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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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EPIDEMIOLOGICAL AND ETIOLOGICAL ASPECTS RELATED TO AEROBIC BACTERIAL SURGICAL SITE INFECTION

A. Kirtcheva[1], D. Radev[2], T. Kantardjiev[3], M. Todorova[3], M. Petrov[3], Tz. Velinov[3], A. Bachvarova[3]

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SUMMARY

Nosocomial infections (NI) and surgical site infections (SSI) in particular constitute a serious problem in infectious pathology. Surgical wound infection (SWI) occupies constantly the first place among nosocomial infections in the Multi-profile Hospital for Active Therapy (MPHAT) „St.Anna" - Varna AD, varying from 35.29% to 51.09%. Gram-negative microorganisms predominate as major etiologic agents - 67.43%. The leading causative agents are E.coli - 17.78%, Staphylococcus aureus - 17.25%, Enterobacter spp - 9.15%, Pseudomonas spp - 8.98%, Proteus spp - 8.01%.

Data are reported as regards the dominating resistance and susceptibility to antibiotics of the isolated microorganisms for the 1994-2001 period. The results are presented of a conducted cross-sectional study, comprising 114 patients from First Surgical Ward having developed SWI, a relation being made with wound class according to CDCP.

Key words: bacterial infection, nsocomial infection, Gram negative bacteria

Despite the definite achievements in infection control, nosocomial infections (NI) continue to be an essential problem of infectious pathology. They are related to a complex and multifactor epidemic process comprising varied etiologic agents, sources and modes of transmission; affect highly susceptible hospital contingent subject to multiple invasive procedures and different standard of medical care (1). Their importance is determined by the impact over the patient and treatment costs as well - by changing the course of underlying disease and the treatment undertaken (surgical one involving) and causing complications, these infections prolong hospital stay as well, raise the treatment costs as a result of introduction of additional highly expensive drugs, and diminish the usability of hospital beds (4). According to calculations of F.D.Daschner, NI have cost to Germany from 500 millions to 1 milliard marks annually, and G.A.J. Ayliffe and B.J.Collins have determined economical losses of 30 million pounds for the public health in Great Britain in case of 5% of NI (2). In our country the problem is often underestimated, or concealed by the hospital settings. Nevertheless, the newly formed branch of knowledge "hospital epidemiology" gained a considerable material and experience in NI evaluation, the methods for their surveillance and control.

The aim of the present study is to clarify the frequency of surgical site infection (SSI) among NI registered as a total in the Multi-profile Hospital for Active Therapy "St.Anna" (MPHAT) - Varna AD; the type of aerobic microbic flora isolated in case of SSI development; the patterns of changes in antimicrobial resistance of the strains isolated; and the role of wound class for surgical wound infection (SWI) development through a cross-sectional study of 114 operated patients.

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ABBREVIATIONS USED IN THIS PAPER:

NI-nosocomial infections, SSI-surgied site infection, SWI-surgical wound infection

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MATERIALS AND METHODS

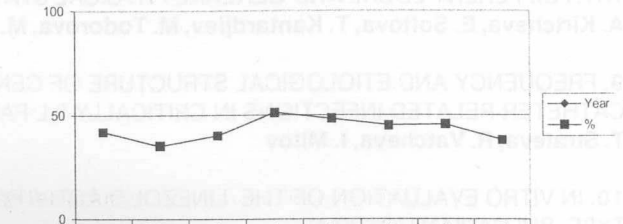
The study is conducted in MPHAT "St.Anna" - Varna AD for the period 1994-2001. Data from the available hospital documentation have been used, patients' charts, operative protocols including, and a "SSI Registration Card" as well. The clinical materials from wound secretions have been tested by means of standardized methods for microbic flora identification, tested for antibiotic resistance with Bauer-Kurby disc diffusion method respectively, and since 1999 tested for the presence of aerobic flora with the "miniAPI" system of the French firm "BioMerieux". The microbiological tests using the above-mentioned methods included a total number of 6085 surgical-wound secretions, 3895 of which with bacterial growth and antibiogram (antibiotic resistance) determination. Specified epidemiologic studies have been performed with regard to SSI frequency according to wound class in 114 patients from the First Surgical Ward of the hospital, using the "SSI Registration Card".

RESULTS

The results achieved indicated that SSI is an actual pathology and occupies a constantly dominating place among NI registered in the hospital, varying from a relative frequency of 35.29% to 51.09% for the period 1994-2001 (Table 1).

Among the clinical materials microbiologically tested, wound secretions occupy repeatedly the third place following urines and naso-pharyngeal secretions. Taking into account that the latter are tested most commonly with a prophylactical aim, and surgical-wound secretions mainly on clinical indications, this fact confirms the dominating percentage of surgical-wound secretions as most important substratum for laboratory analysis (Fig.1).

Table 1. Relative Frequency and Importance of SWI among NI Registered in the Municipal Hospital "St. Anna" of Varna for the 1994-2001 Period



	1	2	3	4	5	6	7	8
Year	1994	1995	1996	1997	1998	1999	2000	2001
%	42,09	35,29	40,09	51,09	48,25	44,84	45,39	38

On tracing the specimens from surgical-wound secretions with growth - 3895 for the period considered, the dominating relative frequency of gram-negative microflora is impressing - 2410 (61.43%), the latter outlining as a leading one among SWI etiologic agents - 110 gram-negative microorganisms (67.43%) (Fig. 2).

Gram-positive microorganisms demonstrate dynamics, as from 1998 till 2001 increase, though slowly, their relative frequency - in surgical-wound secretions from 30.92% to 42.58%, and in the cases of SSI - from 20.95% to 31.87%. These data are consistent with the general trend for the country (3) resulting from the institution of a complex of organisational and hygienic-antiepidemic measures for reducing risk factors for patients, those associated with surgical procedure correspondingly.

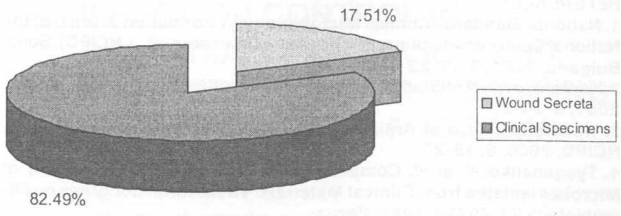


Fig 1 Relative Frequency of Clinical Speciments Tested in the Municipal Hospital „St. Anna" of Varna for the 1994-2001 Period

The generalized data for the period 1994-2001 indicate that among the isolated microflora from surgical-wound secretions of leading role are: E.coli, Staphylococcus aureus, Enterococcus spp, at an increased for the last three years percentage of Acinetobacter spp, Pseudomonas spp and Proteus spp. The generalized analysis of etiologic agents isolated in SWI cases for the same period demostrates some general, but new trends as well in bacterial isolates' spectrum: Leading ones - E.coli - 17.78%, Staphylococcus aureus - 17.25%; of essential importance - Enterobacter spp - 9.15%, Pseudomonas aeruginosa - 8.98%, Proteus spp - 8.01%,

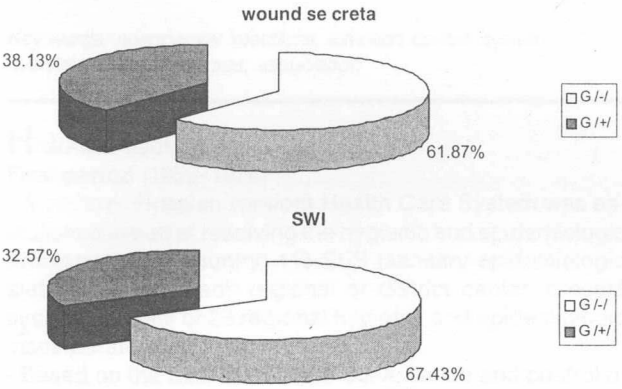


Fig. 2 Portion of Gram (-) and Gram (+) Microflora Isolated from Wound Secreta and SWI

Enterococcus spp - 7.83% and Serratia spp - 7.31%; all the rest are of conditional and more restricted participation with a relative frequency below 6% (Fig.3).

The generalized data of resistance patterns to most commonly used antibiotics for the period 1994-2001 demonstrate high levels of resistance to penicillins - over 70.02%, second-generation cephalosporins - from 61.94% to 83.53% and comparatively low-level resistance in vitro to the commonly used aminoglycosides - below 47.19%. In the last year within the group the susceptibility to Tobramycin has been most considerably increased - from 60.67% to 73.61%.

A very high-level susceptibility demonstrate all strains to Ticarcillin/Clavulanic acid, Piperacillin, Tazocin, carbapenems, third- and fourth- generation cephalosporins and quinolones - an average increase in susceptibility between 72.77% and 97.49%.

Gram-positive cocci retain a high-level resistance besides penicillins (66.67% - 92.90%) to other antibacterials, e.g. in Enterococcus spp isolates a similar high-level resistance is added to Lincomycin, Clindamycin, reaching 94.29%, and to first and second generation cephalosporins - from 67.24% to 87.50%.

In gram-negative bacteria high-level resistance and problematic effectiveness is registered to penicillins' group (combined forms including), Azlocillin, first and second generation cephalosporins, Chloramphenicol and Nalidixic Acid.

The multiply resistant hospital strains Pseudomonas aeruginosa and Acinetobacter spp provide an opportunity for theurapeutical choice - in Pseudomonas isolates: Ticarcillin/Clavulanic acid, Piperacillin (in combination with

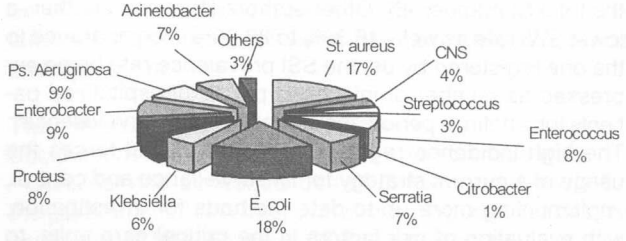


Fig. 3 Relative Frequency of Microflora Isolated from SWI

Tazobactam likewise), carbapenems, fourth-generation cephalosporines, quinolones; in Acinetobacter spp isolates there is a satisfactory susceptibility to carbapenems (63.64% to 98.48%), in the last year the resistance being decreased to Tobramycin and fourth-generation cephalosporins.

The risk of postoperative SSI development is considerably affected by the degree of microbial contamination at the operation site. The classification scheme of wounds developed by the USA National Research Council (clean, clean-contaminated, contaminated and dirty/infected) with CDCP modifications (superficial incisional SSI, deep incisional SSI and organ/space SSI) take into account the initial status of wound with regard to risk of microbial contamination (4,5). Initiating an extended survey of the role of risk factors in SSI development, we set ourselves the task of conducting a cross-sectional study in the First Surgical Ward over 114 operated patients with different surgical diagnosis and cleanness of the operative site. The results are presented in Table 3, data from studies abroad being cited in table 2.

Table 2. Surgical Wound Infection Rates According to Wound Class

Wound Class	Cruse and Foord n=62937	SENIC n=59352	Olson and Lee n=36439	Culver et al. n=84691
Clean	1.5	2.9	1.3	2.1
Clean Contaminated	7.7	3.9	2.4	3.3
Contaminated	15.2	8.5	7.9	6.4
Dirty and Infected	40.0	12.6	-	7.1

Table 3. Cross-sectional Study of SWI According to Wound Class in the 1st Surgical Ward of the Municipal Hospital "St. Ann" of Varna

Wound Class	Number of Patients with SWI	Relative Frequency
Clean	4	3.51
Clean Contaminated	30	26.32
Contaminated	36	31.58
Dirty and Infected	44	38.60
Total	144	100

The percentage of patients with SSI increase in direct proportion to wound class, i.e. universally accepted concept of higher degree of microbial contamination in contaminated and especially heavily contaminated wounds - from 3.51% for clean wounds to 26.32% in clean-contaminated wounds to 38.60% in contaminated wounds and 38.60% in dirty/infected wounds.

DISCUSSION

Nosocomial infections and in particular surgical wound infection are an actual pathology for the polyprofile hospital with predominance of surgical wards and clinics MPHAT "St.Anna" - Varna AD.

SWI occupies continually the first place among NI registered - from 35.29% to 51.09% for the 8-year period studied. In the analyses for the country SSI takes a third place, with almost similar relative frequency by years - of about 11% of

the total NI number (6). Other authors (1) report another, a lower SWI rate as well - 16.30% to 20.00% in comparance to the one registered by us, the SSI prevalence rate being expressed as number of infections per 100 hospitalized patients for a definite period, the so-called "period prevalence". The high incidence registered in our hospital forces the usage of a current strategy for NI surveillance and control, implementing more up-to-date methods for investigation, with evaluation of risk factors in the critical care units, to which surgical clinics pertain (5).

The analysis of microbiological tests' results indicates the important role of Gram-negative microflora, non-fermentative Gram-negative microorganisms including, isolated in SWI. Nevertheless, a trend for restoring the role of Gram-positive bacteria is emerging through a gradual increase in their relative frequency. A similar dependency is reported by other authors as well - N.Ribarova, Tz. Dimitrova 1979; Daschner 1983. The emergence of multiresistant Acinetobacter, Pseudomonas, Enterobacter strains requires a new approach in hospital's antibiotic policy as well. Studies in Great Britain have confirmed that only with a proper hospital policy as regards antibiotics' application, considerable savings may be achieved without restricting theurapeutists in antibiotic choice(10).

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INFECTION CONTROL IN BULGARIA: AN OVERVIEW AND PERSPECTIVES OF THE ONGOING BULGARIAN SWISS PROGRAM

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SUMMARY

In Bulgaria, in parallel with recognition of the need to control nosocomial infections (NI), since the early 1950s a national infection control program has been gradually developed. This report summarizes the information on the key strategies implemented and the lessons learnt from the past experience. Based on the historical background the perspectives of the ongoing Bulgarian Swiss Hospital Hygiene Program (BSHHP) are presented. The following periods are considered: 1952-1974 with efforts focused primarily on the widespread classic infections, 1975-1989 with establishment of the national infection control system, and the 1990s with collapse of the socio- economic infrastructure, incl. health care system and a negative impact on the infection control program. By implementing the BSHHP principles for new professional qualifications and development of quality care standards integrated into clinical practice a future sustainable improvement in the NI prevention and control is expected.

Key words: nosocomial infections, infection control system, overview, training courses, association

Historical background

First period (1952- 1974)

- A vertical (Russian version) Health Care System was established aimed at resolving the hygienic and epidemiologic problems in the country: 115 SES (sanitary-epidemiologic stations) one in each regional or district center; present system consists of 28 regional hygienic and epidemiologic inspectorates - HEI (Figure 1)
- Based on the SES system the surveillance and control of classic infections diseases was organized (Figure 2)
- Substantial reduction or elimination was achieved of severe widespread communicable diseases, such as: diphtheria, poliomyelitis, typhus abdominalis, typhus and malaria (Table 1)
- The main target of infection control were NI caused by enteric pathogens: E. coli, Salmonella, Hepatitis A Virus

Second period (1975- 1989)

- Infection Control System was launched: Instruction No 0-30 (17.05.1975 of Ministry of Health (MoH)
- Statutory reporting of NI was introduced: Order No7403/ 14.12.1979 of MoH
- In 1976 National Infection Control Reference Center was established: Annual Bulletin had being edited to provide regular feedback to HEI and primary health care.
- In 1976 - 1980 a wide spectrum of instructions and guidelines were issued and conferences organized on prevention and control of NI
- Since 1981 a national computerized information system on NI was developed and implemented: quarterly data collated by region, hospital ward, clinical manifestation and etiologic agent.

Third Period (1990- 2002):

Socio-economic crisis in the 1990s characterized with:

- Considerable consequences of the economic crisis for the governmental services: overall drop in public health care expenditures from 65.9% of GDP to 34.0% in 1997.
- Slow economic transition has given rise to widespread poverty in the country: 35% of the population living below poverty line; unemployment reached 17.9% in 2000
- Following the negative economic trends, health status of Bulgarians has generally worsened with a greater deterioration in rural areas;
- Aging of the population (16% aged 65 years and over) due to emigration of young people, low birth and high mortality rates
- Soviet model of Public health care provision hit by the crisis: the downward trend of hospital and community - acquired infections was interrupted
- Health care reforms were initiated: the Law on Health Insurance adopted in 1998 and National Health Insurance Fund established in early 1999 with 28 regional branches

Perspectives of the Bulgarian Swiss Hospital Hygiene Programme (2002-2004)

BSHHP comprises three components (Projects):

- Microbiology
- Hospital hygiene/ Prevention and control of nosocomial infections
- Central Sterilization/ Central Sterile Supplies Department (CSSD)

The overall goal of the Program is to harmonize the practical standards for infection control in Bulgaria with those used in industrialized countries.

Hospital Hygiene Project Aims:

- Improved surveillance of NI
- Increased effectiveness of the system for prevention and infection control
- Formulation of an adequate national antibiotic strategy and cost- effective local antibiotic policies
- Ensuring a higher level of safety for patients, personnel and visitors
- Effective resource allocation

Hospital Hygiene Project Tools:

1) Training courses with attendants from different target groups:

- Infection control nurses from the 6 model hospitals, on a later stage- for all the hospitals
- Epidemiologists/ sanitary inspectors from the regional hygiene-epidemiological inspectorates
- Clinicians

2) Profile specialty "Hospital Hygiene" has been established for nurses and sanitary inspectors (in the context of the Amendment of Regulation 31 of the Ministry of Health)

Table 1. Morbidity of the most widespread communicable diseases in Bulgaria

Disease	Incidence rate per 100 000 in Bulgaria	
	1942	1975
Diphtheria1	60.5	0.1
Poliomyelitis	20.8	0.0
Typhus abdominalis	10.8	0.2
Typhus	2.4	0.0
Malaria[2]	943.6	0.5

1 1940;[2] 1945

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ABBREVIATIONS USED IN THIS PAPER:

BSHHP - Bulgarian Swiss Hospital Hygiene Program

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Fig. 1 Map of Bulgaria with 28 regional HEI centers

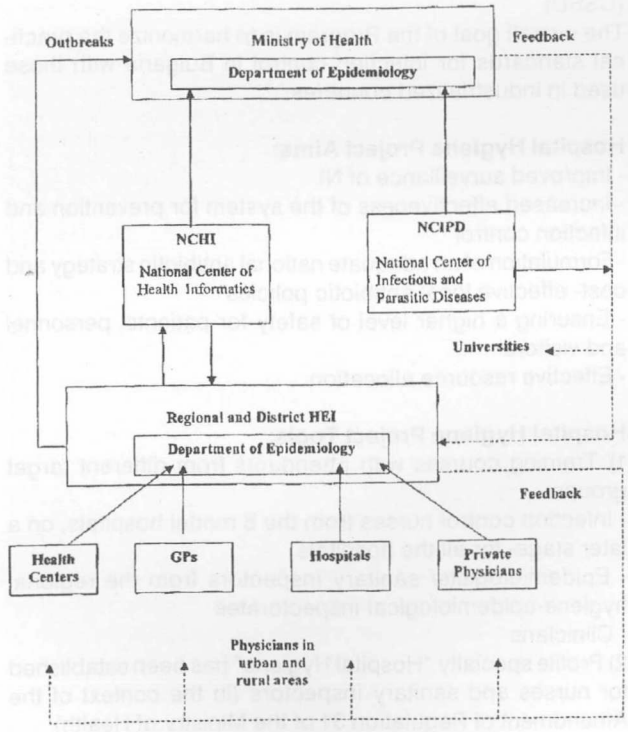

Fig. 2 Flow-chart
of Bulgarian Communicable Diseases Surveillance System

3) Standard for Nosocomial Infections:
- Working group convened by the MoH
- Main topics of the structure defined:
a) Case- definitions
b) Surveillance of NI
c) Universal precautions and isolation measures
d) Disinfection and waste disposal management
e) Sterilization (CSSD)
f) Antibiotic Policy
4) Association for the professionals in infection control was created (Bulgarian Association of Prevention and Infection Control "BulNoso") - inauguration symposium held in October 2003

Hospital Hygiene Project First Results:
- The pilot course for infection control nurses successfully completed in November 2003
- The working group for the National IC Standard has been established
- "BulNoso" joined the International Federation of Infection Control (IFIC)

EPIDEMIOLOGIC CONTROL OF METHICILLIN-RESISTANT STAPHYLOCOCCI IN SURGICAL WARDS

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SUMMARY

The general trend of increasing the relative frequency of staphylococci as an etiologic agent of nosocomial infections and surgical site infection (SSI) in particular, as confirmed by our and foreigh authors, is being established in MPHAT „St.Anna" - Varna as well - 56.40% of all gram-positive microorganisms isolated. Of importance for this is the increased rate of isolation of methicillin-resistant staphylococci (MRS) - 31.81% of all clinical materials tested for the 1996-2001 period, and in SSI - 21.20%. The present study examines the rate of determination of methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-resistant coagulase-negative staphylococci (MR-CNS) on the background of all methicillin-sensitive isolates, an attempt is made of clarifying the specificities of the epidemic process and the risk factors for MRS-associated SSI development. We examined likewise the resistance of the isolated MRS strains to antibiotics routinely used, and the characteristics of the multiply resistant MRSA and MR-CNS strains.

