and stored in dark for 2 h. Cells were collected by FACScan (B-D). The threshold was set on FL3 and 5000 events per FL3 positive population were analyzed by MULTISET software (B-D).

Statistical analysis

The significance of changes between values of immunologic parameters tested was analyzed by Wilcoxon matched paired test. The significance of correlation was analyzed by use of the Spearman rank test.

RESULTS AND DISCUSSION

In the first group of patients (CD4 ≤ 100 cells/ml) the absolute CD4+ count was found slightly elevated 30 days after the onset of dual combination therapy. The highest values of CD4+ T cells were registered at the end of the fourth month. A weak decline of this parameter was observed two months later and persisted till the end of the application of ZDV/3TC therapy, but with values higher than the initial ones. The absolute count of CD8+ and CD3+ T cells was gradually increasing till the end of the second month and after that there was a tendency to a slight decrease till the end of month nine. The CD4/CD8 ratio followed the dynamics of CD4+ T cells till day 90 then one month later slightly decreased and on month six and nine a subsequent increase was measured. After a period of 10 months during which 6 of the patients were free of therapy, just before initiation of HAART, the CD4 cell count was found decreased and was about the level determined before dual combination regimen. Six months later an increase of CD4 + T cells was measured. The dynamics of CD8+, CD3+ T cells and the CD4/CD8 ratio followed the changes of CD4+ T cells (Fig.1).

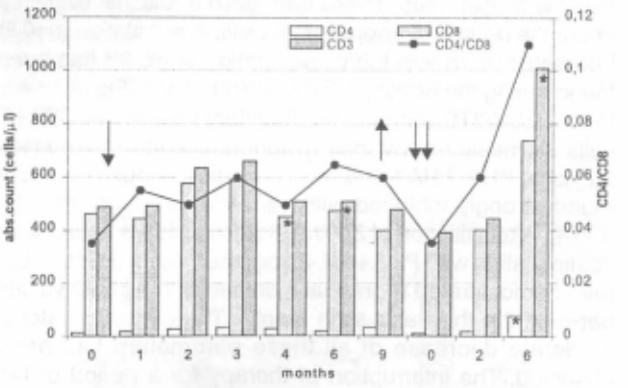

Figure 1. Changes in CD3+, CD4+, CD8+ counts and CD4/CD8 ratio (median values) in patients with baseline CD4+ count <100 cells/μl in the course of dual and triple drug antiretroviral therapy: *p < 0.05, ↓- start of ZDV/3TC therapy, ↑- end of ZDV/3TC, ↓↓ - start of ZDV/3TC/SQV therapy

The degree of cell activation before ZDV/3TC treatment, measured by SLP1h and the concentration of sIL-2R, as well as the long-term SLP (SLP72h, SLP144h) (Fig. 2 and 3) diminished with time, with lowest values on month three. From month six

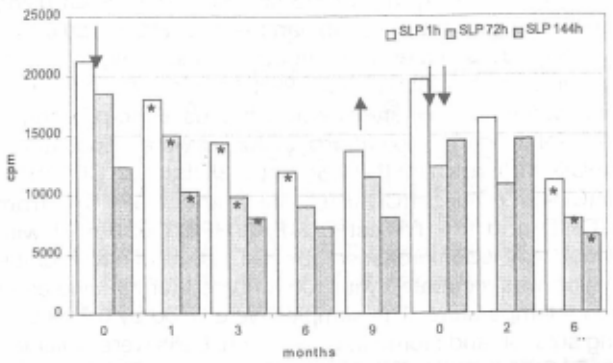

Figure 2. Changes in SLP 1h, SLP 72h and SLP 144h (median values) in patients with baseline CD4+ count <100 cells/μl in the course of dual and triple drug antiretroviral therapy: * p<0.05, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓- start of ZDV/3TC/SQV.

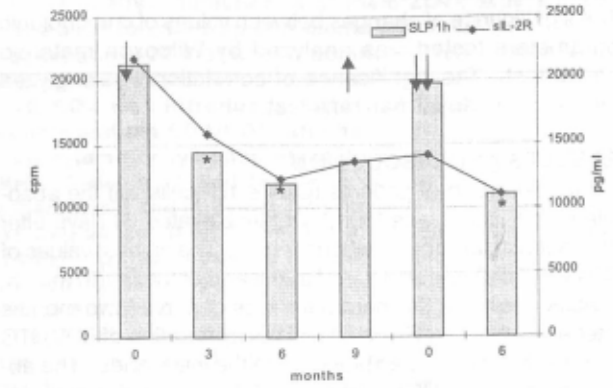

Figure 3. Comparison between the dynamics of SLP 1h and sIL-2R (median values) in patients with baseline CD4+ count < 100 cells/μl in the course of dual and triple drug antiretroviral therapy: *p<0.05, ↓- start of ZDV/3TC,*- end of ZDV/3TC,↓↓- start of ZDV/3TC/SQV.

till the end of dual therapy values of both SLP1h, SLP72h, SLP144h and sIL-2R slightly increased but were still lower than the baseline ones. Before initiation of triple combination the levels of these parameters were found increased again but had not reached the values found at the beginning of dual therapy. Six months later a reduction of cell activation was measured. During both double and triple therapy the values of SLP144h were always lower than the SLP72h and particularly the SLP1h ones. These data gave additional evidence about the degree of apoptosis of cells. A correlation (r=0,9) between SLP-1h and the concentration of sIL-2R has been found during the whole period of investigation (Fig.3).

Before ZDV/3TC therapy, the functional activity of CD4+T cells as measured by their lymphoproliferative responses (LPRs) to PHA, PHA+rIL-2, and the ratio of CoLSI/LSI, were found strongly inhibited with very low values of all LSIs (Fig.4). After initiation of ZDV/3TC therapy, LPRs to PHA and costimulation with PHA+ rIL-2, together with the CoLSI/LSI ratio demonstrated a gradual increase with highest values between the third and sixth month. Three months later a moderate decrease of all these parameters has been observed. The interruption of therapy for a period of ten months apparently abrogated the effect of treatment because at the beginning of the ZDV/3TC/SQV regimen the values both of LSI to PHA and PHA+rIL-2 as well as the CoLSI/LSI ratio decreased. Six months later only the LSI to PHA was found increased. During the hole observation period the values of CoLSI were smaller than those of LSI to PHA and were always under the value of 1.0.

LPRs to mycobacterial antigens and the specific HIV-1 Ag had a similar dynamics (Fig.5). It is worth noting that the values of LSI to HIV-1 Ag were greater than LSIs to PPD M.

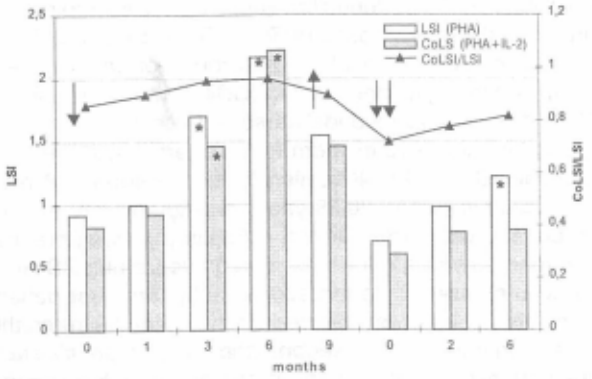

Figure 4. Dynamics of lymphoproliferative responses to PHA, PHA+rIL-2 and the CoLSI/LSI ratio (median values) in patients with baseline CD4+ count < 100 cells/μl in the course of dual and triple drug antiretroviral therapy: * p<0.05, ↓- start of ZDV/3TC, *- end of ZDV/3TC, ↓↓- start of ZDV/3TC/SQV.

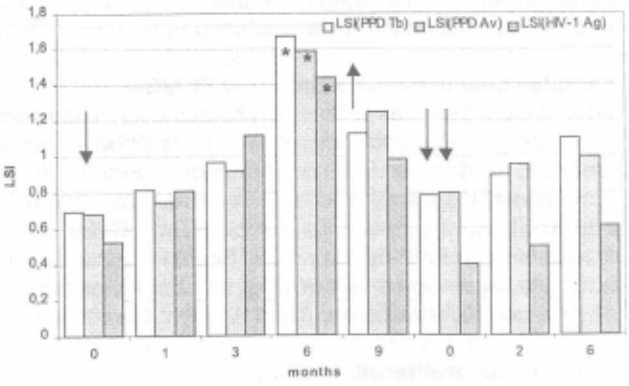

Figure 5. Dynamics of lymphoproliferative responses to PPD M. tuberculosis, M. avium and HIV-1 Ag (median values) in patients with baseline CD4+ count <100 cells/μl in the course of dual and triple drug antiretoviral therapy: *p<0.05, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC,↓↓- start of ZDV/3TC/SQV.

tuberculosis and M. avium at the end of the first and the third month. Between months six and nine all three LSIs had a tendency to decline. Before triple drug therapy the values of these parameters were found decreased and six months later they did not change a lot. In this case LPRs to HIV-1 Ag were always smaller than those to mycobacterial Ags.