Key words: methicillin-resistant staphylococci, surgical wards

Following the isolation of the first methicillin-resistant Staphylococcus aureus (MRSA) strains in 1961, very soon these strains gained wide distribution, at present ranking among the leading causative agents of nosocomial infections (NI) (4,5). The rate of MRSA isolation demonstrates considerable variations in the different countries, and the different hospitals of one and the same country as well (6,7). The MRSA problem in Bulgaria might be considered in its initial stage of organization and control, discussed in single publications for the country and in separate hospitals, predominantly universiy ones.

An AIM of the present study was the rate of isolation to be determined of methicillin-resistant staphylococci (MRS) from clinical materials and patients, having developed surgical site infection (SSI), and some of the factors to be traced, associated with the epidemic process in the high-risk surgical wards of MPHAT "St.Anna" - Varna AD for the 1996-2001 period.

ACCEPTED FOR PUBLICATION: 20.12.2003

ABBREVIATIONS USED IN THIS PAPER:

NI-nosocomial infections; SSI-surgical site infection;
SWI-equivalent to surgical wound infection;
MRS-methicillin-resistant staphylococci ;
MRSA-methicillin-resistant Staphylococcus aureus;
MR-CNS-methicillin-resistant coagulase-negative staphylococci

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MATERIALS AND METHODS

MRS diagnostics in the hospital was initiated in 1996. An object of the study were 823 clinical materials from patients, having developed nosocomial infection, predominantly SSI, with gram-positive microflora isolated as causative agent, within the period of 1996-2001. Staphylococcus aureus was isolated in 453 clinical materials - 55.04%, and coagulase-negative staphylococci (CNS) - in 115 - 13.97%. MRS were confirmed in 118 patients from seven surgical wards with different surgical diagnosis and of large age range - from child's age to the age of more than 60. The infectious process manifested itself in different clinical forms of infection or carrier-state (colonization). In the patients having developed surgical wound infection we observed the course of regenerative process and disease outcome. For the purpose of tracing the reservoirs of staphylococcal carrier-state, besides patients, the whole personnel of the intensive care unit (ICU), accepting the main part of high-risk surgical patients, has been tested as well. In 11 persons among all, transitory nasal S. aureus carriage has been identified.

For microorganisms' identification and determination of antimicrobial susceptibility, routine methodics and the automized system Mini Api (Bio Merieux) have been used.

For MRS determination there have been applied in parallel: Bauer-Kirby disc diffusion method (NCCLS M100A of 2002); Oxacillin-screening agar method with a Becton-Dickinson medium according to NCCLS recommendation for MRS identification; the strip (panel) for staphylococci antimicrobial susceptibility testing with an Oxacillin test included in the automized system Mini Api (Bio Merieux).

RESULTS

The epidemiologic study conducted by us is in support of the global trend for an increase in nosocomial staphylococcal infections and staphylococci as etiologic agent of SSI inclusive (2,4,5,8). MRS demonstrate variations in the rate of isolation during the years, nevertheless retain an average level of 31.81% of all clinical materials tested. Among the MRS isolated, methicillin-resistant Staphylococcus aureus (MRSA) predominates - 36.21%, followed by methicillin-resistant coagulase-negative staphylococci (MR-CNS) - 16.05% of the whole sensitive population tested - Fig.2 (1,11,12).

MRS determination in wound secretions in case of a developed surgical wound infection demonstrates insignificant variations, at an average relative frequency for the period of 21.20% - Fig.3,4 (1,2,3,8).

The epidemiologic survey and control included seven surgical wards with a surgical wound infection registered and MRS isolated - Table 1. Most affected proved to be Orthopedics - 47.17%, Vascular Surgery - 18.87% and General Surgery (First Surgical Ward) - 13.21%. The development of MRS-associated SSI varies within large borders: to the 3rd day of operation - 22.64%, from the 5th to the 10th of operation - 24.53% and after the 10th of operation (to 60 days following the operation) - 52.83% - Fig.5. The age distribution is presented in Fig.6. The cleanness of the surgical wound (according to wound class) as an essential risk factor for infection development is compared to MRS rate of isolation - Table 2. Dominating is found to be the relation with the dirty and contaminated wounds.

Table 1. Wards with MRS related SSI registered

Wards	MRS related	SSIPercentage
Pediatric surgery	3	5,66
Vascular surgery	10	18,87
Orthopedy	25	47,17
GICU	2	3,77
First surgical ward	7	13,21
Second surgical ward	1	1,89
Neurosurgery	5	9,43
Total	53	100

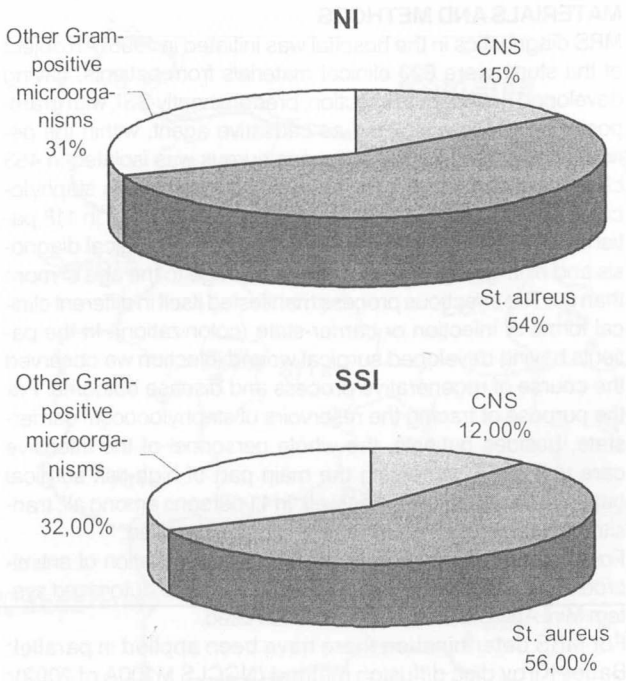

Fig. 1. Relative frequency of staphylococci in NI and SSI etiology for the period 1996-2001

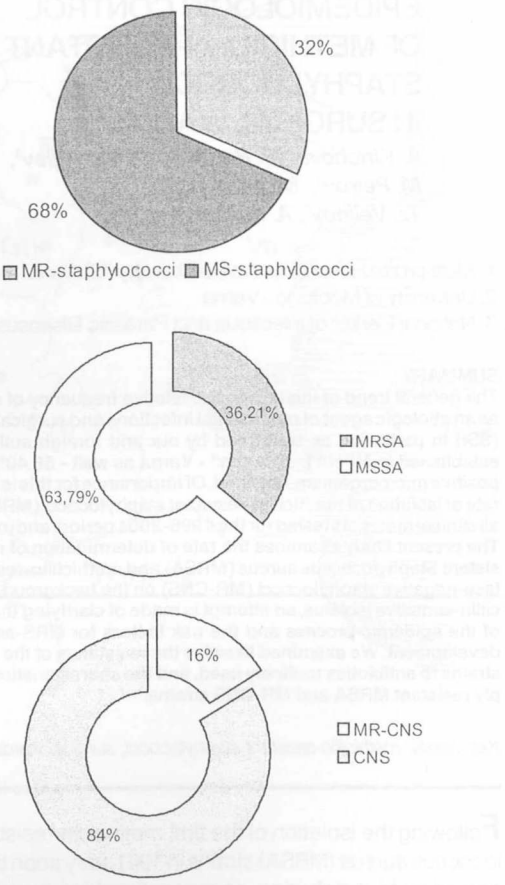

Fig. 2. Rate of isolation of methicillin resistant staphylococci from clinical materials for the period 1996-2001

Among patients having developed a clinically manifested MRS-associated infection (75 as a total), of greatest relative frequency are those with SSI - 70.67%, while those with systemic infection (MRSA isolated from a hemoculture) account for 5.3% - Fig.7. There were two fatal cases among patients having developed MRS-associated SSI, both with an underlying disease of combined trauma, septicemia; and in two patients with a vascular prosthesis, limb amputation proved to be indispensable. The treatment applied for MRS-associated SSI comprised combinations of aminoglycosides and first-generation cephalosporins; second- and third-generation cephalosporins and Azlocillin, Tubocin.
Only in the four patients having developed systemic infection Vancomycin has been used.
MRS carrier state has been confirmed in 36.44% of the traced for the period patients, the nasopharynx being the principal localization - Fig.8. Among the personnel of the intensive-care unit, which a great part of high-risk surgical patients pass through, a transitory S.aureus carrier-state was identified in 11.57%.
The study of MRS strains resistance to antimicrobials revealed higher level of resistance to Erythromycin - 52.83% and a moderate resistance to Tetracycline - 35.85%; Lincomycin -33.96%; Gentamicin and Ciprofloxacin - 28.30%. The susceptibility to second- and third-generation cephalosporins, carbapenems and Piperacillin/Tazobactam is retained - the level of resistance being 1.89%. Multiply resistant strains' characteristics is presented in Table 3.

DISCUSSION

In the trend exhibiting an increase of the etiologic role of staphylococci in nosocomial infections ana SSI in particular, methicillin-resistant staphylococci (MRSA and MR-CNS) participate as well - 31.81% of the registered NI infections for the 1996-2001 period in MPHAT "St.Anna" - Varna. For the period observed, an average of 20% of staphylococcal isolates in the country have been resistant to methicillin (2). The relative frequency for the country indicated is much higher presumably, although not confirmed, due to inaccurate MRSA diagnostics and underreporting (1-4). In comparance, on a conditional basis, of MRSA rate of isolation in Bulgaria with that for the rest European countries (Pan-European Study, 1993), our country ranks in the group of the South-European countries with a considerably higher MRSA rate (Spain - 30.3%, France - 33.6%, Italy - 34.4%, Greece - more than 30%) compared to the North-European countries (2,5). Our data surmount the average ones for the country, which is reason for introducing of a stringent control and an anti-epidemic regime in high-risk wards.
On the background of methicillin-sensitive staphylococci isolated in surgical wound infection for the period of observation, we establish almost equal participation of MRSA - 20.87% of all staphylococcal strains tested, and MR-CNS respectively - 22.73%, by reason of which both are estimated as serious pathogens, the epidemic process of which in hospital conditions needs additional research.
For the purpose of clarifying the risk factors in MRS-associated SSI, we traced its rate in seven surgical wards of different profile.

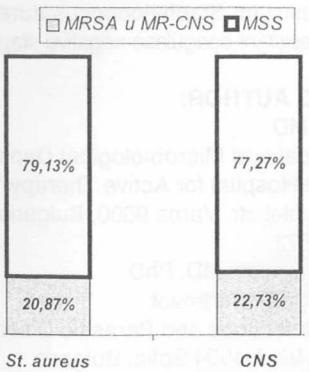

Fig. 3. Relative frequency of MRSA and MR-CNS among SSI staphylococcal isolates for the period 1996-2001

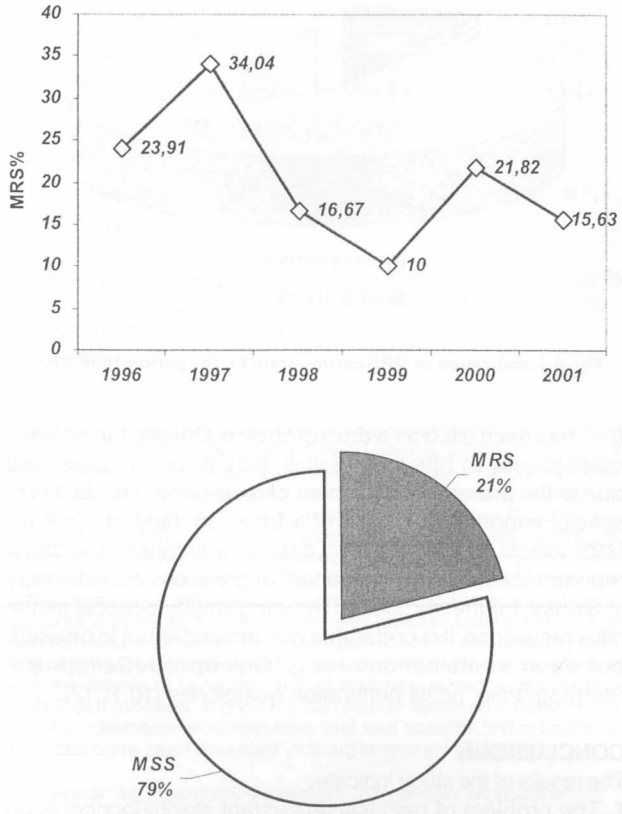

Fig. 4. Relative frequency of MRS isolated in SSI for the period 1996-2001

The highest rate has been the one registered in the Clinic of Orthopedics - 47.17%; Vascular surgery - 18.87% and the First Surgical Ward (General surgery), where a higher SWI "base" rate is observed as well. The rate of MRS isolation to the 3rd day of operation is 22.64% and from the 5th to the 10th day of operation - 24.53%.
Nasal carrier-state is specified by a number of authors as a major reservoir for MRS dissemination in hospital environment (1,3,8,9). The high percentage of MRSA nasopharyngeal carries identified by us in the group of patients traced - 90.70%, as well as the non-negligible percentage of S.aureus transitory carriers among the intensive care unit personnel - 11.57%, confirms the importance of this risk factor in unlocking the epidemic process. The lack of systemic screening in high-risk wards increases still further the epidemic risk. The almost equal relative frequency demonstrated of patients' contamination in the operating room yet, and after that in the ward, confirms the two major mechanisms of infection transmission - autoinfection of the patient-carrier, and the contact route via a transitory contamination of the hands of the medical personnel in direct contact with infected patients, or indirect con-

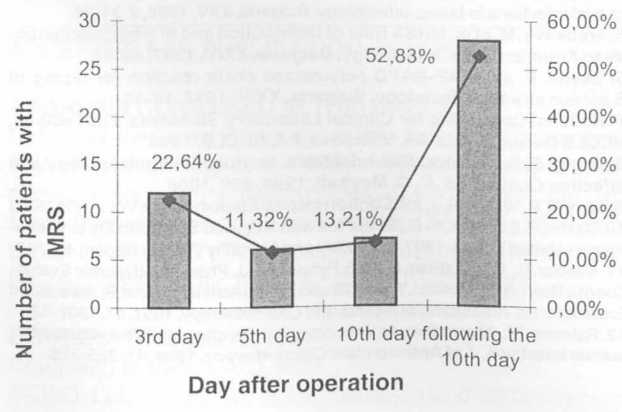

Fig. 5. Time of MRS isolation after operation

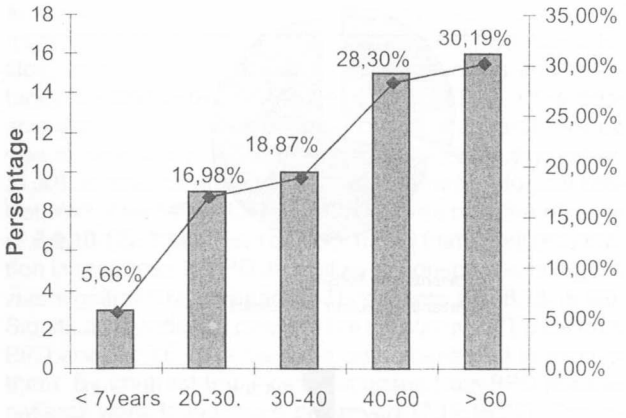

Fig. 6. Age distribution of MRS strains isolated in SSI

tact in the process of using different devices for patient care. The asepsis and antisepsis of poor quality in the surgical wards, and in particular in the presence of a reservoir of infection - patients having developed MRS-associated SSI, fosters the circulation and the maitenance of these highly epidemic strains and is a prerequisite for new cases of colonization and infection.
The later development of SSI associated with MRS isolation following the 10th to 60th day of operation is determined by synergic epidemiologically important factors: prolonged hospital stay, influence of the underlying disease, immunosuppresion, application of different antibiotic combinations, breach in the defensive barriers as a result of medical procedures undertaken (1,5,8,9). MRS strains isolation in SSI increases proportionally to age, mostly after the age of 60 (30.19%). The cleanness of wound (according

Table 2. Relationship between MRS isolation rate and surgical wound class

Surgical wound class	Number of MRS strains	%
Clean	7	13,21
Clean-contaminated		
Contaminated	17	32,08
Dirty/infected	29	54,71
Total	53	100

to wound class) is also a definite risk factor increasing the level of infecting - 54.71% of MRS-associated SSI in case of dirty wounds, followed by the contaminated wounds - 32.08%.
Among the patients traced for the period 1996-2001, the most frequent clinically manifested infection is the wound infection - 70.67%. It terminates with unfavourable outcome only in 4 patients. The rest have been discharged in a good and satisfactory condition.
Since the beginning of observation, there has been a steady increase in MRS resistance to Lincomycin - from 13.2% to 33.9%, Gentamicin - from 9.6% to 28.3%, and Ciprofloxacin - from 2.9% to 28.3% (percentage of resistant strains (R)). A high-level susceptibility is retained to Amikacin (7.55% of resistant strains (R)), to Cefuroxime - 3.77% R, and to Ceftazidime the resistance has fallen from 35.3% to 0%. MRS strains isolated demonstrated most frequently a simultaneous resistance to 3 antimicrobials - 30.19%, followed by those resistant to 5-6 antibiotics - 16.98%.
The epidemiologic study, the analysis and the microbial monitoring of patients, personnel and surrounding environment in the ICU and surgical wards observed, give a ground for rendering account of the general elements, together with some specificities of the epidemic process in the MRS-associated postoperative wound infection. A source (reservoir) of infection are usually the carriers among patients and personnel, and the endogenous flora

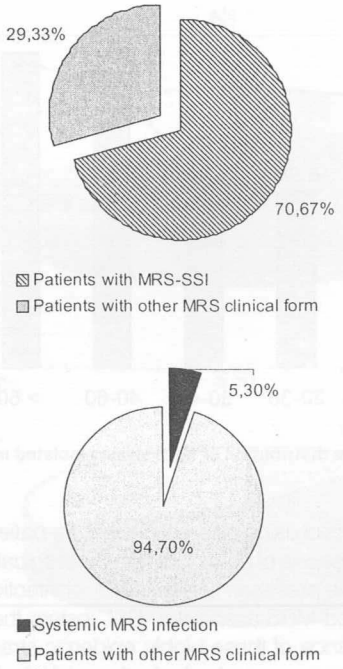

Fig. 7. Relative frequency of patients having developed MRS related SSI and systemic MRS infection

of the patient at the moment of the incident. A basic mechanism of infecting, confirmed in our studies as well, is the auto-infection from the endogenous flora of patients-carriers, and the contact route likewise, through contaminated hands of the personnel, devices and appliances for patients care (S.aureus demonstrated through laboratory control). The most characteristic specificity is the third link of the epidemic process - the highly susceptible hospital population, especially in case of prolonged hospital stay, the latter facilitating the contact with the hospital personnel and the MRS colonized patients. As major risk factors in this type of SSI may be defined: a former antibiotic treatment; presence of a MRS-infected or colonized surgical wound, or personnel and patients' colonization, not being screened routinely and often neglected.

Taking into account the specificities of the epidemic process, it is imperative criteria for surveillance of these infections to be defined and introduced into practice, in order an effective prophylaxis to be achieved.

In therapeutical aspect, the MRS-infected wounds (incisional and deep incisional) are excreting wounds, of slow healing process, with worsening of the general condition of the patient in the course. Our behaviour in case of a milder course of infection comprised an active local treatment with antiseptics (Mupirocin, Octenisept), an early surgical treatment of the wound including, and treatment and isolation of carriers. In protracted course of infection and indications for development of systemic infection, Vancomycin

Table 3. Multiresistance of MRS isolated in SSI

Number of antimicrobials	Number of MRSA strains	Number of MR-CNS strains	Total	%
3	14	2	16	30,19
4	5	1	6	11,32
5	7	2	9	16,98
6	8	1	9	16,98
7	3	1	4	7,55
8	3	2	5	9,43
9		1	1	1,89
10	1		1	1,89
11				
12	1		1	1,89
14	1		1	1,89
	43	10	53	100

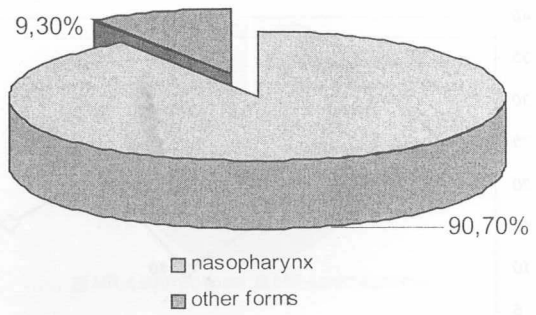

Fig. 8. Localization of MRS carrier-state for the period 1996-2001

(i.v.) has been used as a drug of choice. Despite the retained susceptibility to other antibiotics, they have not been used due to the prompt development of resistance. This fact is of special importance in view of a future therapy of systemic MRS infection. According to data of a number of authors, reported at the 20th International Congress on Chemotherapy in Sidney, for the purpose of avoiding staphylococcal methicillin-resistance, it is preferable glycopeptides not to be used, but other combinations - e.g. Quinuprisin/Dalfoprisin, Streptogramin, novel generation quinolones (10,11,12).