During the course of dual therapy the dynamics of absolute CD4+ counts did not always follow that of LPRs to PHA and HIV-1 Ag. Six months after initiation of triple drug regimen a correlation (r=0,8) was found only between CD4+ counts and the LSI to PHA (Fig. 6). There was a correlation (r=0,9)

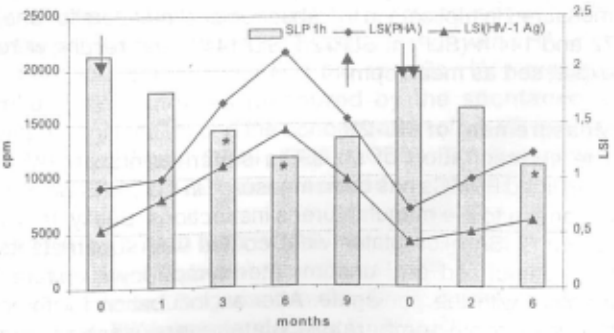

Figure 6. Comparison between the dynamics of SLP 1h and LSIs (PHA, HIV-1 Ag) (median values) in patients with baseline CD4+ count <100 cells/μl in the course of dual and triple drug therapy: *p<0.05, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓- start of ZDV/3TC/SQV

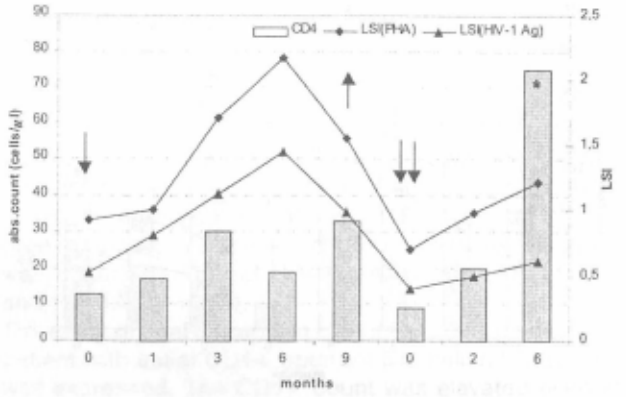

Figure 7. Comparison between the dynamics of CD4+cell count and LSIs (PHA, HIV-1 Ag) (median values) in patients with baseline CD4+ count <100 cells/ml in the course of dual and triple drug therapy: *p<0.05, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓- start of ZDV/3TC/SQV.

between cellular activation as measured by the short-term SLP1h and the recovery of LPR to PHA and HIV-1Ag at all terms of investigation (Fig. 7).

As a whole, the effect of ZDV/3TC combination therapy on immunologic improvement was considered as favorable in all patients from the first group mainly between the third and sixth month after initiation of treatment. It was most expressed in four of the patients who were ZDV-naive before therapy. This conclusion was made according to the degree of reconstitution of T-cell function and the absolute number of CD4+ T cells. The discontinuation of therapy affected adversely all patients from this group. Six months after initiation of the triple drug regimen, immunologic efficacy was determined only in ZDV-naive patients. In the rest of them, including the patient who was ZDV-experienced and who did not stop therapy, the recovery of cell-mediated function was found lower.

In patients from the second group, (CD4 101-400 cells/ml) during the application of dual combination therapy the highest values of CD4+ T cells have been measured between months four and nine. At the end of month nine the values of CD4+ T cells were slightly above those at month six, and both CD3+ and CD8+ counts were found lowered. Ten months later during which patients were free of therapy a decrease of absolute counts of all three cell populations has been determined. At the end of the second month after initiation of triple combination, both CD4+, CD3+ and CD8+ T cells were slightly increased but four months later, they kept the same values (Fig.8).