CONCLUSIONS

The results of the study indicate:

1. The problem of methicillin-resistant staphylococci is an actual one for the country, and in particular for the high-risk group - surgical patients.
2. The high-risk contingents, i.e. patients with SSI and stay in ICU, should be routinely screened for MRS.
3. The MRS-positive patients should be isolated and attended by specially instructed medical staff.
4. For the purpose of eradication of reservoirs, a mandatory measure is the treatment of nasal carrier-state among patients and personnel.
5. In case of MRS-associated SSI development:

in case of mild course of infection a basic curative measure is the local surgical treament of the wound and a treatment with appropriate antiseptic; in case of protracted course of infection and indications for development of systemic infection, Vancomycin is applied parenterally as a drug of choice. The usage of other antibiotics is not recommended because of the prompt development of resistance to them.

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EVALUATION OF CELL-MEDIATED IMMUNE FUNCTION IN THERAPY FREE PATIENTS WITH M. TUBERCULOSIS INFECTION

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SUMMARY

Protective immunity against Mycobacterium tuberculosis is T-cell mediated and requires strong T-cell lymphoproliferative activity and IFN-gamma production. The clinical outcome following TB infection is largely determined by the host immune response. To correlate the degree of impairment of the immune function two groups of patients were set up according their disease stage. Patients with advanced disease demonstrated highly suppressed T-cell responses measured in vitro by unstimulated and stimulated IFN-gamma production and T - lymphocyte proliferation, whereas patients with mildly expressed limited disease showed more preserved immune function. The neutrophil function measured by NBT-test of patients with advanced disease have been found suppressed but the degree of impairment was less expressed in comparison with T-cell function. A positive correlation between cutaneuos induration diameter after Mantoux test and specific IFN-gamma responses have been detected in bought groups of patients.

Key words: M. tuberculosis infection, IFN-gamma production, lymphoproliferative responses, PHA, PPD M. tuberculosis, TST-(Mantoux), NBT-test, QuantiFERON - TB

INTRODUCTION

It is estimated that one third of human population is infected with *Mycobacterium tuberculosis*. Most of infected people (90%) remain clinically healthy and are able to induce life - long protective immune response despite persistence of the pathogen (1). The quality of host defense mechanisms determines the outcome of infection and different disease manifestations. Protective immunity against *M. tuberculosis* requires T-lymphocyte mediated immune responses and strong interferon gamma (IFN-gamma) activity (3). IFN-gamma is the most important cytokine for activation of bactericidal mechanisms of macrophages, granuloma - formation and limitation the spread of the Mycobacterial infection (2,3,16). The main sources of this cytokine are CD4+, CD8+, gamma-delta T-cells and some other unconventional T-cells (3). M. tuberculosis has developed many mechanisms to impair immune function and to survive, spread and cause active disease. This pathogen can inhibit dendritic cell maturation and the ability of this antigen presenting cells to secrete pro - inflammatory cytokines and therefore indirectly suppress the IFN-gamma production by T lymphocytes (3,4).

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ABBREVIATIONS USED IN THIS PAPER:

CMI - cell-mediated immunity; IFN-(- Interferon gamma; PHA - Phytohemagglutinin; PBMCs - peripheral blood mononuclear cells; SI - Stimulation index; SLP1h - short-term (1h) spontaneous lymphocyte proliferation; PPD - purified protein derivative; TST - Tuberculin skin testing; DTH - delayed type hypersensitivity; NBT - Nitroblue Tetrazolium.

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M. tuberculosis limits IFN-gamma induced activation of macrophages by inhibition of phagosome - lysosome fusion, inhibition of acidification of phagosomes and resistance to killing by oxygenated metabolites (3,29). Unresponsiveness to IFN-gamma observed in some patients may be due to deficiency in the IFN-gamma receptor expression (5,30). In patients with Tuberculosis the immunological control over infection is lost and they develop active disease (7,8,9,10,12). It has been demonstrated that T-cell proliferation in response to PPD as well as to nonspecific mitogens was significantly impaired in TB patients (6,7,8,12,15,26). Significantly reduced proliferative responses of T cells from PPD anergic TB patients have been measured in most of them. By contrast the T-cell responses from PPD positive patients were found more preserved (7,12,15,27). The inability of cells to proliferate after specific antigen stimulation is associated with the higher expression of activation markers such as CD69, CD25, HLA-DR and increase of T-cell apoptosis (8,9). Cytokines play an important role in determining the strength and nature of antigen driven immune responses (3,8,10,11,29). Studies have shown that progression of TB infection is associated with a shift from a protective type 1 (IL-2, IFN- gamma) to a type 2 (IL-10) cytokine profile (10,11,). Suppressive T cells secriting IL-10 have been observed in tuberculosis patients with anergic immune reaction (10). The Th 2 type immune response is thought to have immunosupresive antiproliferative effect (12).

To examine cell-mediated immune function in therapy free TB patients with limited and advanced disease we studied lymphocyte function by the *in vitro* spontaneous and stimulated with Phytohemagglutinin (PHA) and PPD *M. tuberculosis* lymphocyte proliferation and IFN-gamma production. *In vivo* DTH responses were measured by the tuberculin skin test. Further studies concerning the restoration of immune function upon effective anti-TB therapy are in progress.

MATHERIALS AND METHODS

Patients

30 individuals with Tuberculosis infection have been enrolled in the study at the Regional dispensary for the prevention and treatment of tuberculosis and the University hospital for lung tuberculosis "St. Sofia" in Sofia (table 1). Two groups of patients were set up according to disease stage. The patients from the first group (n=15) had been diagnosed for pulmonary tuberculosis and were sputum smear and culture TB - positive. The patients from second group (n=15) were sputum smear TB - negative but with clinical and radiographic features consistent with tuberculosis. All patients did not receive therapy at the time of examination. 30 adult healthy volunteers were included in the control group. All subjects were BCG vaccinated. All study subjects participated voluntarily and gave informed consent.

Tuberculin skin testing (TST)

TST was performed to all patients according to the standard procedure. All subjects were skin tested with 0.1ml of 5TU of PPD M. tuberculosis (NCIPD) placed intradermally (Mantoux technique). Readings for response to Tuberculin are taken 72 hours after injection by measuring the diameter of the infiltrate. Positive reactions are those measuring

Table 1. Characteristics of TB patients and healthy controls

Groups of patients	Group I Sputum-smear positive TB patients	Group II Sputum-smear negative patients suspicious for TB	Healthy controls
Total	15	15	30
Sex M/F	11/4	7/8	19/11
Age, yr: mean (range)	43 (20-78)	39 (17-77)	36 (28-50)

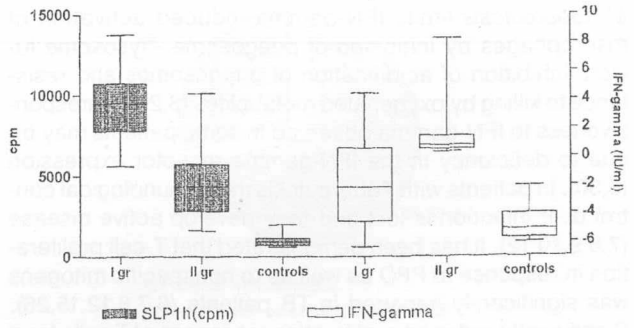

Fig 1. Spontaneous T-cell lymphoproliferation and IFN-gamma production in therapy free TB patients I gr - sputum smear positive TB patients with advanced disease (n=15); II gr - sputum smear negative TB patients with limited disease (n=15); healthy controls (n=30). Median values with range are presented for each group.

5mm or more in diameter. A diameter of over 15mm was considered hyperergic and below 5mm - anergic.

Sample collection and isolation of peripheral blood mononuclear cells (PBMCs).

Whole blood was collected by heparinised vacutainers (B-D). PBMCs isolated by density gradient centrifugation were resuspended at 10^6 cells/ml in RPMI medium (Sigma), supplemented with 10% foetal bovine serum (Sigma), 2mM L-glutamine (Sigma) and 100 U/ml penicillin and 100 μg/μl streptomycin.

Conventional T-cell lymphoproliferation assay

Proliferative responses of isolated PBMCs were measured by incubating 10^5 cells/well in a total volume of 200 μl in 96-well, flat-bottom microtiter plates (Greiner, USA). Cells were cultured in triplicates with PHA μg5 (g-Sigma) and PPD *M. tuberculosis* (10μg-NCIPD) for 72 and 144 hours respectively. 18 hours before the end of cultivation cells were pulsed with 37 Bq 3H-thymidine (Amersham). After cell harvesting the amount of 3H-Thymidine incorporation was determined as counts per minute (cpm) in a beta-scintillation counter (Beckman). Results were expressed as stimulation index (SI). The spontaneous lymphocyte proliferation has been measured in triplicate in the absence of stimulators for one hour cultivation (SLP1h) and results were expressed as mean cpm.

QuantiFERON-CMI and QuantiFERON - TB assays for evaluation of PHA - and PPD - -stimulated production of IFN-gamma.

Concentrations of IFN- gamma were measured by the commercially available IFN-gamma release assays (QuantiFERON-CMI and QuantiFERON-TB, Cellestis) according to the manufacturer's instructions. Briefly: aliquots of fresh heparinized whole blood were dispensed into 24 well tissue culture plates and incubated overnight with PHA (5 μg/ml), PPD *M. tuberculosis* (10μg/ml). Plasma samples were harvested and the concentrations of IFN-gamma were measured in ELISA. ELISA was generally performed according to the manufacturers specifications. Briefly, 96 well plates pre-coated with an anti-IFN-gamma monoclonal antibody were purchased. Each well was filled with 50μl of anti-human IFN-gamma horseradish peroxidase conjugate and 50μl of the test specimen. The plate contents were thoroughly mixed and incubated for 1h at room temperature. The plates were washed for 6 cycles with 300ml wash buffer. A 100ml portion of substrate was added to each well. The admixture was allowed to develop for 30 min (room temperature), at which time 50ml of enzyme stopping solution (1 N H_2SO_4) was added to halt the reaction. Absorbance was measured at 450nm and 620nm using a plate reader. A standard curve was generated using Genesis V.3.05 software. The IFN-gamma values (in international units per milliliter) of the test samples were determined from the standard curve.

NBT (Nitroblue Tetrazolium) test.

The NBT test (unstimulated and stimulated) was performed as follows. For the unstimulated test in a vial of 200 μl heparinized blood were added 120μl NBT solution (Sigma); for the stimulated - to 50 μl heparinized blood 100mcl NBT μl (Sigma) solution and 5μl stimulant (SIGMA) were added. After incubation at 37° C for 10 min 2 smears were prepared and examined under a light microscope (x40). The number of formazan-positive cells per 100 neutrophils were count in each smear.

STATISTICAL ANALYSIS

Statistical analysis was done by the Mann-Whitney test and the Kruskal-Wallis test for determination of differences between subject groups. A Spearman correlation test was performed to analyze the association between antigen-specific lymphoproliferation and IFN-gamma production.

RESULTS

Levels of spontaneous short -term lymphocyte proliferation (SLP 1 h) and IFN-gamma production

In patients from both groups the levels of the short-term spontaneous lymphoproliferation and spontaneously released IFN-gamma were found significantly greater than in healthy controls. Patients with advanced disease included in the first group showed the highest values both of SLP1h and IFN-gamma. A strong correlation (r=0.80) between the spontaneous SLP1h and IFN- gamma production was found in all patients (fig.1).

Lymphoproliferative and IFN-gamma responses to the polyclonal mitogen Phytohaemagglutinin (PHA)

Lymphoproliferative responses to PHA have been found significantly lower in both groups of patients in comparison to healthy controls but in patients with advanced disease the inhibition of the PHA-stimulated lymphoproliferation was more pronounced (fig.2). The PHA - stimulated IFN-gamma production was impaired in all patients too and particularly in patients from the first group. A correlation between SI(PHA) and PHA- stimulated IFN- gamma production was found in all subjects tested (fig. 2).

Lymphoproliferative and IFN-gamma responses to PPD *M. Tuberculosis*

The PPD - induced lymphoproliferation was significantly impaired in patients from the first group. In analogy with it the PPD - stimulated IFN- gamma production was impaired too in the same group of patients. In contrast to that the PPD - stimulated lymphoproliferation and IFN - gamma production was found increased in patients from the second group (fig.3).

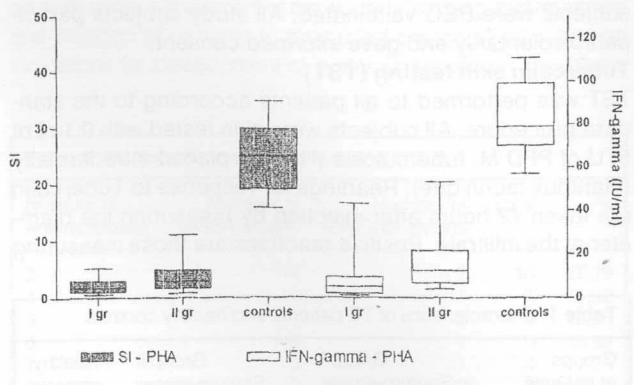

Fig 2. T-cell lymphoproliferative and IFN-gamma responses to the polyclonal mitogen Phitohemagglutinin (PHA) in therapy freeTB patients. I gr - sputum smear positive TB patients with advanced disease (n=15); II gr - sputum smear negative TB patients with limited disease (n=15); healthy controls (n=30). Median values with range are presented for each group.

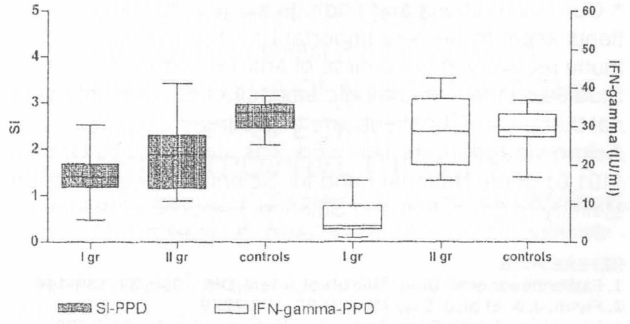

Fig 3. T-cell lymphoproliferative and IFN-gamma responses to PPD *M. tuberculosis* in therapy freeTB patients. I gr - sputum smear TB patients with advanced disease (n=15); II gr - sputum smear negative TB patients with limited disease (n=15); healthy controls (n=30). Median values with range are presented for each group.

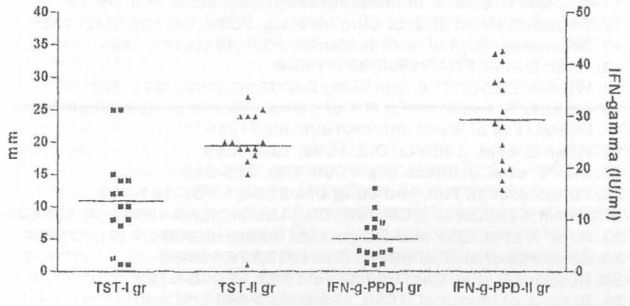

Fig 4. Correlation between the size of cutaneous induration and PPD - stimulated IFN-gamma production in therapy freeTB patients. I gr - sputum smear positive TB patients with advanced disease (n=15); II gr - sputum smear negative TB patients with limited disease (n=15); healthy controls (n=30). Individual values with medians are presented for each group.

Tuberculin skin testing (TST-Mantoux test) for determination of *in vivo* DTH responses

The results obtained by the Mantoux test demonstrate different tuberculin reactivity in patients from the first and second group. In the group of patients with advanced disease three of them were anergic in response to PPD Tuberculin, two were hyperergic and the remaining 10 showed positive responses with a size of induration between 5 to 15 mm. All patients but one (who was found anergic) from the second group, with limited disease were hyperergic in response to PPD Tuberculin with size of induration over 15mm (fig.4). An agreement between the TST response and the PPD - stimulated lymphoproliferation and IFN-gamma production was found in patients from both groups (fig 5).

Functional activity of polymorphonuclear phagocytes

The results from the non-stimulated and stimulated NBT test showed an impaired neutrophil function. It was greater in patients with advanced than in patients with limited disease.

DISCUSSION

In the present study we examined the cell-mediated immune function in 30 therapy free patients with tuberculosis infection set up in two groups according to the stage of disease. 15 of them were sputum smear TB - positive with advanced disease and the rest of them were sputum smear TB - negative with mildly expressed, limited disease.

In agreement with the results of other investigators (7,9,11,15,18,20,21,22,23,26) our results demonstrate several important findings. We assessed high levels of chronic immune activation as measured by the in vitro spontaneous short-term lymphocyte proliferation and IFN-gamma production in all patients studied. This activation was more expressed in patients from the first group with advanced disease. The high level of spontaneous cellular activation was in inverse correlation with non-specific and specific T-cell

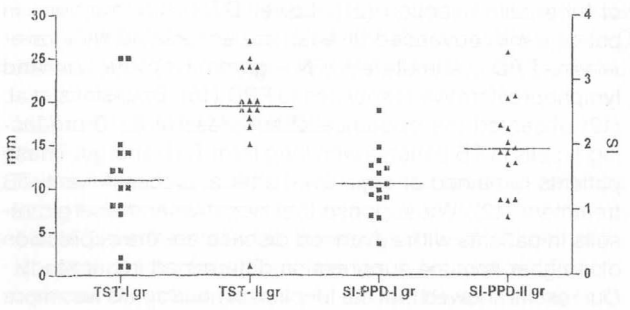

Fig 5. Correlation between the size of cutaneous induration and T-cell lymphoproliferative responses (SI-PPD) to PPD *M. tuberculosis* in in therapy freeTB patients. I gr - sputum smear positive TB patients with advanced disease (n=15); II gr - sputum smear negative TB patients with limited disease (n=15); healthy controls (n=30). Individual values and medians are presented for each group.

responses especially in patients with advanced disease. Our results demonstrate that PBMCs of patients from the first group proliferated poorly after stimulation with PHA and PPD *M. tuberculosis* and secreted low quantities of IFN-gamma. Similar are the findings of Delgado et al (7), Turner et al. (20), Rohit K. et al. (22), Al-Attiyah et al (26), Pathan et al (27), who reported increased spontaneous T-cell activation and suppressed T-cell responses to mitogens and specific antigens in anergic TB patients due to apoptosis and cytokine dysregulation. The reduction of IFN-gamma production and the low lymphoproliferative responses could be also explained by high levels of cytokines like IL-10 and TGF-beta as described by Hussain R. et al. (11), Boissiotis V. et al. (12), Hirsh C. et al. (14), to sequestration of antigen - specific IFN-gamma - secreting T-cells at the site of infection as established by Dieli F. et al. (10), Schwander S. et al. (13), or to unresponsiveness or anergy of IFN-gamma - secreting T-cells as reported by Delgado J (7). The higher bacterial burden in those patients could also be the reason for depressed T-cell immune responses and inability of infected individuals to generate effective control over infection (25).

In patients from the second group with limited disease *in vitro* lymphoproliferative and IFN-gamma responses to PHA were found depressed too but less impaired than in patients with advanced disease. At the same time both lymphoproliferative and IFN-gamma responses to PPD *M. tuberculosis* were preserved.

The results from the tuberculin skin testing showed that the number of anergic reactions was greater in patients with advanced disease. Patients with limited disease responded better to PPD and the size of induration in these patients was greater than in patients with advanced disease. There was a positive correlation between the *in vitro* IFN-gamma production and the diameter of induration in patients from both groups. These findings are consistent with previously reported ones by Delgado J. et al. (7), Dieli F. et al. (10), Wilsher M. et al. (15), Desem N. et al. (21), Boussiotis V. et al. (12), Rohit K. et al. (22), Converse P. et al. (23). Our results are in agreement with data of other investigators (7, 10, 12, 21) showing strongly depressed *in vitro* T-cell immune responses in TST anergic patients. Anergy in TB infection is associated with paradoxical absent of dermal reactivity to intradermal injection with tuberculin PPD in infected persons. It occurs in about 15% of TB patients. Some of the tuberculosis patients are TST negative or anergic despite sputum positive results and go on to develop TB disease (15). At the same time the ability to mount a delayed type hypersensitivity response (DTH) does not result in protective immunity. The DTH reaction, as measured by the tuberculin skin test, has been shown to depend on the production of cytokines, including IFN - gamma, at the site

of tuberculin injection (21). Lower DTH skin reactions in patients with advanced disease are associated with lower *in vitro* PPD - stimulated IFN - gamma production and lymphoproliferative responses to PPD (15). Boussiotis et al. (12) observed the existence of suppressive IL-10-producing T-cells in TB patients with long term TST-anergy. These patients remained anergic even after a successful anti-TB treatment (12). We suppose that negative and anergic results in patients with advanced disease are the expression of a higher immune suppression determined in our study.

Our results showed that the function of neutrophils was more disrupted in patients with advanced disease. The observed suppression of neutrophil function was not as high as the dysregulation of lymphocyte function assessed by us. We suppose that the inhibited function of these cells could be due also to high levels of apoptosis during the phagocytosis of mycobacteria by neutrophils as it was reported recently by Perskvist et al. (28).

In summary, our results stimulated us to make the following conclusions:

* Cell - mediated immune function as measured by T - cell proliferative and IFN-gamma non-specific and specific responses was found inhibited in all TB patients enrolled in this study;
* The *in vivo* tuberculin skin reactions strongly correlated with the *in vitro* specific lymphocyte proliferation and IFN-gamma production;
* The function of neutrophils was found inhibited, particularly in patients with advanced disease;
* A correlation between the clinical manifestation of the disease and the degree of impairment of cell-mediated immune function was found in all patients;
* Our observations and findings made in therapy free patients seem to be very important for the evaluation of immune recovery in the course of anti-TB treatment.

Studies on the immunologic efficacy of specific antibacterial therapy in TB patients are in progress.

Acknowledgements This work was supported by Grant L-1101/01 of the National Fund for Scientific Research of the Ministry of Education and Science, Republic of Bulgaria.

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ASSESSMENT OF CELL - MEDIATED IMMUNITY IN HIV-1 INFECTED PATIENTS BEFORE THE ONSET OF SPECIFIC ANTIRETROVIRAL THERAPY

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SUMMARY

The hallmark of HIV-1 infection is the progressive loss of CD4+T cells together with functional impairment of the remaining lymphocyte populations: chronic immune activation, failure in T-cell proliferative responses to recall, specific antigens and mitogens as well as a critical Th1 - Th2 switch in cytokine profile. The aim of this study was to analyze T-cell function in therapy-naive chronically HIV-1-infected patients before inclusion in HAART. 29 individuals were set up in two groups following CD4+T-cell count. Each group consisted of two subgroups according to lymphoproliferative responses to PHA: group A with SI(PHA)< 3.0 and group B with SI(PHA)>3.0. The degree of chronic immune activation was measured by the short-term (1 hour) spontaneous lymphocyte proliferation and the non-stimulated IFN-gamma production. Functional activity of CD4+ T cells was assessed by lymphoproliferative and IFN-gamma responses to PHA, PPD *M. tuberculosis* and the specific HIV-1 p24 antigen. An increased spontaneous T-cell activation was found in all patients and the highest values were measured in those with lowest CD4+T cell count and SI(PHA)<3.0. An impaired T - cell function was determined in all individuals, which correlated inversely with the spontaneous lymphocyte activation, as a marker of chronic immune activation.

Key words: HIV-1 infection, lymphoproliferative responses, IFN-gamma production, CD4+ T cells, PHA, PPD M. tuberculosis, HIV-1 p 24 antigen, HAART.

INTRODUCTION

Although the hallmark of progressive HIV-1 infection is the decline of the number of CD4+T helper cells, quantitative impairment of CD4+ T lymphocyte function occurs very early in the course of infection (30, 3, 2, 27). HIV-1 antigen - specific responses are among the first proliferative responses that are lost during progressive infection, followed by the loss of responsiveness to recall antigens and polyclonal mitogens (29).

Cytokines play an important role in determining the strength and nature of antigen - driven immune responses and can affect the expression of HIV-1 (35). Studies have shown that HIV-1 disease progression is associated with a shift from a dominant type 1 (IL-2 and IFN-gamma) to a type 2 (IL-4 and IL-10) cytokine environment (8) and it is thought that it has an immunosuppressive anti-proliferative effect (28). Th1 responses enhance mainly cell-mediated immunity and in long-term non - progressors HIV-1 specific CD4+ T cell proliferative responses are associated with type 1 cytokine production (30, 35, 16, 31).

In the present paper we evaluateded T cell function in therapy-naive chronically HIV-1 - infected patients before the start of specific antiretroviral therapy. Data from the analysis of lymphoproliferative and IFN-gamma responses to the polyclonal mitogen Phytohemagglutinin (PHA), the classic recall antigen PPD *M. tuberculosis* and to the specific HIV-1 p24 antigen are presented.

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ABBREVIATIONS USED IN THIS PAPER:

SLP1h-short-term (1h) spontaneous lymphocyte proliferation; SI-Stimulation index; IFN-gamma - Interferon gamma; PHA-Phytohemagglutinin; PBMCs-peripheral blood mononuclear cells; PPD - purified protein derivative; HAART - highly active antiretroviral therapy.

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MATERIALS AND METHODS

Patients

Twenty nine chronically HIV - 1 - infected therapy - naive patients before inclusion in highly active antiretroviral therapy (HAART) were enrolled in the study at the ADIS unit of the Hospital of Infectious Diseases "Prof. Ivan Kirov". Patients were set up in four groups (IA, IB, IIA, IIB) according to the absolute of CD4+ T cell count and the lymphoproliferative response to PHA. 13 patients with CD4+ count < 200 cells/mm^3 were included in the first group. This group was divided into two groups as follows: IA - 8 patients with $SI_{(PHA)}$ < 3 (7 males and 1 female, median age 34,2 years) and IB - 5 patients with $SI_{(PHA)}$ > 3 (4 males and 1 female, median age 37 years). In the second group 16 patients with CD4+ count > 200 cells/mm3 were included and further set up in two groups: IIA - 8 with $SI_{(PHA)}$ < 3 (6 males and 2 females, median age 37,8 years) and IIB - 8 patients with $SI_{(PHA)}$ > 3 (3 males and 5 females, median age 31.4 years) (Table 1). 30 adult healthy volunteers were included in the control group. All study subjects participated voluntarily and gave informed consent.

Isolation of PBMCs

Blood samples were collected in heparinized vacutainer tubes (B-D). Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Histopaque (Sigma Diagnostics, ICN). Cells were resuspended in RPMI 1640 medium (Sigma), supplemented with 10% foetal bovine serum (Sigma), 2mM L- glutamine (Sigma) and 100 U/ml penicillin and 100 μg/ml streptomycin.

Lymphocyte proliferation assay

Proliferative responses of isolated PBMCs were determined by incubating 10^5 cells/well in a total volume of 200 μl using 96-well, flat-bottom microtiter plates (Greiner) at 37°C in a humidified 5% CO^2 atmosphere. Cells were cultured in triplicates with PHA (5 μg/ml - Sigma), PPD *M. tuberculosis* (10 μg/ml - NCIPD) and HIV-1 p24 Ag (0.25 μg/ml - Protein Science) for 72 and 144 hours 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 of radioactivity was determined as counts per minute (cpm) in a beta-scintillation counter (Beckman). Results were expressed as stimulation index (SI), calculated by dividing the mean cpm of 3 replicate-stimulated wells by the mean cpm of the non-stimulated ones. The spontaneous non-stimulated short-term lymphocyte proliferation (SLP1h) has been measured in triplicate in the absence of stimulators for one hour and results were expressed as mean cpm.

Table 1. Characteristics of HIV-1 infected patients before the start of spesific antiretroviral therapy

Groups of patients	No of patients	CD4+count (cells/mm^3) (median with range)	SI to PHA (median with range)
IA group $SI_{(PHA)}$<3.0 CD4+count<200 cells/mm^3	8	54 (2 - 146)	1.41 (0.47 - 2.02)
IB group $SI_{(PHA)}$>3.0 CD4+count<200 cells/mm^3	5	20 (3 - 62)	3.98 (3.30 - 5.54)
IIA group $SI_{(PHA)}$<3.0 CD4+count>200 cells/mm^3	8	349 (212 - 530)	1.49 (0.67 - 2.64)
IIA group $SI_{(PHA)}$>3.0 CD4+count>200 cells/mm^3	8	334 (202 - 840)	5.15 (3.40 - 9.65)

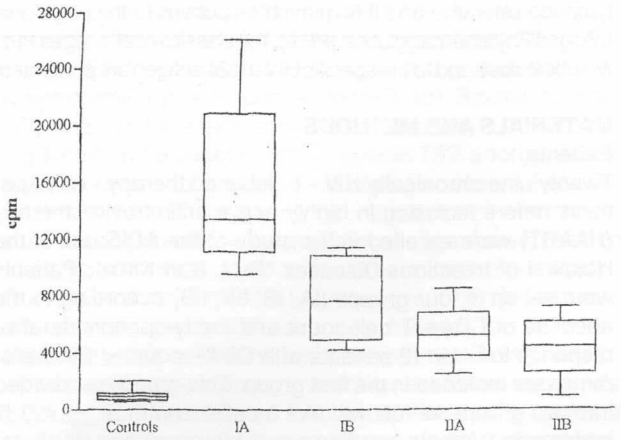

Fig. 1. Levels of the short-term lymphoproliferation (SLP 1h) in HIV-1infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

QuantiFERON - CMI assay for evaluation of PHA-, PPD- and HIV-1 p24 Ag stimulated production of IFN- gamma

Concentrations of IFN-gamma were measured by the commercially available IFN-gamma release assay QuantiFERON-CMI, Cellestis. Briefly: fresh heparinized whole blood was dispensed into a 24 well tissue culture plate and incubated overnight with PHA (5 μg/ml - Sigma), PPD *M. tuberculosis* and HIV-1 p24 antigen (0.25 μg/ml). Plasma samples were harvested and the concentrations of IFN-gamma were measured in ELISA. Results were expressed as IU/ml.

ELISA was generally performed according to the manufacturers instructions. Briefly, 96-well plates, precoated with an anti-IFN-gamma monoclonal antibody, were purchased. Each well was filled with 50 μl of anti-human IFN-gamma horseradish peroxidase conjugate and 50 μl of the test specimen. The plates were incubated for 1h at room temperature and then were washed for 6 cycles with 300 μl wash buffer. A 100 μl portion of substrate was added to each well. The admixture was allowed to develop for 30 min (room temperature), at which time 50 μl of enzyme stopping solution (1 N H_2SO_4) was added to halt the reaction. Absorbance was measured at 450 nm and 620 nm using an automated plate reader. A standard curve was generated using Genesis V3.05 software. The concentration of IFN-gamma (in international units per milliliter) in the test samples was determined from the standard curve.

Immunophenotyping

Absolute CD4+ T cell counts were determined by the CD3/CD4/CD8 TriTEST with TrueCOUNT tubes (B-D), using the FACSCalibur flow cytometer (B-D).

Statistical analysis

Statistically significant differences between values of immunologic parameters tested were analysed by Mann-Whitney non-parametric t test. Spearman rank order correlation test was applied to evaluate the significance of correlation.

RESULTS

Levels of short-term (one-hour) spontaneous lymphocyte proliferation (SLP1h)

To evaluate the degree of activation of the immune system in HIV-1 chronically infected subjects we have analysed levels of short-term spontaneous lymphoproliferation. Increased levels of SLP 1h were established in patients from all groups in comparison to the healthy control subjects. In patients with CD4+ count < 200 cell/mm³ we found the highest values of SLP 1h (groups IA and IB). In patients from the group with CD4+ count > 200 cell/mm³ the SLP1h values were also high but below the levels obtained from patients included in the first group. (Fig.1).

Levels of spontaneous IFN- gamma production

Increased levels of IFN-gamma production were determined in all patients. The highest values were measured in patients from the first group with CD4+ count < 200cell/mm³ and a SI to PHA below 3.0 (Fig. 2).

A correlation (r=0.7) between SLP 1h and the spontaneous IFN-gamma production was found in all patients.

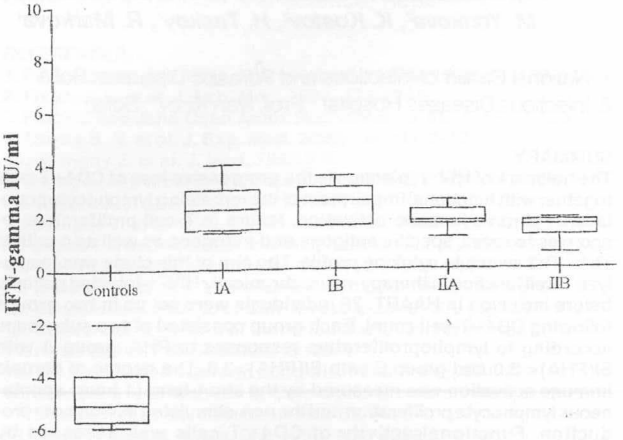

Fig. 2. Non-stimulated IFN-gamma production in HIV-1 infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

Lymphoproliferative responses to the polyclonal mitogen Phytohemagglutinin (PHA)

T helper cell function as measured by lymphoproliferative responses to PHA was substantially impaired in all subjects studied but in different degree. When comparing the four groups of patients we found that the PHA lymphoproliferative response was most inhibited in patients with absolute CD4+ T cell count < 200 cells/mm³ where the $SI_{(PHA)}$ was below 3.0 (IA group). In IB group including the rest of patients with CD4+ T cell count < 200 cells/mm³ the PHA response was more preserved and the $SI_{(PHA)}$ was found above 3.0 (Fig.3). Eight of the patients with CD4+ T cell count > 200 cells/mm³ (IIA group) showed depressed PHA responses and the SI to PHA was measured between 0.67 - 2.64. These results were similar to the results obtained in patients from group IA. The other 8 patients with CD4+ T cell count > 200 demonstrated better preserved responses to PHA and like in IB group the SI to PHA was found above 3.0 (Fig.3).

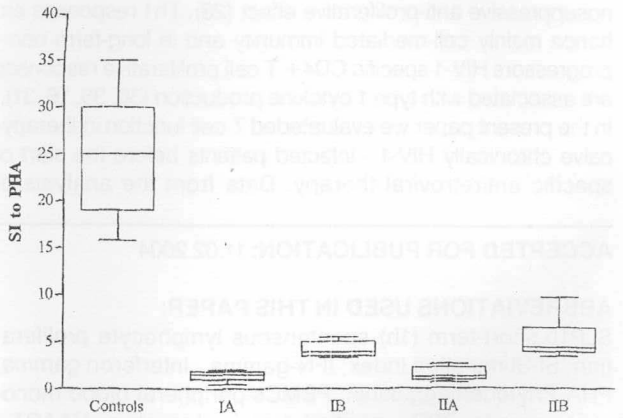

Fig. 3. Lymphoproliferative responses to the polyclonal mitogen Phytohemagglutinin in HIV-1infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

In all patients included in groups IB and IIA the correlation between lymphoproliferative responses to PHA and CD4+ T - cell counts was found weak (r=0.3).
A correlation (r=0.7) between the short-term spontaneous lymphocyte proliferation and PHA responses was found in all groups of patients and particularly in patients from the IIB group.

PHA - stimulated IFN-gamma responses

The PHA - stimulated IFN- gamma production was most significantly suppressed in patients from groups IA and IIA. IFN-gamma responses to PHA were better expressed in patients from groups IB and IIB where lymphoproliferative responses to PHA were found more preserved (Fig.4).

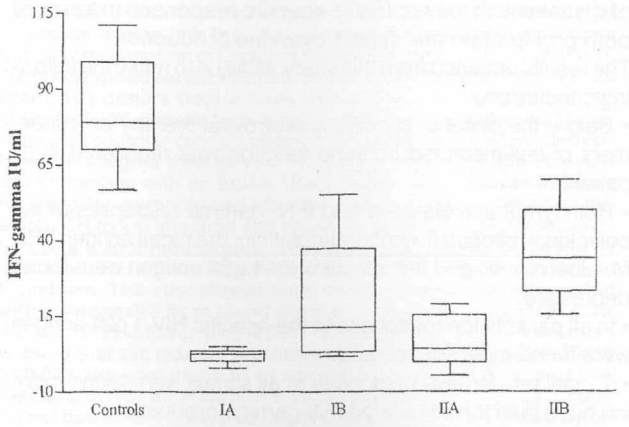

Fig. 4. Phytohemagglutinine stimulated IFN-gamma production in HIV-1infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+ count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

Lymphoproliferative responses to PPD *M. tuberculosis*

The impairment of lymphoproliferative responses to the recall antigen PPD *M. tuberculosis* was greater than that to the polyclonal mitogen PHA in all patients. The lowest values of SI to PPD *M. tuberculosis* were found in patients from groups IA and IIA and the highest ones in patients from groups IB and IIB (Fig.5).

PPD - stimulated IFN-gamma responses

The PPD-stimulated IFN-gamma production was suppressed predominantly in patients from groups IA and IIA who were with less preserved immune function as measured by lymphoproliferative responses to PHA (Fig.6). In all patients the PPD-stimulated IFN-gamma production was found lower than the PHA-stimulated one.

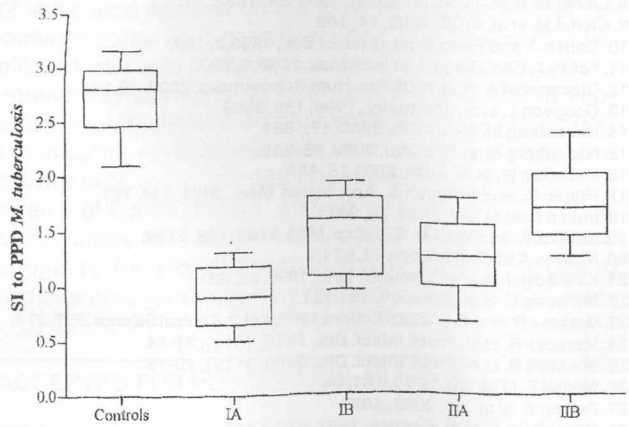

Fig. 5. Lymphoproliferative responses to the PPD *M. tuberculosis* in HIV-1infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+ count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

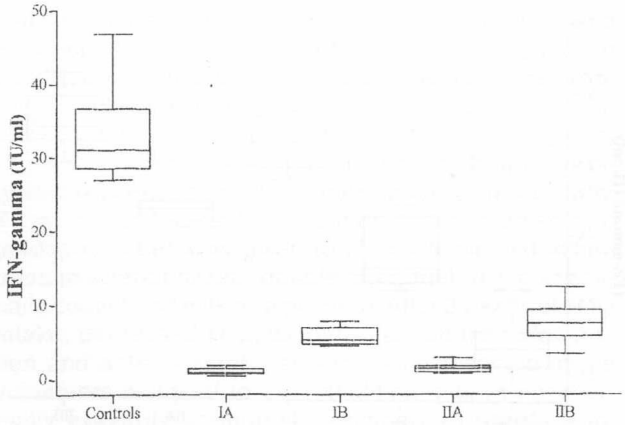

Fig. 6. PPD *M. tuberculosis* stimulated IFN-gamma production in HIV-1infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+ count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

Lymphoproliferative responses to the specific HIV-1 p24 antigen

In patients from the four groups lymphoproliferative responses to the specific HIV-1 p24 antigen were found very poor but the lowest SIs to p24 Ag were measured in groups IA and IIA (Fig.7).

HIV-1 p24 Ag - stimulated IFN-gamma responses

Like lymphoproliferative responses to the specific HIV-1 p24 Ag, the p24 - stimulated IFN-gamma production was strongly suppressed at the time of examination in all patients. The poorest IFN-gamma responses were measured again in patients from groups IA and IIA where the lowest lymphoproliferative response to PHA have been established (Fig.8).

DISCUSSION

The results describing cell-mediated immune function assessed by non-specific and specific lymphoproliferative and IFN-gamma responses in therapy-naive chronically HIV-1 - infected patients before their inclusion in specific antiretroviral therapy are presented. Twenty nine HIV - 1 positive patients set up in four groups according to the absolute CD4+ T cell count and the SI to PHA were included in this study.
Our results demonstrate increased levels of chronic immune activation as measured by the short - term lymphoproliferation and the spontaneous IFN - gamma production in all patients. The highest values of SLP1h as compared to healthy control

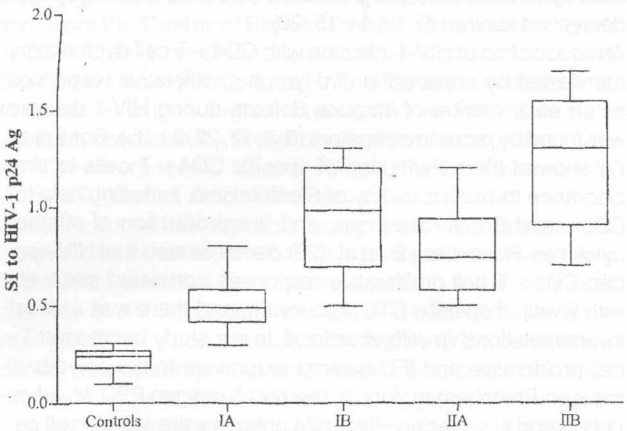

Fig. 7. Lymphoproliferative responses to the specific HIV-1 p24 antigen in HIV-1infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+ count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

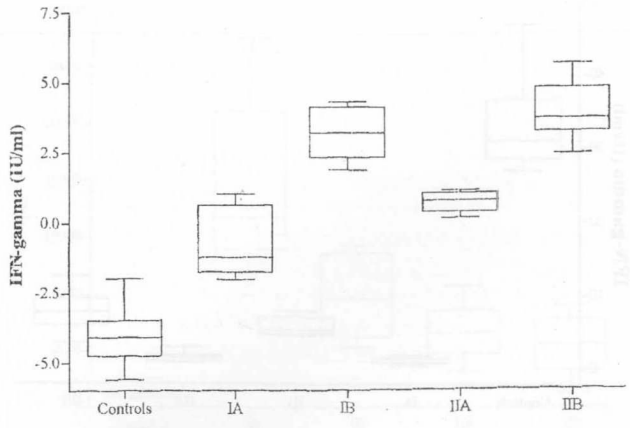

Fig. 8. HIV-1 p24 antigen stimulated IFN-gamma production in HIV-1infected patients before the start of therapy. Median values with range are presented for each group of patients: IA - CD4+count 200< cell/mm³ and SI (PHA) <3.0 (n=8); IB-CD4+count< 200 cell/mm³ and SI (PHA) >3.0 (n=5); IIA-CD4+count> 200 cell/mm³ and SI (PHA) 3.0< (n=8); IIB-CD4+count >200 cell/mm³ and SI (PHA) >3.0 (n=8);

subjects were found in the group of patients with CD4+ T cell count < 200 cells/mm³ and a SI to PHA < 3.0. In patients with CD4+ T cell count < 200 cells/mm³ and a SI to PHA > 3.0, SLP1h values were measured significantly lower. No significant differences in SLP1h values were found between the two groups of patients with CD4+ T cell count > 200 cells/mm³. However the lowest SLP1h values were found in patients with CD4+ T cell counts > 200 cells/mm³ and a SI to PHA above 3.0. The results obtained are in agreement with our previous results and the data of other investigators (4, 5, 11, 13, 33, 33, 32, 34). The state of chronic immune activation characterises HIV-1 infection and might be the primary mechanism responsible for premature cell death in AIDS and accounts for disease progression since the activation of host immune cells (primarily CD4 + T cells, macrophages and dendritic cells) facilitates multiple steps of the viral life cycle and cellular factors that are directly or indirectly connected with it (18). These include up-regulation of viral co-receptors (CCR5 and CXCR4), decreased beta-chemokine secretion (21), enhanced viral entry and integration as well as viral assembly and/or release (32). Immune activation also leads to profound changes in the cytokine network, with increased secretion of tumor necrosis factor-alpha (TNF-alpha), IL-4, IL-6 and IL-10, and affects the cell cycle (4, 10, 11). Immune activation is also associated with various degree of immune dysfunction, hyporesponsiveness and apoptosis, all leading to enhanced progression of immune deficiency and decreased survival (5, 12, 14, 15, 26).