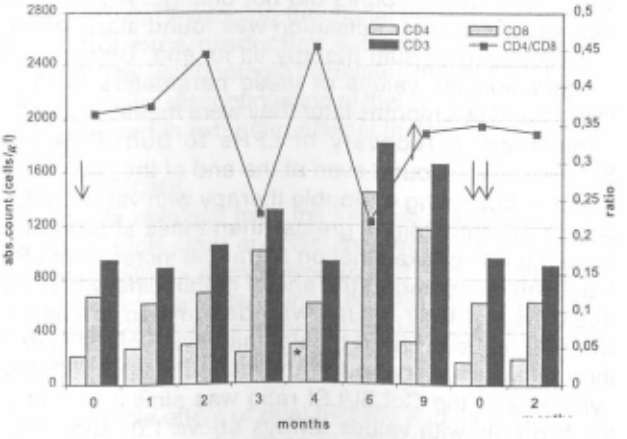

Figure 8. Changes in CD3+, CD4+, CD8+ counts and CD4/CD8 ratio (median values) in patients with baseline CD4+ count 101-400 cells/ml in the course of dual and triple drug antiretroviral therapy: *p < 0,05; ↓- start of ZDV/3TC therapy; ↑- end of ZDV/3TC therapy; ↓↓- start of ZDV/3TC/SQV therapy

The effect of ZDV/3TC combination on the reduction of cellular activation as measured by SLP1h, SLP72h, SLP144h (Fig.9) and the concentration of sIL-2R (Fig.10) was found most expressed between months three and nine, with progressively falling values. It is interesting to note that at the end of dual therapy values of SLP72h and SLP144h were greater than the SLP1h ones and this fact figured out the decreased tendency to apoptosis of cells at that moment. A correlation (r=0,95) has been observed between the dynamics of SLP1h and the concentration of sIL-2R (Fig.11). The recovery of LPRs to PHA and PHA+rIL-2 was evaluated as greatest between months three and nine. The CoLSI was measured higher than the LSI to PHA from the end of the third till the end of the sixth month and on month nine the values were about the same level (Fig.12).

LSIs to PPD M. tuberculosis, M. avium and HIV-1Ag had a similar dynamics during dual therapy. At the end of the sixth and ninth month LPRs to HIV-1 Ag were 6-7 times greater than the initial ones and were much higher than LPRs to mycobacterial Ags. After discontinuation of treatment and at the beginning of the triple combination, all three LSIs have been found substantially decreased. After a slight increase, at the end of the second month, on month six no changes in LPRs had been observed, even values were at a lower level (Fig.13). As in the first group of patients, there was a correlation (r=0,85) between the marker of activation SLP1h and the LPRs to both PHA and HIV-1Ag which was better than that found between the absolute CD4+ count and the responses to the polyclonal mitogen PHA and the specific HIV-1Ag (r=0,6) (Figs.14).

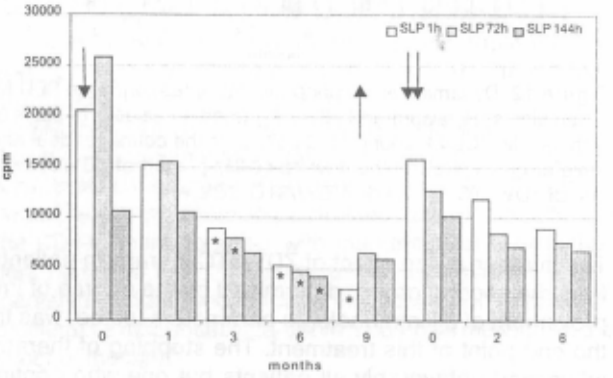

Figure 9. Changes in SLP 1h, SLP 72h and SLP 144h (median values) in patients with baseline CD4+ count 101-400 cells/ml in the course of dual and triple drug antiretroviral therapy: * p<0.05, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓ - start of ZDV/3TC/SQV.

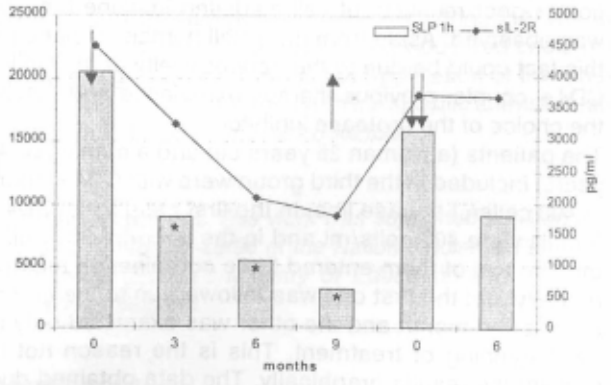

Figure 10. Comparison between the dynamics of SLP 1h and sIL-2R (median values) in patients with baseline CD4+ count 101-400 cells/ml in the course of dual and triple drug antiretroviral therapy: * p<0.05, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓ - start of ZDV/3TC/SQV.

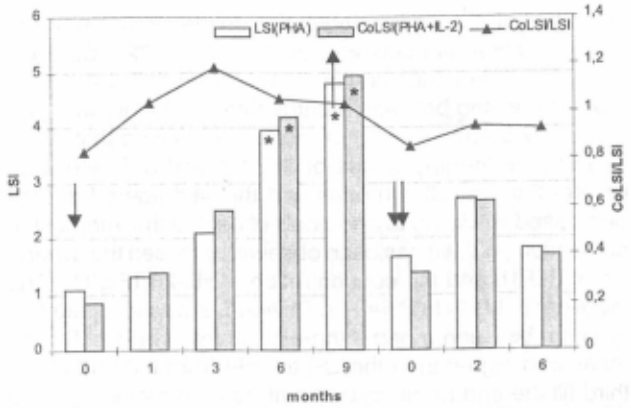

Figure 11. Dynamics of lymphoproliferative responses to PHA, PHA+rIL-2 and the CoLSI/LSI ratio (median values) in patients with baseline CD4+ count 101-400 cells/μl in the course of dual and triple drug antiretroviral therapy: * $p<0.05$, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓ - start of ZDV/3TC/SQV.

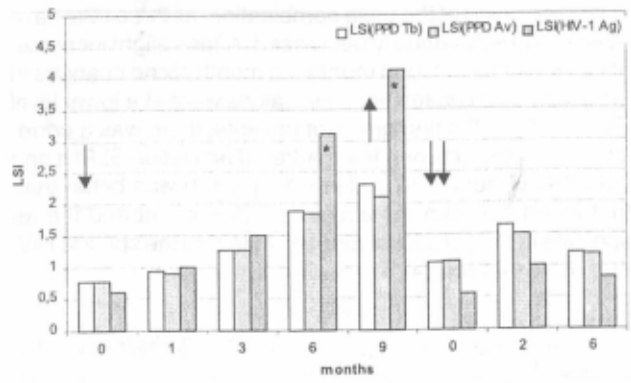

Figure 12. Dynamics of lymphoproliferative responses to PPD M. tuberculosis, M. avium and HIV-1 Ag (median values) in patients with baseline CD4+ count <100 cells/μl in the course of dual and triple drug antiretroviral therapy: *$p<0.05$, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓ - start of ZDV/3TC/SQV.

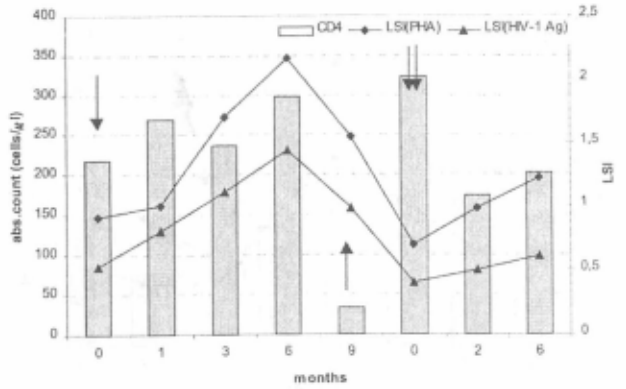

Figure 13. Comparison between the dynamics of CD4+cell count and LSIs (PHA, HIV-1 Ag) (median values) in patients with baseline CD4+ count 101-400 cells/μl in the course of dual and triple drug therapy: *$p<0.05$, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓- start of ZDV/3TC/SQV.