An association of HIV-1 infection with CD4+ T cell dysfunction, manifested by impaired *in vitro* lymphoproliferative responses as an early marker of immune defects during HIV-1 disease was found by many investigators (3, 9, 17, 22, 34, 35). Boaz et al. (7) showed the contribution of specific CD4+ T cells to viral clearance through a variety of mechanisms, including help for CD8+ and B cell responses, and the production of effector cytokines. Rosenberg E. et al. (28) demonstrated that HIV-specific CD4+ T cell proliferative responses correlated positively with levels of specific CTL precursors, and there was a direct inverse relationship with virus load. In our study decreased T - cell proliferative and IFN-gamma responses to the polyclonal mitogen Phytohemagglutinin, the recall antigen PPD *M. tuberculosis* and the specific HIV-1 p24 antigen were found in all patients enrolled in this study. The lowest responses to PHA were measured in patients from the first group with CD4+ T cell count < 200 cells/mm³ where very small values (under 3.0) of SI to PHA were found. Similar were the results in some of the patients with CD4+ T cell count > 200 cells/mm³. Like PHA responses, the responses to PPD *M. tuberculosis* were significantly lower again in patients from both groups with a SI to PHA below 3.0. Our results demonstrate a weak correlation between the absolute number of CD4+ T cells and their capacity to proliferate and secrete IFN-gamma under stimulation with mitogen and recall antigen.

The poorest lymphoproliferative and IFN-gamma responses of T-cells were found after in vitro stimulation with the specific HIV-1 p24 antigen in patients from all groups. These responses were significantly lower than those to the recall antigen and the polyclonal mitogen PHA. Our data are in agreement with the findings of other investigators (18, 19, 27) who report CD4+ T helper dysfunction at earliest stages of disease with loss of HIV-1 specific responses in terms of both proliferation and type 1 cytokine production.

The results obtained from this study allow us to make the following conclusions:

- Before the onset of specific antiretroviral therapy an impairment of cell-mediated immune function was registered in all patients.
- Both lymphoproliferative and IFN- gamma responses to the polyclonal mitogen Phytohemagglutinin, the recall antigen PPD M. tuberculosis and the specific HIV-1 p24 antigen were found depressed.
- In all patients the responses to the specific HIV-1 p24 antigen were found most suppressed.
- T - cell proliferative responses to all stimuli were found more inhibited than the stimulated IFN-gamma production.
- The absolute number of CD4+ T cells did not always correlate with the functional activity of these cells.
- The levels of the spontaneous lymphocyte proliferation and IFN-gamma production as markers of chronic immune activation were found increased in all patients.
- An inverse correlation between the spontaneous lymphocyte activation and the specific and non-specific lymphoproliferative and IFN-gamma responses was found in all cases.
- The results obtained in our study give a description of cell-mediated immune function in patients with HIV-1 infection before their inclusion in therapy.

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IS THERE A PREVENTIVE EFFECT OF LACTOBACILLUS BULGARICUS IN YOGHURT TO THE CLINICAL OUTCOME OF H. PYLORI INFECTION?

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SUMMARY

Lactobacilli exert inhibitory effects on H.pylori in vitro. The aim of this study was to link the clinical outcome of H.pylori infections with the consumption of Lactobacillus bulgaricus (found in yoghurt). We investigated serum samples of 307 blood donors aged over 20 years: 151 donors from a town with a lower consumption of yoghurt and 156 donors from a rural area in front of Balcan Mountains with a higher consumption of the same milk product, derived from the vilagers themselves. They were tested for anti-H. pylori IgG antibodies with an ELISA (Roch Diagnostic). A questionnaire for sociodemographic and lifestyle possible risk factors of acquisition of H.pylori infection and about the quantity of milk consumption was also administrated. The observed overall seropositivity rate was 88% in Stara Zagora and 92% in the rural area of Balkan Mauntains. The educational level of the parents was associated with a seropositivity of blood donors in the same region (OR 6.00; 95%CI: 1.31-55.38; p<0.009). Sociodemographic variables showed that the manual profession (OR 6.58; 95%CI: 1.01-25.62; p<0.02), the consumption of uncooked milk (OR 5.91; p<0.01), the contact with animals (OR 6.58; p<0.004), the eating from communal bowls (OR 3.54; p<0.03) are strong predictors of H. pylori seropositivity in the Balkan rural area as in the region of Stara Zagora. Although the seroprevalence of H. pylori is higher, the prevalence of the gastroduodenal diseases is significantly lower in the Balkan area than in Stara Zagora: 46 of 151 donors have duodenal ulcer in Stara Zagora and 10 of 156 patients - in the Balkan area (p<0.000001); 98 of 156 blood donors have not complains about the gastroduodenal tract in the rural area and 50 of 151- in the town of Stara Zagora (p<0.000002). Therefore the growth and clinical outcome of H. pylori may be suppressed by Lactobacilli and their fermented food products, (esp. Lactobacillus bulgaricus in yoghurt) in vivo.

Key words: Helicobacter pylori, Lactobacillus bulgaricus, gastroduodenal diseases

Treatment of *Helicobacter pylori* infection with antibiotics has been shown to be the best way to eradicate this bacterium. However, some drawbacks have been highlighted: cure rates tend to be lower in clinical practice than in clinical trials, antibiotic resistance is increasing in most countries, triple therapy is cumbersome, and retreatment after eradication failure is difficult. For these reasons, a search for better therapies against H. pylori is needed. The effects of lactic acid bacteria on the local and systemic host immune response have been described. Potential health benefits of lactic acid bacteria include: 1) protection against enteric infections (1), 2) use as oral adjuvants to vaccines, and 3) prevention of chemically induced tumors. Lactobacillus ac/dopMuswas first shown to inhibit the growth of H. pylori'm 1989 (1). Midolo *et al.* tested eight strains of L acidophilus, and six of them were found to be inhibitory to *H. pylori* NCTC 11637. This in vitro study suggests that the inhibition of *H. pylori* growth is due to organic acid production by the probiotics (2). Commercially available yoghurt preparations contain lactic acid in concentration ranges which may inhibit H. pylori in vivo. Lactobacillus bulgaricus also has the same property (3). The oral administration of lacto-bacilli in a gnotobiotic murine model showed a beneficial effect against H. pylori colonization (4,5). Probiotics are frequently ingested with the food product that supported their growth. To reproduce this real life situation, milk fermented by L acidophilus La1 was given to *H. pylori*-infected volunteers in a randomized, double-blind trial, using chemically coagulated milk as a placebo. After 3 weeks of daily intake, decreased *H. pylori* densities, an-tral inflammation and antral gastritis activity were observed in the volunteers exposed to La1- acidified milk (a commercially available yoghurt) as compared to a placebo. These effects persisted for several weeks after stopping milk intake (6). In another trial, supplementation with *L. acidophilus* GG significantly reduced the incidence of antibiotic associated gastrointestinal side effects common in the course of standard triple therapy (7). In addition to their antimicrobial activity, probiotics have been claimed to exert anti-carcinogenic activities, e.g. inhibition of tumor growth in rodents. A preparation obtained from
L. bulgaricus strain LB51 was especially studied in this respect and has even been used for anti-cancer therapies in humans in Eastern countries (8). This property has been related to the ability of this product to induce TNFa secretion.

OBJECTIVE

To investigate the prevalence of H. pylori infection, possible risks of acquisition and modes of transmission. To examine if there is an association between a high consumption of *L. bulgaricus* in yoghurt and a decreased load of *H. pylori* in the stomach.
To confirm the impact of yoghurt consumption on the prevalence of gastroduodenal diseases.

MATERIALS AND METHODS

Cross-sectional study performed on subjects attending a blood bank in 2 areas of Bulgaria with different consumption of yoghurt.
The selected areas are: Stara Zagora, a big city in Bulgaria with a moderate consumption of yoghurt, and a rural area in the foothills of the Balkan Mountains with a high consumption of yoghurt.
Serum samples from 307 blood donors aged 20 years or older (5 groups: 20-29,30-39,40-49,50-59, over 60): 151 donors from the Center of Blood Transfusion of Stara Zagora (a town with a moderate consumption of yoghurt) and 156 donors from a rural area, in the foothills of the Balkan Mountains, with a high consumption of the same milk product, prepared by the villagers themselves, were studied. They were tested for anti-H. pylori IgG antibodies with an ELISA (Cobas Core EIA, Roche Diagnostics, Switzerland). A questionnaire was completed for possible risk factors of acquisition of H. pyforiinfection concerning sociodemographics and lifestyle and also the type and quantity of milk consumption. Each blood donor was investigated for: age, gender, birthplace, place of residence, occupation, smoking, drinking and dietary habits, health status, socioeconomic status (income, education, living conditions), etc. An informed consent was also obtained. These groups of Bulgarian blood donors were compared by the test and the Mantel Haenszel x^2. A logistic regression was performed to identify the risk factors for the acquisition of *H. pylori* infection.

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ABBREVIATIONS USED IN THIS PAPER:

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RESULTS

The observed overall seropositivity rate was 88% in Stara Zagora and 92% in the rural area of Balkan Mauntains. There was a significant difference between male and female patients of the Balkan (OR 4.73; 95%CI: 0.95- 45.51; $p<0.03$), but not in Stara Zagora. When the place of residence in childhood and the parent's place of living is village, a significant association was found in Stara Zagora (resp. $p<0.0001$; $p<0.003$).

Sociodemographic variables showed that the manual profession (OR 4.32; 95%CI: 1.01-25.62; $p<0.02$), the consumption of uncooked milk (OR 5.91;$p<0.01$), the contact with animals (OR 6.58; $p<0.004$), the eating from communal bowls (OR 3.54; $p<0.03$) are strong predictors of H.pylori seropositivity in the Balkan rural area. Although the seroprevalence of *H. pylori* was almost identical in the 2 regions, the prevalence of the gastroduodenal diseases was significantly lower in the Balkan area than in Stara Zagora: 46 of 151 donors reported peptic ulcer disease in Stara Zagora vs 10 of 156 patients in the Balkan area ($p< 10^{*}$); 100 of 151 had reported gastroduodenal symptoms in Stara Zagora vs 58 of 156 in the Balkan area ($p< 10^{-6}$)

From the National Cancer Register, the prevalence of the gastric cancer for the year 1998 was 68.2 per 100,000 in Stara Zagora vs 42.8 per 100,000 in the Balkan area, and for the year 1999, 77.5 per 100,000 vs 46.7 per 100,000, respectively. One possible interpretation of these results is that repeated consumption of L. bulgaricus'm traditional yoghurt may lead to a decrease in bacterial cell load in the stomach, which would decrease gastric inflammation and associated diseases.

DISCUSSION

The growth and clinical outcome of H.pylori infections could be suppressed by Lactobacilli and their fermented products (esp. Lbulgaricus in yoghurt) in vivo. Probiotic organisms could play a role in *H. pylori* treatment both through a direct action against the organisme and in case of clinical side effects associated with antibiotics, or to use as an oral adjuvant to a vaccine.

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SUPPURATIVE-DESTRUCTIVE PNEUMONIA IN CHILDHOOD: TREATMENT EXPERIENCE

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SUMMARY

Despite the new antibiotics the problem of suppurative-destructive pneumonia in childhood is still valid at present and needs timely and thorough diagnostics. Infection of a pleura! effusion, particularly as consequence to an underlying pneumonia, results in significant morbidity to the patient and health care costs to the family and payers. A considerable group of children has been directed to surgical treatment from pediatric wards of peripheral hospitals too late and in bad condition, with destructive pulmonary processes and severe intoxication effect. The problem of the treatment of children with suppurative-destructive pneumonia in Bulgaria is one of the basic issues in pediatric surgery and pulmonology. Early surgical intervention has been demonstrated to hasten recovery and reduce morbidity.

Key words: pneumonia, empyema, childhood

INTRODUCTION

Current treatment of empyema in children is highly variable due to in part both provider experiences and a wide spectrum of clinical presentations. Empyema thoracis is defined as a pleu-ral space suppurative fluid collection. Pleura! space infections may complicate thoracis injury or arise secondary to a subjacent pneumonia. In the pediatric population, parapneumonic effusion is the most frequent etiology for empyema (4. 5). The management of postinfectious empyema historically involved aspiration for diagnosis, repeated aspirations if warranted, and tube or open drainage procedures once the empyema cavity was stable.

MATERIAL AND METHODS

We observed 34 children aged from 3 months to 7 years - 19 girls and 15 boys, which were under treatment from 1995 to 2003 in Departments of Pediatric surgery Stara Zagora and Plovdiv (see table 1).

With those patients we applied integrated diagnostic and curative complex includings: clinical setting of febrile illness in children with pulmonary symptoms-e. g. cough, tachipnea, sputum production, desaturations, and leukocytosis; X-ray examination of the lungs, blood tests, immunity status /T- and B-lymphocytes), Phagocytes-activity of leucocytes, Ig A, G, M, some hormones (ACTH, STH, cortisone), Timus image. Radiographic studies include anteroposte-rior and lateral chest radiograph, which demonstrate parenchymal infiltrates or consolidations, and pleural space fluid. Computed tomography and ultrasonography we perform for cases with difficult diagnosis. After the puncture and the complete evacuation of suppurative pleural fluid we set an intrathoracic drainage for a permanent draining of the pus out of the pleural cavity. Needle thoracocentesis for chemistry analysis and culture is usually the initial step coincident with the initiation of intravenous antibiotics. We used

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ABBREVIATIONS USED IN THIS PAPER:
ESS - empyema severity score

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Table 1

Age/Sex	3 - 12 months	1 - 2 years	2-3 years	4-7 years
Boys	3	5	4	3
Girls	2	10	6	1

an integrated methodology: antibiotic therapy (Ampicillin + Gentamicin + Ciprofloxacin) in the beginning and after the antibiograms data/records we used antibiotics according to the bacterial strains sensitivity. Each patient underwent a pleural puncture with aspiration of empyema and antiseptic solution installation /1:100 solution of Povidon-jodine (Braunol) or Ventrosteril (Hibiscrub) for intra-pleural and intraabdominal induction with subsequent draining of the liquid we put in. We observed indicators of immune and hormonal status. As intoxication syndrome indexes we applied evidences of general condition of the patients: respiratory problems, tachycardia, electrocardiogram, pulse, body temperature, general condition of the patient, dimensions of the so-called medium molecules.

RESULTS

All the patients have one or more clinical setting of febrile illness: cough, tachipnea, sputum production, desaturations, and leukocytosis.

The X-ray examination in two projections revealed several forms of the disease:

- Pyopneumothorax - in 11 of the cases (32. 3 %)
- Pyothorax-m21 of the cases (61. 7 %)
- Pneumothorax with destructive infiltrate - in 2 cases (58 %)

Bilateral pyopneumothorax we observed in two patients. The left lung was more frequently injured (19 of the cases - 59. 06 %) while the right lung was affected in 13 of the children - 40.6%. See table 2 for the content of the hormones observed. Table 3 submits records of the immunity status of the patients under observation.

In 20 patients we examined fagocitar-activity of the leucocytes through defining of the phagocytar number and phagocytar index.

The fluid of the pleural space is fibrinopurulent (intermediate stage of parenchymal infiltrates), and content thicker, opaque fluid (5%). or fluid with positive cultures (95%). In determining this activity we applied cultures of Staphylococcus aureus with 1.000.000,000/1 ml³ density of bacterial substances. The research revealed severe reduction of the phagocytar activity accordingly to the serious pathologic process. Microbiologic examination of the empyema revealed Staphylococcus aureus presence in all 34 patients 7100 %/. Mono infection of Staphilococcus aureus was found in 27 children 779. 4 %/. In other 7 patients we examined poliinfection of Staphylococcus aureus and streptococcus Haemoliticus - 2, Staphilococcus + Klebsiella - 3, Staphilococcus aureus +Streptocoecus Haemoliticus + Pseudomonas aeruginosa - 2. The poliinfection amounted to 20,6 %.

The level of the so-called "medium molecules'" observed in all 34 patients has increased in all cases. See the facts in table 4. All examinations were dynamically conducted and all the hormonal and immunity status breaches were in proportion to the seriousness of the clinical situation /status/. Two patients died /lethality - 5. 8 %/. We stand by the complex

Table 2.

Observed group	ACTHNg/ml	STHNg/ml	Cortical Ng/ml	IIOCS µg/ml
Statistical index for all groups M+M,P<0. 05				
Control - healthy children n=10	384±47	1. 13±6. 14	157±7.9	14.2±0.4
Patients with destructive pneumonia and hyperplasia of the timus n=29	123±12.5	3.04±0.6	48±7. 1	6.6±0. 7

Table 3. Immunity Status

Observed group	T-99 lymphocytes Number - %	B-lymphocytes Number - %	Ig G	Ig M	Ig A
		Statistical Index m+m; p<0. 05			
Control - healthy children	2490±39 69±1.2	843±27 18.7±0.9	1317±120	72±3.7	74±4, 1
Patients with destructive pneumonia and hyperplasia of the timus	1347±64 30±2. 1	323±19 6. 7±0. 7	547±70	35±3.4	38±3.7

treatment of the suppurative-destructive pneumonia in childhood. After patients' entering the hospital and diagnosing the disease we set a system for intravenous infusion and put a system for permanent mtrathoracic aspiration and laving drainage. In the individual antibiotics therapy we applied according to the testifications Tercef, Azlocilin, Ampicillin + Metronidazol + Immunovenin intravenously, small doses (20-50-100 ml) haemotransfusion 2 times a week and intravenous electrolytic and macromolecular solutions daily. The aspiration drainage we removed after the complete opening of the lung and disappearing of the suppurative fluids approximately 5-7 days after its setting.

Patients who fail to respond to intravenous antibiotics with defervescence and improving pulmonary symptoms, whose pleural fluid does not aspirate with needle or tube thoracotomy, presents for surgery. We perform closed-tube thoracostomy for oft-employed primary surgical maneuver in 21 children. The drainage stay 3 to 5 day and we perform X-ray or chest sonog-iaphy for follow-up. Decortication in open surgery we perform for two children.

DISCUSSION

Evaluation of the child with suspected empyema includes clinical stabilization (intravenous fluids, antibiotics, supplemental oxygen and/or ventilatory support), and radiographic evaluation (I, 3). Plain films confirm pleural fluid and may suggest the presence of loculations. Needle thoracocentesis may aid in confirming the diagnosis of a suppurative pleural effusion and yield useful specimens for culture and antibiotic selection. An empyema severity score (ESS) was derived by Hoffet al (5) based on pleural pH, glucose, radiographic findings (scoliosis, peel with significant entrapment), and anaerobic infection. Chest sonography may be useful in localizing loculated pleural fluid prior to aspiration or tube thoracostomy. Computed tomography may be useful in distinguishing pleural peel from consolidated parenchyma and identifying lung abscesses (2, 3, 6).

The development of suppurative-destructive pneumonia in childhood and hyperplasy of timus, revealed in X-ray examination, is characterized by serious general condition with progressing intoxication. In this pathological process there occurs severe immunity status disruption towards its prolonged insufficiency as a result of the reduction of T and B lymphocytes as well as 1G G, A and M. Phagocytar activity of the leukocytes is disrupted and this process is accompanied with severe reduction of the phagocytar number and fagocytar index. The immune protection is seriously disrupted/damaged for a long period of time and recovers accordingly to the process of coming through the illness. There is also a disorder in the hormonal status with a sharp reduction of ACTH, STH, Cortisol and 11-oksiketosteroids secretion. Its recovery is accompanying the healing process. The increased STH level shows intensified metabolism of immunity process activation and stress interaction caused by the activation of the timus function/The indicators of cells and humoral immunity reveal deep violation of the hormonal function of hypophysis; timus and suprarenals glands and confirm the significance of the timus in the immunity process and its influence through the hormones over the activity status of other endocrine glands. In staphylococcus- pneumonia destruction in childhood we observe a severe intoxication syndrome which can be overcomed by means of complex therapy: aspiration drainage for a short time, antibiotics of the last generation + Hinolones and metronidazole, intravenous infusions of macro- and micromolecular solutions, haemotransfu-sion and immunotherapy with general stabilizing treatment (4). With 3 patients we applied extracorporal desintoxication by means of haemabsorbtion which had excellent results and allowed recommending the haemabsortion application in cases of severe intoxication. The changes of cell and humoral immunity in serious timomegaly prove the insufficiency of immunity protection in cases of hyperplasy of the timus. The hormonal status disruption show the expedience of patients' immunity and hormonal status examination in suppurative-destructive pneumonia in childhood and the advisability of active surgical and therapeutical treatment.

Table 4 - Level of the "medium molecules"

Patients' ageLevel of "medium molecules"	3-12 months	1 -2 years	2 -3 years	4-7 years
Control group - starting level: 0. 17 conventional /conditional/ units, H=10				
0. 17-0. 30	1	3	0	0
0.31-0.50	1	6	2	1
0.51-0.8	0	3	5	3
0.81-1.07	0	2	3	0

Recently, there is a worldwide increase in the number of loculated empyema resistant to conservative management the usefulness of thoracoscopic surgery in the management of empyema in children. American Thoracic Society presents three stages for empyema: early exudative phase, an intermediate phase and a late organizing phase (2). As the stage progress, there are formation of loculi in the fibrinopurulent phase, with lung parenchyma consolidation-this is second phase. An early surgical intervention is best performed before empyema reaches the organization stage.

The therapeutic aim is to remove all the infected material after removing the fibrous septae so as to restore expansion. Many modalities for management have been described, which include tube drainage, tube drainage and thoracotomy and open debridment and thoracoscopic debridment. Thoracotomy and open debridment is still the gold standard for patients who present late, and are as would be seen on a CT scan. Open thoracotomy also permits lung resection if necessary for nonresponsive necrotizing and fungal pneumonias, and parenchymal abscesses. Thoracoscopy would not be useful in these cases and should be avoided.

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COURSE OF SURGICAL WOUND HEALING PROCESS IN PATIENTS WITH DIFFERENT LOCAL AND GENERAL PHYSICAL STATUS

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SUMMARY

Surgical cutaneous wound healing submits to both the general regularities of wound healing process, and the influence of a number of risk effects. The general risk factors related to patient and surgical procedure are being extensively examined and evaluated. The local factors, inhibiting or stimulating regeneration are being still only mentioned, but there lack studies of their detailed research and objectification. In the present work we investigated 24 operated patients of different surgical diagnosis, age, surgical wound class and physical status. After the termination of operation we took a biopsy material for histological evaluation of wound tissues' status. On this ground we made a general conclusion of the course of the healing process in the four patients groups as regards surgical wound class.

Key words: wound-healing process, Surgical cutaneous wound , Surgical wound infection, Histomorphological testing

INTRODUCTION

Wound healing is based on biological regularities strictly determined in the course of evolution of man. The surgical cutaneous wound is of the type incisional wound and submits to the general regularities of wound healing process. Concentrated on the operation itself, the surgeons often neglect the processes related to wound healing, which are in most cases dependent on their surgical skill and a possibility of ensuring of optimal local conditions for that. Majority of factors affecting the course of surgical wound healing process have been described in the literature, which often transfers into an obstacle to evaluation of their importance (1,2,5). In most cases is accentuated over the action of the general factors, being the best studied as well. The latter, however, may have an effect only through "the key local factors" - inhibiting or stimulating regeneration (2,3). Those factors are being still only mentioned, practically we are far from their detailed research.

Aim of the study With the present study we set ourselves the task to trace the course of wound healing process in operated patients with different class of surgical wound: preoperative and postoperative status of the surgical site, and burdened to a different extent general physical status.

MATERIALS AND METHODS

24 operated patients (volunteers) with different clinical diagnoses and of different age groups were included into a histomorhological testing. An object of the study was the four basic classes of surgical wounds: clean, clean-contaminated, contaminated and dirty wounds. The biopsy material was taken after the termination of operation, through incision to the adjacent sane tissue, before the treatment with antiseptic, and the setting of the suturing material. In some patients having developed during the hospital stay a surgical site infection, a specimen for microbiological testing has been taken as well. The biopsy materials were classified into four groups: biopsy materials taken from clean surgical wounds - 6; materials from clean- contaminated wounds - 5; from contaminated wounds - 6 and from dirty / infected wounds - 7. The clinical course of the underlying disease and the wound healing process were observed during the whole period of the hospital stay and up to the 30th day following hospitalization by means of a questionnaire of the patients. Methods used: 1.Over paraffin sections of a thickness of 5 microns were applied the following routine histological stainings: with Hemalaun-eosin; with Azan for connective tissue; Gomori's silver impregnation for reticular fibres.

2. Histochemical methods: 0.05% solution of Tolouidin blau, for determination of mastocytes; 0.1% solution of Alcian blue of pH=1.2, for determination of sulphured glycosaminoglicanes (GAG); McManus' PAS reaction under the control of, and out the control of alpha-amylase, for determination of glycoproteins; Gram-Weigert's method for staining and positification of bacteria.

RESULTS AND DISCUSSION

In all the 24 patients the taking of a material for histological testing has been estimated even before the operation, the supporting criteria for that being: the surgical wound class, the local preoperative status and the condition of the general physical status. Patients admitted with an infection and those having developed surgical wound infection in the course of a number of reoperations were of interest in the study too. Most of the patients were admitted for surgical treatment for the first time, and several ones - for reoperation. Through an assessment in detail of the results of the histomorhological testing of wound healing status following the termination of operation, as well as the impact of additional risk factors, we'll try to characterize the development of wound healing process in the 4 groups of patients formed.

I. Histological result in patients with a clean surgical wound: The derma and the hypoderma are of an intact histological structure. In isolated cases hyperaemia is revealed in the blood vessels of the fatty tissue.

II. Histological result in patients with a clean-contaminated surgical wound: The derma and the hypoderma are of an intact histological structure. Slight infiltrations of mononuclear cells into the derma and small haemorrhages into the fatty tissue are observed.

III. Histological result in patients with a contaminated surgical wound: The epithelium is papillomatoseously hyperplastic, at places with acanthosis and vacuolization of the epithelial cells. In one of the patients Gram-positive bacteria are found. More often in orthopaedic blood repositions, haemorrhages are observed in the fatty tissue, and the rest soft tissues. The granulations formed are rich in leukocytes, macrophages and mononuclear cells. The collagen fibres in the derma are thick, not well formed, or thin, rather loose. The substance found between them contains non-sulphurated acidic glycosaminoglicanes. The blood vessels are with thickened walls or are thin-walled with a perivascular mononuclear infiltration. Small groups of blood vessels are observed as well with a swollen endothelium and a strongly thickened wall. Necrotic changes are identified in the muscular cells next to the granulations.

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ABBREVIATIONS USED IN THIS PAPER:
glycosaminoglicanes (GAG)

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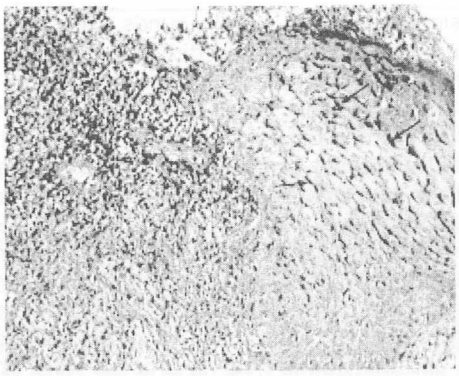

Fig. 1

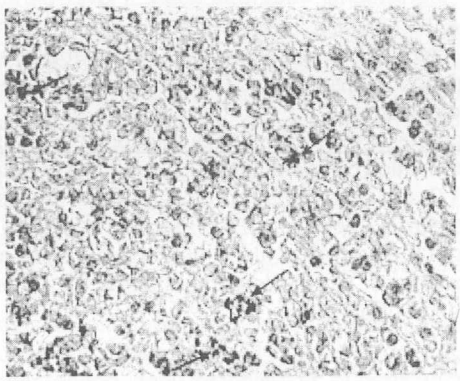

Fig. 2

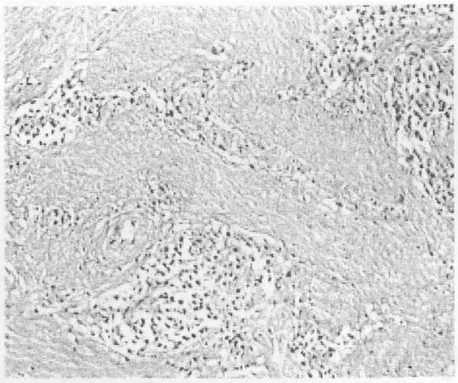

Fig. 3

IV. Histological result in patients with a dirty/infected surgical wound: * Cicatrix - Collagen fibres of irregular orientation predominate, some with necrotic changes. Foci of lymphoid and leukocyte infiltration are observed. Bands of connective tissue penetrate between the muscular fibres, some of which are with degenerative changes.

* The granulations are weak, of great number of lymphocytes, plasmocytes and macrophages. Zones of necrosis and haemorrhages are identified. In the intercellular substance irregularly situated glycoproteins are positified, such with sulphurated groups been missing.

* The blood vessels are of a small number, with a swollen endothelium, some with laminated adventitia and inflammatory infiltration. In the wall of separate vessels we observe fibrinous necrosis. In some of the sections recanalized thrombi and a perivascular oedema are found.

* The collagen fibres are of irregular orientation, thin ones, at places with a perpendicular situation, islands of mononuclear cells being revealed between them. A small number to a lack of fibroblasts is observed.

* Epithelium - The epithelium is of irregular thickness, scanty quantity of glycogen in the cell cytoplasm, at places papillomatoseously hyperplastic, with acanthosis (Fig.1). There is a leukocyte inflammatory infiltration between the epithelial cells. In three patients Gram-positive bacteria are visualized (Fig.2).

In 21 of the patients observed the physical status has been estimated of a 2nd degree according to ASA score, and in three patients - of a 3rd degree according to ASA score. Additional procedure related risk factors having an effect on clean wounds are: used foreign material, resorbing suturing material, drainages, articular prosthesis. In clean-contaminated surgical wounds of risk effect are the factors - obesity, age of more than sixty, neoplasms (related to the patient risk factors). In contaminated and dirty surgical wounds the additional patient related risk factors are obesity, diabetes, concurrent surgical wound infection (in 5 of the patients), cardiac and venous insufficiency, and on the part of the surgical procedure - the usage of a foreign material, drainages, the accomplishment of multitude operations. In 7 of the patients the surgical wound closed primarily, and in the others revisions were imposed, related to a secondary closure and prolonged hospital stay. Two of the patients with a dirty surgical wound were discharged with a nosocomial infection of the surgical site and a confirmed causative agent - a multiresistant strain Enterobacter cloacae. Both received twice a free plasty (Plastica cutis libera) from a neighbouring and remote site with an unsuccessful result (outcome). The rest patients were discharged in a good general condition and a recommendation for a dressing every 3 days. Finally we could generalize the results achieved in the following conclusions:

1. The histological testing accomplished of surgical patients with different surgical wound class are indicative of the inhibitory effect of the local factors over the wound healing process (1,3). 2. The pathological changes found in the tissues of the cutaneous surgical wound are most demonstrative in contaminated and dirty wounds, and partly - in clean- contaminated wounds. 3. The deviations observed in the morphogenesis of the reparative process are related to: - a delay in regeneration; - collagen formation disorder; - changes in the epithelium, impeding cell proliferation; - immature granulations; - microcirculation disturbed related to changes in the blood vessels (3,4,6); (Fig.3). 4. The local pathology confirmed, leading to a tissue hypoxia, increased vascular permeability, poor nutrition of tissues, the inflammatory changes and objectified bacterial presence in the wound area are an important prerequisite, which hampers and prolongs the healing period of the surgical wound (2,5). 5. The recognition of the local and general risk factors and their unidirectional unfavourable effect on the wound healing process is of primary importance in giving a new meaning to surgical practice.

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FREQUENCY AND ETIOLOGICAL STRUCTURE OF CENTRAL VENOUS CATHETER-RELATED INFECTIONS IN CRITICALLY ILL PATIENTS

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SUMMARY

To determine the frequency and etiological structure of central venous catheter (CVC)-related infections among critically ill patients, hospitalized in a surgery-trauma Intensive Care Unit in Sofia (2001, March - 2002, June). Patients, materials and methods: 90 CVC from 71 patients, mean age 51.4 (range 18-81) and APACHE II averaged sum 14 (2-29), were studied. The used catheters were: Cavafix (76.6 %), Balton (16.8 %) and Vygon (6.6 %). Microbiological methods: (1) A semiquantitative culture method of Maki et al.: rolling the distal catheter segment across blood agar. (2) Determination of the time to growth of blood cultures taken simultaneously from the CVC and a peripheral vein, or „differential time to positivity" of paired samples. CVC-colonization- 47 catheters (52.2 %); CVC-sepsis- 22 episodes (24.4 %). Etiology- (1) CVC-colonization- gram-negative bacteria (mainly K. pneumoniae and P. aeruginosa) - 40.3 %, coagulase-negative staphylococci (CNS)- 29.8 %, S. aureus- 17 %, Candida spp.- 8.5 %, and E. faecalis- 4.3 %. (2) CVC-sepsis- Gram-negative bacteria (mainly K. pneumoniae)- 45.4 %, S. aureus- 32 %, CNS- 13.6 %, and E. faecalis- 9 %. The frequency of CVC-related infections in the present study was above 3 times higher than that worldwide. The major etiological pathogens were Gram-negative bacteria, different from the leading pathogens in the USA and Europe (CNS, S. aureus and Candida spp.). In the base of these considered differences is an underestimation of the role of this serious complication for the increasing mortality among critically ill patients and thence the absence of contemporary strategy for the prevention of intravascular catheter-related infections in Bulgaria.

Key words: central venous catheter, catheter-related infections, frequency, etiology.

INTRODUCTION

The use of central venous catheters (CVC) for vascular access and hemodynamic monitoring has become indispensable in modern-day clinical practice and their advantages are beyond doubt (3, 4). Unfortunately, the application of these devices is associated with a variety of complications, the most common being infection (3, 4, 5). Catheter-related infection (CRI) is a major cause of nosocomial infection and may be life-threatening (19).

More than 20 million (over 50 %) of inpatients in the USA receive intravenous therapy every year (3, 14, 20) and almost 5 million require central venous catheterization (3, 13).

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ABBREVIATIONS USED IN THIS PAPER:

CVC- central venous catheter, CRI- catheter-related infections, CR-BSI- catheter-related bloodstream infection, CFU- colony-forming units, MRSA- methicillin-resistant Staphylococcus aureus, CNS- coagulase-negative staphylococci, ESBLs- extended-spectrum β-lactamases, ICU- Intensive Care Unit.

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According to the statistics, colonization is established in approximately 850 000 (17 %) of the placed CVC (6) and 200 000 of inpatients develop an episode of catheter-related sepsis (20). In different European studies catheter-related bloodstream infections (CR-BSI) account for 23.5- 66 % of all bacteremic episodes (22, 23). The increased cost per survivor in Intensive Care Unit (ICU) patients with CR-BSI has been estimated at $28 960, with a 25 % mortality (3, 17). In Bulgaria the problem of CRI is not investigated in detail. In one study, carried out at the National Center for burns, the reported frequency of CVC-colonization, CVC-bacteremia and CVC-associated mortality was respectively: 31 %, 19.5 % and 14 % (10).

Infection related to intravascular devices may develop by the following major mechanisms: contamination of the catheter at the time of insertion; migration of skin organisms along the catheter external surface; contamination of the catheter hub from extrinsic or endogenous sources passing through the lumen; contaminated infusion solutions and hematogenous seeding from a distant infection (3). For short-term catheters (< 8 days), skin contamination is the most likely mechanism of pathogenesis; whereas in the case of long-term catheters (> 8 days), hub contamination is more frequent (3, 16). The adherence properties of microorganisms also are important in the pathogenesis of CRI. For example, S. aureus can adhere to host proteins (e.g., fibronectin) commonly present on catheters. Many strains of coagulase-negative staphylococci (CNS) produce an extracellular polysaccharide often referred to as "slime". In the presence of catheters, this slime potentiates the pathogenicity of CNS by allowing them to withstand host defense mechanisms (e.g., acting as a barrier to engulfment and killing by polymorphonuclear leukocytes) or by making them less susceptible to antimicrobial agents (e.g., forming a matrix that binds antimicrobials before their contact with the organism cell wall). Certain Candida spp., in the presence of glucose-containing fluids, might produce slime similar to that of the bacterial strains (27).

The etiology of CRI depends on the type of patient, the underlying condition, the type of catheter, its location and use. The purpose of the present study was to determine the frequency and etiological structure of central venous catheter-related infections (CRI) among critically ill patients, hospitalized in a surgery-trauma ICU in Sofia during the 16-month period of March 2001 through June 2002.

MATERIALS AND METHODS

Patient population and catheters: 90 CVC from 71 patients, mean age 51.4 (range 18-81) and APACHE II averaged sum 14 (2-29), were studied. The used catheters were: Cavafix (76.6 %), Balton (16.8 %) и Vygon (6.6 %).

Blood cultures: During the study period, the blood culture results were reviewed for each patient from whom a catheter(s) was obtained and cultured. Blood cultures from samples taken simultaneously from the catheter and a peripheral vein were performed by using the BACTEC system (Becton Dickinson).

Microbiological methods:

1. Maki's semiquantitative culture method- it consists of rolling the distal 3-5 cm of the catheter (its tip) back and forth across the surface of a blood agar plate at least four times using a slight downward pressure (12). The threshold of colony-forming units (CFU) per plate is more than 14 (a significant value). Siegman-Igra et al. reported an 85 % sensitivity and an 85 % specificity in a meta-analysis of the diagnosis of CR-BSI (24).
2. Determination of the time to growth of blood cultures drawn through the CVC and by venepuncture, or "differential time to positivity" of paired samples- Flynn et al. have

used differential quantitative blood cultures from samples taken simultaneously from the catheter and a peripheral vein for the diagnosis of CR-BSI (8). Blot and colleagues have applied a recent addition to this technique- "differential time to positivity" of blood cultures drawn through the CVC and by venepuncture (1, 2). A cut-off value of +120 min was established.

Criteria for diagnosis

Catheter-related infections (CRI) include colonization of the device, skin exit-site infection and catheter-related bloodstream infection (CR-BSI).

1. CVC-colonization- it is defined as more than 14 CFU by the roll plate method in the absence of any clinical signs of infection at the insertion site of the vascular access (7, 24).
2. Exit-site infection- it is characterized as a positive semiquantitative catheter culture in the presence of clinical signs of infection (erythema, tenderness, induration or purulence) (7, 24).
3. CR-BSI- it includes: CVC-colonization + evidence of local infection with isolation of the same organism (i.e. identical species, antibiogram) from the distal catheter segment, pus around the insertion-site and from the paired samples of blood cultures and differential time to their positivity > 120 min + clinical evidence of sepsis (e.g., fever > 38 0C, hypotension, altered mental status, leukocytosis, tachycardia) that does not respond to antibiotic therapy, but resolves once the catheter is removed + no other apparent source of infection (7, 24).

RESULTS

In the present study the semiquantitative culture method described by Maki et al. showed a significant value of microbial growth in 47 from all 90 studied CVC- i.e. the established frequency of CVC-colonization was 52.2 %. The frequency of CR-BSI was 24.4 % (i.e. 22 episodes of sepsis) and the mortality rate in the patients developed CVC-sepsis was 63.6 % (i.e. 14 died from 22 ill patients). Microorganisms isolated from CVC tips are presented in table 1.

Table 1. Microorganisms isolated from central venous catheter tips in a surgery-trauma ICU in Sofia (2001, March- 2002, June).

Microorganism	Number of isolates (n=47)	Percentage (%)
CNS	14	29.8
K. pneumoniae	11	23.4
S. aureus	8	17
P. aeruginosa	5	10.6
Candida spp.	4	8.5
E. faecalis	2	4.3
A. baumannii	1	2.1
E. coli	1	2.1
S. marcescens	1	2.1

ICU- Intensive Care Unit, CNS- coagulase-negative staphylococci.

As it is shown, Gram-negative rods (mainly K. pneumoniae and P. aeruginosa) accounted for 40.3 % of all pathogens isolated from the distal segment of CVC according to the method described by Maki et al. (12). Some of K. pneumoniae strains were producers of extended-spectrum ?-lactamases (ESBLs). Coagulase-negative staphylococci (CNS)- mainly S. epidermidis, were the most frequently isolated Gram-positive cocci in the cases of CVC-colonization- 29.8 %, followed by S. aureus-17 %, and E. faecalis- 4.3 %. All isolated S. aureus strains were methicillin-resistant (MRSA), and susceptible only to vancomycin and rifampicin. The colonization-rate of Candida spp. was 8.5 %.

The etiology of CR-BSI is summarized in table 2. The leading pathogens were Gram-negative rods (mainly K. pneumoniae)- 45.3 %, followed by MRSA- 31.8 %, CNS- 13.6 %, and E. faecalis- 9 %.