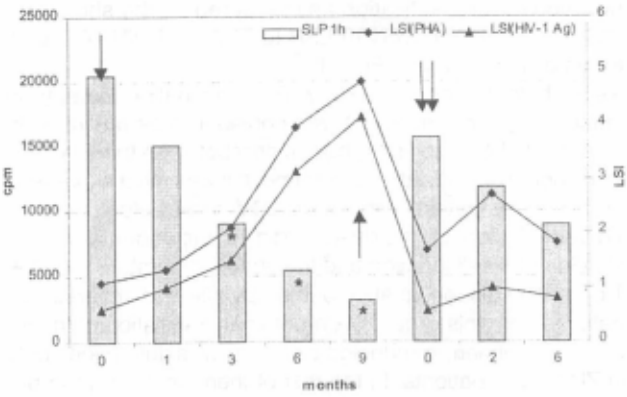

Figure 14. Comparison between the dynamics of SLP 1h and LSIs (PHA, HIV-1 Ag) (median values) in patients with baseline CD4+ count 101-400 cells/μl in the course of dual and triple drug therapy: *$p<0.05$, ↓- start of ZDV/3TC, ↑- end of ZDV/3TC, ↓↓ - start of ZDV/3TC/SQV

The duration of the effect of ZDV/3TC therapy in patients from the second group, determined by the degree of improvement of all immunologic parameters tested, was till the end point of this treatment. The stopping of therapy influenced unfavorably all patients but one who continued treatment with the combination ZDV/3TC/IDV(Crixivan). Six months after the replacement of Crixivan by Invirase in this patient, the previous immunologic improvement was disrupted considerably. In the rest of patients, sixth months after initiation of triple combination, a poor recovery of cell-mediated immune function was observed. Aside from the small number of patients, this fact could be due to the heterogeneity of their initial CD4+ counts, previous therapy experience and maybe the choice of the protease inhibitor.

The patients (a woman 25 years old and a man aged 49 years) included in the third group were with CD4+ count > 400 cells/μl at base line. In the first case initial CD4+ T cells were 402 cells/ml and in the second -503 cells/ml. The two of them entered triple combination therapy recently and the first one was followed-up to the end of the second month and the other was examined only at the beginning of treatment. This is the reason not to present the results graphically. The data obtained during the whole treatment of the patient with initial CD4+ T cells 402 cells/ml are the following. During ZDV/3TC therapy a gradual increase of CD4+ T cells was observed till the end of the third month. One month later, after a slight decrease, these cells with high values, persisted till the end of dual therapy. The dynamics of CD3+ T cells was similar but of CD8+ T cells was a little bit different. Like CD4+ T cells, they sharply increased three months after the set up of therapy, then smoothly decreased till the end of the sixth month and three months later were slightly elevated. At the beginning of ZDV/3TC/SQV therapy and two months later absolute CD4+, CD8+ and CD3+ counts did not change. A very great decrease of cellular activation was found starting from the beginning of dual therapy till its end. Before triple combination the values of these parameters were elevated and two months later they were measured feebly diminished. A recovery of LPRs to both PHA and PHA+rIL-2 was found even at the end of the first month after the beginning of double therapy with values of LSI and CoLSI three times greater than those at base line. At next terms of examination a gradual increase of LSIs has been measured till the end of month nine, when a 5-6-fold rise of their values was determined. Values of CoLSI have been found greater than those of LSI to PHA through the whole period of application of ZDV/3TC. The dynamics of the CoLSI/LSI ratio was similar to that of the two LSIs with values always above 1.0. After interruption of therapy a decrease of LSI, CoLSI and the CoLSI/LSI ratio has been measured. Two months after the beginning of ZDV/3TC/SQV regimen a very slight increase has been observed.

The dynamics of recovery of LPRs to mycobacterial Ags and the HIV-1 Ag was very similar, with peak values at the end of month nine. During the application of ZDV/3TC therapy the values of LSI to the specific Ag were always greater than LSIs to mycobacterial Ags. At the start of triple combination all three LSIs were found pritty low and two months later a slight increase was registered. As in all other cases the correlation (r=0,90) between the degree of cellular activation and LPRs to PHA and HIV-1 Ag was better than that of CD4+ counts and LSIs to PHA and HIV-1Ag (r=0,60).