Table 2. Etiology of catheter-related bloodstream infections in a surgery-trauma ICU in Sofia (2001, March- 2002, June).

Microorganism	Number of isolates (n=22)	Percentage %
K. pneumoniae	7	31.8
S. aureus	7	31.8
CNS	3	13.6
E. faecalis	2	9
P. aeruginosa	1	4.5
E. coli	1	4.5
S. marcescens	1	4.5

ICU- Intensive Care Unit, CNS- coagulase-negative staphylococci.

Finally, there were 3 from all 90 studied CVC (i.e. 3 %), which did not show microbial growth by the roll plate method, but the blood cultures taken simultaneously from CVC and a peripheral vein were positive, differential time to their positivity was > 120 min (firstly the blood culture taken from CVC showed bacterial growth, followed by the blood culture obtained by venepuncture) and there were all clinical symptoms of sepsis. In these cases 2 S. aureus and 1 A. baumannii were isolated and the strains from paired samples were the same organisms- with identical antimicrobial susceptibility.

DISCUSSION

In our study the determination of CVC-colonization was performed by the semiquantitative culture method of Maki et al. (12). According to the opinion of many authors, this method is sufficiently effective in CRI-diagnosis and therefore its routine use in the daily practice should proceed (3, 11, 24). Its combination with another method possessing sufficiently high sensitivity, specificity and cost-effectiveness levels would improve the precision of microbiological examination of CVC used in CRI-diagnosis. The most important disadvantage of Maki's method is false negative results in the cases when only the lumen of CVC is colonized (11, 24). Probably the undiagnosed cases of CVC-colonization in the present study were due to this disadvantage.

The half of the studied central venous catheters were colonized, a quarter of them were associated with an episode of CVC-sepsis and only 8 from 22 patients developed CVC-sepsis survived. The frequency of CRI in the present study was above 3 times higher than that worldwide (e.g. the reported frequency of CVC-colonization, CVC-sepsis and CRI-mortality is respectively: 7-15 %, 3-7 % and 10-30 %) (6, 21). More over, the established frequency of CRI was higher than that in another bigger Bulgarian research, carried out in 1996-1998 at the National Center for burns (10, 11). The major etiological pathogens were Gram-negative bacteria, different from the leading pathogens in the USA and Europe (CNS, S. aureus and Candida spp.) (3, 6, 18, 25, 27). For comparison, in a recent study at the University Hospital Vienna (1998-2000), Gram-positive cocci were the most frequently isolated organisms from central venous catheter tips (9), but our results were similar to these in India, where the most common pathogens causing CVC-colonization were Gram-negative organisms (26). According to the literature, microorganisms the most frequently responsible for CR-BSI are CNS and S. aureus (3, 6, 9, 18). Because CNS are ubiquitous on the skin, their isolation from blood often reflects specimen contamination rather than true infection. Historically, these microorganisms were regarded as relatively avirulent. However, true bloodstream infections due to CNS are common (9, 25). All the more, CR-BSI due to CNS can produce life-threatening complications in adults, such as prosthetic heart valve endocarditis (9). Before 1986, S. aureus was the most frequently reported pathogen in the cases of CRI (data from the National Nosocomial Infection Surveillance (NNIC) system, USA) (3).

It must emphasize that a large part of isolated microbial strains from CVC tips and blood cultures in this study were multiresistant- for example, all S. aureus strains were resistant to oxacillin and susceptible only to vancomycin and rifampicin; and some of K. pneumoniae strains were producers of ESBLs. According to the NNIS system, the percentage of enterobacteria that produce ESBLs, particularly K. pneumoniae, increases in ICUs. Such organisms not only are resistant to extended-spectrum cephalosporins, but also to frequently used, broad spectrum antimicrobial agents. In 1999, for the first time since NNIS has been reporting susceptibilities, > 50% of all S. aureus isolates from ICUs were resistant to oxacillin (27).

In conclusion, in the base of established differences about the frequency and etiology of CRI in the present study and worldwide is an underestimation of the role of this serious complication for the increasing mortality in critically ill patients and thence the absence of contemporary strategy for the prevention of these infections in Bulgaria. In general, the measures aimed at decreasing the incidence of CRI include: educational programs; strict adherence to hand-hygiene measures; full barrier precautions during CVC-insertion; use of antiseptics at the skin insertion site; antiseptic hubs and antibiotic prophylaxis strategies, such as systemic antibiotic prophylaxis, antibiotic ointment applied to skin insertion site (e.g., triple antibiotic ointment including polymyxin, bacitracin and neomycin or topical mupirocin), catheter flushing with solutions containing vancomycin and heparin or minocycline and EDTA and use of antimicrobial impregnated catheters (chlorhexidine and silver sulfadiazine or minocycline and rifampin) (4, 7). All these measures should strike a balance between patient safety and cost effectiveness (27). The development of preventive strategies to reduce the incidence of CRI should be a major goal for all health care providers.

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IN VITRO EVALUATION OF THE LINEZOLID ACTIVITY IN GENERAL TYPE BULGARIAN HOSPITAL

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SUMMARY

Treatment of hospital-acquired infection is made difficult by increasing incidence of Gram-positive bacteria, many of them resistant to available therapy.Linezolid is a novel oxazolidinone antibiotic that has a bacteriostatic activity against staphylococci and enterococci, and bactericidal activity against streptococci. We performed a study to assess the in vitro activity of linezolid against common Gram-positive clinical isolates in our hospital. A total of 301 nosocomial isolates from the last two years were included: Staphylococcus aureus - 79 MSSA and 86 MRSA; Coagulase negative Staphylococcus - 37 CoNMS and 43 CoNMR; Enterococcus faecalis- 52; Enterococcus faecium-24. We used Mini API (Bio Merrieux, France) System and conventional Kirby-Bauer disk diffusion test according NCCLS. We presented comparative susceptibility data about oxacillin, clindamycin, ampicillin,erythromycin, levofloxacin, vancomycin, teicoplanin, quinipristin/ dalfopristin. All tested strains exhibited 100% linezolid susceptibility. The results demonstrated linezolid to be active against most important Gram-positive cocci in our hospital, including those resistant to other antibiotics.

Key words: Gram-positive bacteria, linezolid, methicillin-resistant, staphylococci, enterococci, disk diffusion

Over the past two decades there has been a noticeable change in pattern of nosocomial infections with Gram positive microorganisms re-emerging as important pathogens. Methicillin-resistant staphylococci (MRS) are ones of the most important multiresistant pathogens causing hospital infection throughout the world and MRS incidence is still increasing in many countries (2, 13). The frequency of methicillin-resistant Staphylococcus aureus (MRSA) strains and coagulase-negative staphylococci (CoNS) has increased markedly (14,16). In 1994 the frequency of MRSA in European countries ranged from less 1% in Scandinavia to more than 30 % in Spain , France and Italy (16). The data from our hospital shows an increasing of the rate of MRSA as a cause of nosocomial infections from 6% in 1996 to 48% in 1998, as well (11). Concerning with the increasing incidence of nosocomial infections caused by Gram positive organisms, there is a trend towards increasing levels of resistance to available antimicrobial agents among this organisms (8). Antimicrobial resistance among Gram-positive cocci have emerged and disseminated in the hospitals and the community setting (12).

The impact of antimicrobial resistance can differ significantly from country to country and from hospitals within one country (4).

In a low frequency, strains of MRSA with intermediate susceptibility of vancomycin and other glycopeptides have been identified. There has been an increasing in the rates of resistance with four great problems: MRSA and MRCoNS; in a low frequency strains of MRSA with intermediate susceptibility of vancomycin and other glycopeptides (VISA and GISA); penicillin-resistant Streptococcus pneumoniae; Enterococcus faecalis and E. faecium resistant to vancomycin and other glycopeptide antibiotics (3, 9).

Options for treatment have remaind limited. Linezolid is the first of the first new class of antibiotics, the oxazolidinones, for more than 30 years (9,17). The oxazolidinones have an unusual chemical structure that is unrelated to any other class of antimicrobials available. Linezolid acts at a site unique from other antibiotics, inhibiting initiation of protein synthesis at the ribosomal level and exhibiting no cross-resistance with existing antimicrobial agents (1,4,10).

The aim of this study is to evaluate the in vitro activity of linezolid against common Gram-positive clinical isolates in our hospital comparative other antimicrobials.

MATERIAL AND METHODS

A total of 301 non repeated clinical isolates collected last two years (2002 and 2003) were included. The number of the tested isolates is as follows: S.aureus- methicillin susceptible (MSSA)-79, MRSA-86; CoNS-methicillin susceptible-37, methicillin resistant-43; Enterococcus faecalis vancomycin susceptible -52; E.faecium vancomycin susceptible-24.

All of them were isolated from surgical wounds, abscesses, blood cultures,tips of i.v. catheters, urinary tract,respiratory tract. The isolates were identified and their susceptibility was recovered by Semi Automated system Mini API (Bio Merrieux, France) and conventional methods according NCCLS (7).

MRSA were detected by oxacillin screen agar (BD,USA).

The isolates were tested by standardized Kirby-Bauer disk diffusion test for their susceptibility against linezolid 30 mkg (BBL,USA), quinipristin/dalfopristin 15 mkg (Oxoid,UK), vancomycin 30 mkg (BBL, USA), teicoplanin 30 mkg (Oxoid,UK), levofloxacin 5 mkg (BBL,USA), erythromycin 15 mkg (Bul Bio,BG), ampicillin 10 mkg (Bul Bio, BG), oxacillin 1 mkg (BBL,USA), clindamycin (BBL,USA).

The susceptibility category criteria are those, published in NCCLS (2000). Criteria for linezolid are S> 20 mm, R<20 mm (Linezolid product package insert, 2000).

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ABBREVIATIONS USED IN THIS PAPER:
Methicillin-resistant staphylococci (MRS)
methicillin-resistant Staphylococcus aureus (MRSA)
coagulase-negative staphylococci (CoNS)

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Table 1. The results from Kirby-Bauer testing of 301 clinical isolates Staphylococcus and Enterococcus against linezolid and 7 other antimicrobials

Microbial species	Antimicrobial agent (n)	% of susceptibility category: Susceptibility n	Susceptibility %	Intermediate n	Intermediate %	Resistant n	Resistant %
S. aureus	Linezolid	79	100.0	-	-	-	-
MSSA	Quinipristin/dalfopristin	79	100.0	-	-	-	-
n= 79	Clindamycin	71	89.8	2	2.5	6	7.5
	Erythromycin	46	58.2	4	5.06	29	36.74
	Levofloxacin	65	82.3	2	2.5	12	15.2
	Teicoplanin	79	100.0	-	-	-	-
	Vancomycin	79	100.0	-	-	-	-
MRSA	Linezolid	86	100.0	-	-	-	-
n= 86	Quinipristin/dalfopristin	85	98.8	1	1.2	-	-
	Clindamycin	24	27.9	1	1.2	61	70.9
	Erythromycin	4	4.6	2	2.3	80	93.1
	Levofloxacin	10	11.6	4	4.7	72	83.7
	Teicoplanin	86	100.0	-	-	-	-
	Vancomycin	86	100.0				
CoNS	Linezolid	37	100.0	-	-	-	-
MSCoNS	Quinipristin/dalfopristin	37	100.0	-	-	-	-
n= 37	Clindamycin	31	83.8	1	2.7	5	13.5
	Erythromycin	22	59.5	-	-	15	40.5
	Levofloxacin	29	78.4	2	5.4	6	16.2
	Teicoplanin	37	100.0	-	-	-	-
MRCoNS	Vancomycin	37	100.0				
n= 43	Linezolid	43	100.0	-	-	-	-
	Quinipristin/dalfopristin	43	100.0	-	-	-	-
	Clindamycin	23	53.5	1	2.3	19	44.2
	Erythromycin	8	18.6	1	2.3	31	79.1
	Levofloxacin	20	46.5	3	6.9	20	46.6
	Teicoplanin	43	100.0	-	-	-	-
	Vancomycin	43	100.0				

Microbial Species (n)	Antimicrobial agent	% of susceptibility category: Susceptibility n	Susceptibility %	Intermediate n	Intermediate %	Resistant n	Resistant %
Enterococcus	Linezolid	52	100.0	-	-	-	-
faecalis	Quinipristin/dalfopristin	2	3.8	1	2.0	49	94.2
vancomycin	Ampicilin	51	98.08	-	-	1	1.92
susceptible	Erythromycin	9	17.3	6	11.5	37	71.2
n= 52	Levofloxacin	27	51.9	2	3.8	23	44.3
	Vancomycin	52	100.0				
Enterococcus	Linezolid	24	100.0	-	-	-	-
faecium	Quinipristin/dalfopristin	16	66.7	5	20.8	3	12.5
vancomycin	Ampicillin	5	20.8	-	-	19	79.2
susceptible	Erythromycin	4	16.6	2	8.4	18	75.0
n=24	Levofloxacin	8	33.3	1	4.2	15	62.5
	Vancomycin	24	100.0				

RESULTS AND DISCUSSION

The results are shown in table 1.

All tested Staphylococcus isolates, both S.aureus and CoNS, are susceptible to linezolid (diameter of inhibition zone>21 mm). The rates of oxacillin resistance is 52,12% for S.aureus and 53,8% for CoNS. MRSA and MSSA isolates with reduced susceptibility to the glycopeptides vancomycin and teicoplanin were not found.

Quinipristin/dalfopristin also has very wide spectrum of activity-100% sensitivity isolates. Only one MRSA isolate shows intermediate susceptibility.

Among methicillin-resistant isolates 93,1% of MRSA and 79,1% of MRCoNS are erythromycin resistant. For clindamycin the spectrum is wider for methicillin susceptible strains, but reduced for methicillin-resistant isolates.

The levofloxacin is less active against methicillin resistant strains (11,6% MRSA; 46,5% CoNMR), compared with methicillin susceptible (82,3% MSSA; 78,4% CoNMS).

The results of our study show 100% susceptible E. faecalis and S. faecium strains to linezolid. Quinipristin/dalfopristin showed very good activity versus E. faecium-66,7%, but not versus E. faecalis (3,8%).

Ampicillin has very good activity against E. faecalis (98,8%). E. faecium strains are resistant to ampicillin in 79,2%. The results for erythromycin and levofloxacin have restricted areas of activity to tested Enterococcus strains.

E. faecalis remains susceptible to ampicillin (98.08%) and may supplement an aminoglycoside ampicillin activity.

E.faecium is more difficult to treat. The resistant rate to ampicillin is 79.2%.It has natural resistance to penicillins, aminopenicilins and an intrinsic adenylating enzyme for gentamycin (15).

IN CONCLUSION

Our results demonstrate that the oxazolidinone linezolid possess an in vitro very good activity against gram-positive isolates, including most difficult to treat bacterial pathogens.Resistance to linezolid there is not select .

The strains S. aureus and Coagulase-negative staphylococci that are resistant to methicillin are also resistant to most other antibiotics and are great problem of treatment. In our study MRSA and MR CoNS demonstrated in vitro 100% susceptibility of linezolid. All Enterococcus strains also exhibited 100% susceptibility to linezolid.

Linezolid represents an important new option for the treatment of Gram-positive infections in the hospital in the era of rapidly increasing resistance. Further studies are recommented in order better determination the clinical application and effectiveness of linezolid in hospital.

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FIRST PCR DETECTION OF FRANCISELLA TULARENSIS IN RESERVOIR ANIMALS FROM ENDEMIC AREA IN BULGARIA

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SUMMARY

PCR method is a new, sensitive, rapid, and specific method for diagnosis and identification of microorganisms. It eliminates risk for personnel of working with bacteria at high risk, such as Francisella tularensis. PCR investigation of rodents in endemic for tularemia region was conducted and 21% positive for F. tularensis rodents were detected. There were some differences in positivity of different organs: higher in spleen and liver samples, lower in kidney, heart and lung. Positive PCR reaction in considerable number of rodents suggests their epidemiological role in transmission of the infection. Our data correlate with results from serological studies on domestic animals.

Key words: Tularemia, Francisella tularensis, PCR, reservoir animals1

INTRODUCTION

Tularemia is endemic in many parts of the world linked to a variety of sources such as ticks, mammals, water etc. Francisella tularensis is a putative biological warfare agent and is extremely infectious, with as few as 25 organisms capable of causing disease. The tularemia etiologic agent is a small, Gram negative, obligately aerobic, facultative intracelular bacteria. It was subcultivated in 3 biogroups: type A tularensis is strongly virulent, met in North America, type B holartica is more weakly virulent, met in Europe and Asia, rarely in North America, and Francisella novicida (2). Main factor of virulence of F. tularensis is the precence of capsule-like layer with structural differences in virulent and avirulent strains.

The pattern of distribution of tularemia is much more limited to endemic foci, where mainly three genera of ticks, such as Ixodes, Dermacentor and Haemaphysalis were found to support their long-lasting persistence. In Bulgaria some sporadic clinical cases of tularemia occurred in 1952, but they were not microbiologically described. A focus of tularemia existed since 1972 in Srebarna reserve. In 1997, a tularemia focus appeared in the Slivnitza region. Clinical cases were microbiologicaly confirmed as tularemia by Velinov et al. (7). The focus enfolded the regions of Breznik and Pernik and some other parts of the country later.

F. tularensis is fastidious and grows slowly. That is why, it requires days for cultivation. Serology is the most common method used to diagnose tularemia, but a specific antibody response in serum is not detectable until two weeks or more after infection. There are serological cross-reactions between F. tularensis and other microorganisms, such as Brucella, etc.

Virulence of many biological warfare agents results in disease within hours to a few days and can kill the organism shortly after symptoms appear. That is why development of PCR technology to diagnose these agents offers an opportunity to establish rapid diagnosis and recovering of the host. The PCR is highly specific and sensitive method based on amplification of the specific gene of F. tularensis. The PCR results are available within 4 hours whereas results from cultivation take 72 hours, thus revealing the advantage of the PCR method.

MATERIALS AND METHODS

Rodents: This study is based on detection of F. tularensis in the spleen, liver, lungs, heart and kidneys of different species of rodents, trapped in endemic area of tularemia (Meshtitza, Pernik area). The rodents are live-trapped in 2003 in agricultural terrains and in the houses and yards of the people in this area. There were 42 rodents: 8 of them were specified as Mus musculus and 34 as Rattus rattus.

Extraction of DNA: PCR required extraction of DNA from animal tissue. The DNA extraction procedure was carried out by Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham Biosciences). The eluted DNA was frozen at
-20°C until use.

Primers: Tul4-435 and Tul4-863 primers are used for amplification. They amplyfied 400-bp fragment of tul4 gene, encoding 17-kDa lipoprotein (6).

PCR: The puRe Taq Ready-To-Go PCR Beads (Amersham Biosciences) were used for PCR analysis. Each batch of PCR beads was able to generate a specific PCR product. When brought to a final volume of 25 µl, each reaction contain ~ 2.5 units of puRe Taq DNA polymerase, 10mM Tris-HCl (pH 9.0), 50 mM KCl, 1,5 mM MgCl2, 200µM dATP, dCTP, dGTP, and dTTP, and stabilizers, including BSA.

Each PCR reaction tube contained 5µl DNA from rodent tissue. Negative control contained 5µl steril distilled water instead of DNA, positive control - 5µl DNA from F. tularensis, strain "Srebarna 19".

PCR reaction was conducted under following conditions: 94°C for 3 min, 35 cycles of 93°C for 1 min, 65°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min and 4°C for cooling afterward.

Amplified products are vizualized by electrophoresis in 1.5% agarose gel stained with ethidium bromid.

RESULTS AND DISCUSSION

Spleen samples of 9 (21%) from 42 rodents were PCR positive. Eight of them were Rattus rattus (89%), one was Mus musculus (11%). DNA was extracted from the other organs of the PCR positive rodents - liver, heart, lung, and kidney. PCR positive were 89% from the liver samples, 33% of the heart samples, 33% of the lung, and 22% from the kidney samples. More detailed these data are present on Table 1.

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ABBREVIATIONS USED IN THIS PAPER:
PCR- Polymerase Chain Reaction

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Table 1. PCR detection of F. tularensis in organs of the nine positive rodents from endemic area of tularemia

DNA sample from	PCR - positive rodents									Number of infected samples	% infected samples
	1	2	3	4	5	6	7	8	9		
Spleen	+	+	+	+	+	+	+	+	+	9	100
Liver	+	+	+	-	+	+	+	+	+	8	89
Heart	-	-	-	+	-	-	-	+	+	3	33
Lungs	+	+	-	+	-	-	-	-	-	3	33
Kidney	+	+	-	-	-	-	-	-	-	2	22
Total number of infected organs	4	4	2	3	2	2	2	3	3	25	56

This study represents the first investigation by PCR on F. tularensis infection of reservoir animals in endemic area of tularemia in Bulgaria. Positive PCR reaction was found in considerable number of rodents - 21% suggesting their epidemiological role in transmission of the infection. PCR method is specific, sensitive, rapid and eliminates risks for laboratory personnel working with agents at high risk, because it requires DNA, but not live strains. Serological studies in this focus of tularemia established 24% significant serologic reactions in domestic animals (5).