The effect of dual therapy on immune reconstitution in the patient with initial CD4+ count of 530 cells/μl was very well expressed. The CD4+ count was elevated even at the end of the first month after the onset of treatment and with similar values persisted till month six. At the end of month nine a very slight decrease was measured. The movement of CD8+ and CD3+ counts was very likely from the beginning till the end of the application of this regimen with highest values of CD8+ and CD3+ T cells on month nine. The lowest values of SLP and sIL-2R have been measured at months six and nine, when the values of SLP144h were greater than those of SLP1h and SLP72h. In this patient the highest degree of recovery of LPRs to PHA, PHA+rIL-2, PPD M. tuberculosis, PPD M. avium and HIV-1Ag has been determined during dual therapy. At the end of month nine the greatest values of LSI to PHA, PHA+rIL-2 and HIV-1Ag were found. As in all other patients the stopping of therapy was detrimental to all immunologic parameters. This was illustrated by the results obtained at the beginning of the triple combination regimen. At that moment CD4+ count was under its base line level before ZDV/3TC therapy, the degree of cellular activation was increased almost twice and all LSIs were lower than those measured nine months after the onset of dual therapy.

Our data are in agreement with the results received by other working teams describing the improvement in cell-mediated immune function during the application of potent anti-HIV drug regimens (14, 15, 16, 17, 18, 19, 20, 21, 22). These authors show that the extent of immune recovery during HAART has been shown to depend on the achievement of adequate suppression of viral replication and that the long-term immunologic reconstitution is a very gradual process and might not be complete in a number of HIV-infected persons. They also address the important question of when to initiate HIV-infection treatment, which has to be precisely controlled. The early treatment may be the key to developing functional immunosurveillance to control virus production without permanent drug therapy. The immunologic correlates that predict control of viremia after discontinuation of therapy have to be carefully analyzed in extended clinical trials.

CONCLUSIONS

The results obtained from the immunologic monitoring of dual and triple drug specific antiretroviral therapy in 13 Bulgarian patients, even preliminary and from a small number of patients are very interesting. The evaluation of the immunologic efficacy of this treatment allows the following more general conclusions to be made.

The favorable effect of dual therapy and its duration depended mainly on the stage of HIV-1 infection and previous therapy experience (12, 13). In patients with initial CD4+ count < 100 cells/μl the recovery of cell-mediated immune function was registered three months after the onset of therapy and lasted till month six; in patients with CD4+ count 101-400 cells/μl - it was detected between the second and the third month and persisted till the end of the application of ZDV/3TC combination, and in patients with CD4+ count > 400 cells/μl - earlier, from the end of the first month and continued throughout dual therapy. It must be emphasized that ZDV-nanve patients responded better and longer to dual therapy. Treatment-induced changes were observed in responses indicative of sustained improvement of T cell function. T-cell proliferative responses reconstituted first to the polyclonal mitogen PHA and then to mycobacterial and the specific HIV-1 antigens, but in individuals who responded better to therapy an earlier and greater recovery of the response to HIV-1 Ag was found. The favorable effect of dual therapy was also confirmed by the marked decrease of cellular activation as measured by the spontaneous lymphoproliferation (mainly SLP1h) and the concentration of sIL-2R. The interruption of this drug regimen had a detrimental effect on cellular immunity in all patients independently of their baseline CD4+ count.

It is very difficult to define the effect of triple combination therapy till that moment. The main reason is the smaller number of patients who were tested till the end of month six after its initiation. More definite conclusions could be made for patients from the first group since all of them were followed-up till the last term of observation, where only CD4+ counts and lymphoproliferative responses to PHA, even pretty low, were found increased. In three of the patients from the second group, who entered triple therapy earlier, no changes in CD4+ counts have been observed. Also the responses to PHA and HIV-1Ag even slightly have been measured depressed. In patients from the third group triple therapy was recently introduced and we are not able to discuss its influence on immune reconstitution.

Finally, the results from this prospective ongoing study show that the implementation of the immunologic monitoring of antiretroviral therapy is of great importance for the evaluation of its effects. From all parameters tested in addition to the CD4+ count, together with markers of cellular activation, particular attention deserves the assessment of cell-mediated immune function since the recovery of lymphocyte-proliferative responses may serve a potential measure of the effect of therapy.

All data obtained till now are both intriguing and ambiguous. They give rise to a variety of questions, which could be answered with time when a greater number of patients receiving potent antiretroviral therapy for a longer period of time will be covered. At the same time highly informative will be the results obtained from the follow-up of patients who will appear therapy-naive before entering HAART. In future time it is worth thinking of an individualized choice of the drug regimen. The idea of the application of immune-based therapy in combination with the specific antiviral treatment should be taken into account too.

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