PCR investigations on reservoirs of F. tularensis have been conducted in Slovakia, Austria, USA, Sweden (3, 6, 8). Challenges of BALB/c and C57BL/6 mice have shown that aerosol infection of F. tularensis causes weak changes in the lungs (1). Another study revealed increase in number of bacteria at the place of infection, in lymph nodes, and in spleen of laboratory infected mice with F. tularensis containing extract of salivary glands of the ticks (4). It correlates well with our PCR results revealing lower infection of the lung samples and higher infection in spleens.

Due to population dynamic and number of the small rodents, reservoir of this infection might be a factor with significant epidemiological importance. The PCR methods provide a complementary tool in approach for detection or diagnosis the presence of biological agents. These methods are rapidly developed in the last years and correspond to the increased requirements of the time.

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DEVELOPMENT OF AFLP AND RAPD METHODS FOR TYPING OF CLINICALLY SIGNIFICANT CANDIDA SPESICES

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SUMMARY

Two molecular epidemiology typing approaches RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism) have been applied for better evaluation of genetic diversity among Candida isolates with clinical significance. Main objectives of the study were to develop typing methodologies and to investigate the reproducibility, discriminatory power and typeability of each method applied. By using six short oligomer primers (10-15 mers) with arbitrary chosen sequences, distinctive and reproducible sets of RAPD products were observed. The AFLP technique was applied by amplifying specific *PstI/BamHI* restriction fragments. With the applied typing methods it was found that the isolates obtained from a single person with chroniocal recurrent vaginal candidosis, but isolated from different body sites show polymorphic fragments.We performed an attempt to study possible transmission of *Candida* strains in hospitalized HIV positive patients. In conclusion, AFLP is a method with good reproducibility, but laborious. RAPD needs strict performance criteria.

INTRODUCTION

During the last 10 years clinically significant Candida isolates are a serious medical problem, especially for immunocompromised patients. The diseases caused by these microorganisms develop as viscerous (superficial) or systemic infections. Superficial infections are widely distributed, but the scientific community could not answer to fundamental questions: *L* it is not clear to what extent vaginal candidosis is a sexually transmitted disease and *IL* there are no clear evidences whether strains colonizing the oral cavity and the colon lead to vaginal candidosis. The second group of infections are the systemic mycoses. The genetic homogeneity of commensal and infecting populations has not been adequately explored to make definitive conclusions about how a clonal population evolves or is replaced during continuous commensal carriage in the transition from a commensal to pathogenic state or in recurrent infections. With the recent development of DNA fingerprinting techniques which allow assessment of strain relatedness at genetic level, many epidemiological questions can now be examined (1,2,3). Our goal with this study was to develop reliable molecular typing methods for clinically significant Candida species. Two molecular epidemiology typing approaches - RAPD (AP-PCR) and AFLP have been applied for better evaluation of genetic diversity among Candida clinical isolates. We performed an attempt to demonstrate transmission of Candida strains in hospitalized patients and strain carriage.

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ABBREVIATIONS USED IN THIS PAPER:

RAPD - Random Amplified Polymorphic DNA; AFLP - Amplified Fragment Length Polymorphism

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MATERIALS AND METHODS

The investigated strains were:

L Candida albicans isolated from vagina, oral cavity and anus from women with chronical recurrent vaginal candidosis, *H. Candida rugosa* strains isolated form a patient with prosthetic valve endocarditis, *Hi. Candida spp.* isolated from patients with HIV infection. All strains were of Bulgarian origin and biochemicaly identified by Vitek32.

The RAPD typing has been performed with arbitrary chosen oligomer primers. UBC734-5'-GGAGAGGGAG -3-'; **(GACA)$_4$-5'-** GACAGACAGACAGACA-3'; AP3- 5'-TCACGATGCA-3'; M13-5'-GAGGGTGGCGGTTCT-3'; CI-5'-ACGGGCCAGT-3'; **AP12H-5'-** CGGCCCCTGT-3'. For the 10-mer primers, **UBC734, AP3, CI and AP12H** the annealing temperature *(Tm)* was 37C. For the longer primers, **(GACA)$_4$ and M13** the *Tm* was 45C.

In our previous studies (4) we have described the development of an AFLP typing strategy based on DNA digestion with *BamHI* and *PstI* restriction enzymes, ligation of appropriate linkers (adaptors) to the restriction sites and PCR amplification of the polymorphic fragments with ^{32}P labeled BamHI primer. Briefly, for complete digestion with *BamHI,* about 3 μg DNA were placed in microcentrifuge tube with 5 U/μl *BamHI* (MBI Fermentas, Vilnus, Lithuania) and put at 37 °C for overnight incubation. Ethanol precipitation of DNA is performed for sample desalting and then the same procedure is repeated with *PstI* restriction enzyme (MBI Fermentas, Vilnus, Lithuania). The adaptors construction was performed second to manufacturer instructions (Invitrogen, Paisley, UK). 25 μl of each sample restricted with *BamHIand PstI was* placed together with 10 μl BamHI adaptor, 10 μl PstI adaptor, 5 ul ligation buffer and 2,5 U/μl T4 ligase (Amersham Bioscience, Piscataway NJ, USA) per sample in sterile microcentrifuge tube, vortexed shortly and left for 16 h at room temperature. Subsequent precipitation and washing of DNA was performed and the pellet dissolved in 50 ul of dH_2O. The PCR was performed with two primers - BamP 19 and PstP 18 both incorporating *A* (adenine base) as selective nucleotide at 3' site (Fig. 1). The BamHI primer was 5 end-labeled with ^{32}P using T4 polynucleotide kinase (Amersham Bioscience, Piscataway NJ, USA). The labeling reaction was performed in 50 ul volume containing: 5 μl lOx T4 PNK buffer (0,5 mM Tris-HCl pH 7.6,100 mM $MgCl_2$, 10 mM 2-mercaptoethanol), 10 μl [γ-^{32}P]ATP 100 uCi (Amersham Bioscience, Buckinghamshire, UK), 90 pmol BamHI9 oligonucleotide, 3 U/μl T4 PNK and sterile water to the final 50 μl reaction volume. The PCRs were performed with Ready-To-Go PCR beads (Amersham Bioscience, Piscataway NJ, USA). 5 μl template DNA, 10 pmol BamHI9 primer, 10 pmol PstP 18 primer and dH_2O up to 25 ul total reaction volume, were placed per tube. The PCR program was: 3 min at

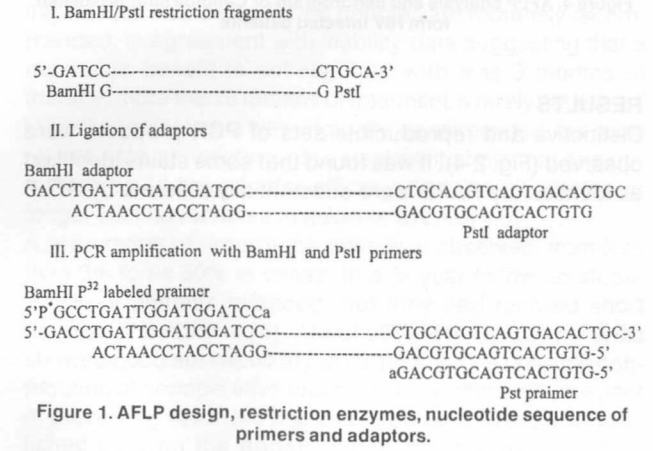

Figure 1. AFLP design, restriction enzymes, nucleotide sequence of primers and adaptors.

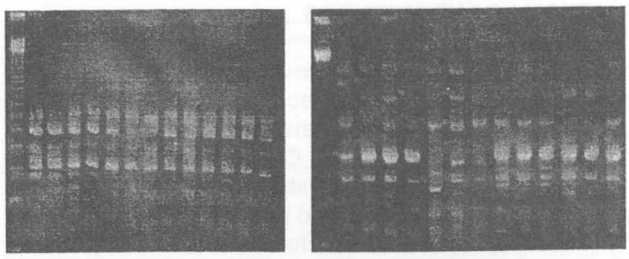

Figure 2. RAPD typing with $(GACA)_4$ (left) and with M13 primers (right) of *C. albicans* strains isolated from different body sites from women with recurrent vaginal candidosis.

94 °C followed by 35 cycles of amplification with 45 sec at 94 °C, 45 sec at 58 °C, 45 sec at 72 °C and termination 7 min at 72 °C. These were done on PERKIN-ELMER -480 thermocycler. The PCR product was dyed with 4 µl formamide dye (98% formamide, 10 mM EDTA pH 8.0 and 0.1% bromphenol blue) and 5 µl loaded on 6% denaturing (sequencing) polyacrilamide gel. Electrophoresis was performed at 1800 V/40 mA for 3 hours.The gels were dried and left for exposure with AGFA (Agfa, Gevaert, Belgium) X-ray films at -20 °C overnight.

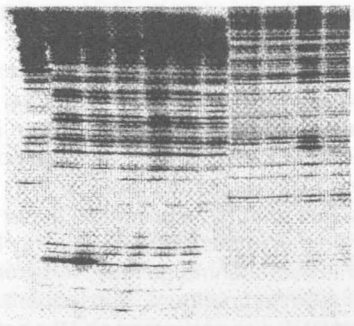

Figure 3. AFLP typing of C *albicans* strains isolated from different body sites from women with recurrent vaginal candidosis

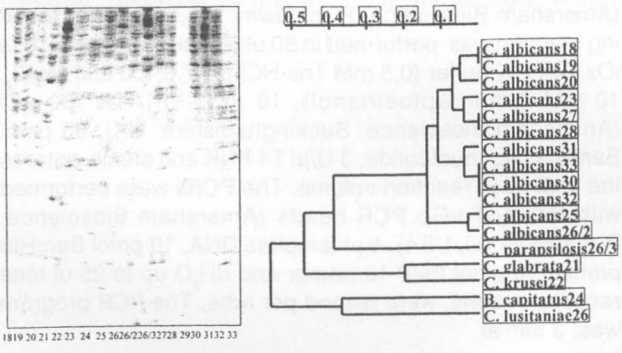

Figure 4. AFLP analysis and dendrogram *of Candida* strains isolated form HIV infected patients

RESULTS

Distinctive and reproducible sets of PCR products were observed (Fig. 2-4). It was found that some stains identified as identical by RAPD were substantially different by AFLP

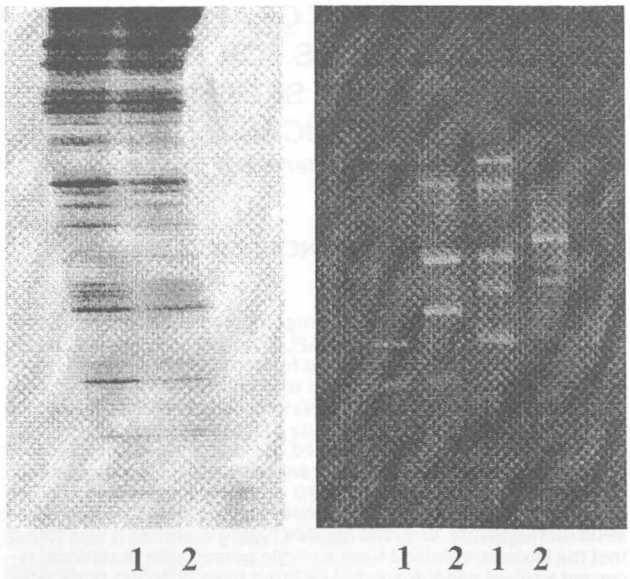

Figure 5. AFLP (left) and RAPD (right) analysis of *Candida rugosa* strains form a patient with prosthetic valve endocarditis. AFLP patterns of both strains are almost identical. RAPD performed with CAN and MI 3 primers show band polymorphisms. 1 - *Candida rugosa* isolate before treatment, 2 - *Candida rugosa* isolate during the follow-up treatment

or *vice versa*. Investigated strains of Candida rugosa isolated during the treatment course from a patient with prosthetic valve edocarditis displayed discrepant results (Fig. 5). Problems of different kind have been encountered: DNA quality, residual oligo RNA molecules, set up of primer annealing temperature.

DISCUSSION AND CONCLUSIONS

DNA fingerprinting of the clinically significant Candida species and strains has become an important subdiscipline of medical mycology (5). To obtain good and reproducible RAPD or AFLP profiles it is essential that DNA is isolated from fresh cultures and free of RNA (6). It was found that residual RNA molecules could seriously inhibit PCR amplification with short oligo-primers at low non specific hybridization temperatures. Residual non ligated adaptors inhibit AFLP analysis. Both techniques are not equally effective in typing. Some results can lead to misinformation. No one of the (AFLP or RAPD) methods could evolve as a dominant method. In fact each method has it's own set of assets and limitations.

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CHEMOTHERAPY OF CYSTIC EHINOCOCCOSIS-INDICATION AND REVIEW OF LITERATURE

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SUMMARY

Chemotherapy is a noninvasive treatment and is less limited by the patient's status than surgery or PAIR but is not ideal when used alone. Albendazole, the drug most often used, appears to have the greatest eficacy of any agent used so fan nevertheless, apparent cure (shrinkage or disappearance of cysts) rangeds only between 20% and 30%. Another important indication for chemotherapy is the prevention of secondary echinococcosis. There is not yet formal consensus, as the efficacy and safety of some of the methods require further evaluation before we can establish comprehensive guidelines for the medical treatment of hydatidosis.

Key words: hydatidosis, chemotherapy

Chemotherapy is certainly indicated for inoperable patients with primary lung or liver cysts, those with multiple cysts-specially in multiple organs-making operative resection difficult or impossible and the patients with peritoneal cysts which usually are multiple and result of secondary echinococcosis [1,9]. The following short review discusses only noninvasive treatment of echinococcosis. Until the early 1980s and the first treatment attempts with benzimidazole compounds-albendazole (ABZ), mebendazole (MBZ) [I, 2]-surgery was the only treatment, and the general progress made in antiparasitic therapy (especially that of helminthiasis) had no beneficial impact on human echinococcosis. It has long been known that effective parasiticidal, or at least parazitostatic, chemotherapy is required.

The use chemotherapy alone as the primary treatment of cystic echinococcosis in other patients suggested by some authors [9,11,13] but not widely accepted because of relatively low and unpredictable cure rate. Chemotherapy also should be considered in patients with relapse of hydatid cyst after surgical treatment because of technical difficulties and relatively high complication rate associated with repeat surgery [10].

The choice of benzimidazole earbamates was primarily related to their mode of action and pharmacologic properties. The mode of action of benzimidazole earbamates includes a direct effect pn the cumulus oophorus and perhaps also on the wall of the cyst, whose permeability might be increased [1,10]. the pharmacology of these drugs also influences their action. MBZ was designed as a broad-spectrum anthelmintic drug active against intestinal nema-todes. To limit risk of adverse reaction, it had to be poorly absorbed. ABZ proved to be a more interesting product
showing better absorption and tissue distribution than previous benzimidazole molecules [2,3,7].It is absorbed at a higher rate than MBZ, and it undergoes almost total first-pass metabolism to its effective protoscolicide metabolite ABZ sulfixid, whereas liver metabolism of MBZ leads to inactive and a loss of antiparasitic activity [8]. Albendazole is widely used as adjunct pre-and post-OP chemotherapy with both surgery and percutaneous aspiration therapy to reduce incidence of secondary hydatidosis. Preoperative chemotherapy not only decrease the number of variable protoscoleces, but also results in significant reduction of intracystic pressure, which facilitates surgical removal and percutaneous aspiration [6]. Different dosages and durations of preoperative chemotherapy given in different studies from single dose of drug given 4 hour before procedure (to attain maximum serum concentration at time of procedure) to 1-3 months of operative treatment [7, 9]. Although WHO recommends preoperative chemotherapy for least 4 days followed by continuous postoperative treatment for 1 and 3 months for ABZ and MBZ respectively [5,6], prospective studies showed that increasing the duration of preoperative chemotherapy significantly increases the percentage of non viable cysts and even 3 mounths of treatment was significantly more effective than one mounth. Uncontrolled trials of preoperative chemotherapy with ABZ and continued postoperative treatment for 2-7 mounths have recurrence rates as low as 0-4% within 30 months of follow-up[4,8], which compares favorably with recurrence rate of 10-30 % associated with conservative surgical methods.

Further controlled trials clearly needed to determdne the best timing and duration of preoperative chemotherapy, but based on currently available data, preoperative treatment of 1-3 months followed by 1-month postoperative chemotherapy with ABZ can be recommendable whenever possible. Combined regimen of ABZ plus praziquantel is also useful as preoperative therapy and apparently was more successful in descreasing the number of viable cysts compared with ABZ alone [7].

Today ABZ is the drug most often used. In 1997 Morton reviewed the data collected or published during 12 years of expirienee with ABZ [3] since the initial publication. Chemotherapy seems to be more effective in young than in older patients. Small cysts with a thin wall, without infection or communication, and secondary cysts are mostly susceptible to chemotherapy; chemotherapy may, however, be less effective on daughter cysts within a mother cyst. The different studies for pre- and postoperatively medical therapy are showed in Table 1.

Contraindications and precaution for medical treatment: large cysts, and risk of rupture (spetially those superficially located or infected); pregnancy and lactation; bone marrow suppression; some liver disease with portal hypertension and cholestasis. The serum glucose levels of diabetics should be carefully monitored during treatment with these drugs [5,12].

Clinical and laboratory examinations liver function test are necessary initially every two weeks and then monthly. Leucocyte count should be checked at 2 weeks intervals during the first 3 mounths. Three courses are routenely recommended, in agreement with viability data suggesting that a maximum benefit is not reached with less 3 months of therapy; more than 6 months of treatment is rarely necessery [13]. Cyclic treatment was originally recommended, but more recent data on uninterrupted treatment show that this approach could have better efficacy over 3 to 6 months or longer with no increase in adverse events [4,6].

A wide range of recurrence rates was observed, from less than 3% to as 30% of cases. In a 5- year follow-up studu, 22,7% of patients relapsed, but they had recivied short courses of treatment [6], About 95% of the recurring cysts showed good susceptibility to treatment. The possible contribution of perioperative chemotherapy offers the prospect of preventing recurrent disease [6,9], but there are no published data on the added benefit of postoperative ABZ

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ABBREVIATIONS USED IN THIS PAPER:
ABZ-Albendazole; MBZ-Mebendazole

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Table 1. Results of treatment of cystic echinococcosis with ABZ (adapted from the WHO guidelines [2]and [9])

Autor	Country	Age	Dose	Week		Outcome			
Keshmiri	Iran	15-18	400 mg/bid	42-126	14	36,7	42,8	NG	21,4
Nachmias	Israel	36-90	400 mg/bid	112	68	41	57	27	13
Davis	Mulicent	<24	800 mg/day	6-90	30	16,6	13,3	46,6	23,3
Horton	Multicen	<24	15mg/kg	30-365	253	29	NG	51	18
Derosa	Italy	6-24	12mg/kg	90	46	9	NG	58	21
Todorov	Bulgaria	>33	10mg/kg	120	23	43,4	43,4	NG	13
Agrawal	China	-	15-20 mg/kg	360-540	58	24,1	NG	50	25.8
Cocremani	Libya	9-13	400 mg/bid	84-1 68	40	NA	81	NA	49
HAo	Italy	12-72	400 mg/bid	90	47	63,40	21,2	NA	10
Elmulti	India	3-14	10 mg/kg	56	10	NA	NA	NA	100
Morris	UK	44-72	10 mg/kg	30-90	23	22	NG	52	26
Golematis	Greece	NG	10 mg/kg	60-150	33	21	NG	58	21

therapy in patients undergoing a complete surgical cure. It requires more long-term controlled clinical trials to establish that pre-or postoperative chemotherapy prevents recurrence.

Currently it is recommended only when there is cyst spillage at surgery, partial cyst removal or biliary rupture. Acording to the WHO guidelines, preoperative treatment with bezimidazoles should begin at least 4 days before surgery and last one month (ABZ) is 10-15 mg\kg\day in two divided dosesfor several 1-month courses separated by 14-day intrvals; or 3 to 6 months (MBZ)-5QO mg tablets in daily doses of 40 to 50 mg\kg (in three divided doses) [2].

CONCLUSIONS

Chemotherapy is a noninvasive treatment that can be used in patients of any age (although there is little experience in children); it is less limited by the patient's status than is surgery or PAIR. Chemotherapy is the preferred treatment when thedisease is inoperable whatever the reason (e.g., patient's status, multiple cysts in several organs, peritoneal cysts), when surgery or PAIR is not avaible, or if the cysts are too numerous. ABZ appears to have the greatest efficacy of any agent used so far, although the apperent cure (shrinkage or disappearance of cysts) rate is only 20 to 30%. The WHO guidelines (1996) is not yet formal consensus, as the efficacy and safety of some of drugs for medical treartment of hydatidosis.

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ASYMPTOMATIC CASES OF HUMAN HYDATIDOSIS

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SUMMARY

The diagnosis of uncomplicated hydatid cyst depends on clinical suspicion. Nonspecific symptoms may also be present. There exist scarce information about the natural history of the illness in non-treated patients. At the present time, these aspests in non-symptomatic carriers detected by ultrasonography (US) are studied. Ultrasonography, a noninvasive, readily available, sensitive, cost-effective imaging technique, should be the diagnostic method of choice. 47 of these cases are detected in 331survey by US made in South-Eastern Bulgaria between 1990 and 2003 years. 40 of these are investigated by way of interviews, review of clinical histories, clinical control, US and serology; and other cases - only by US. 75% did not present clinical evidence of hydatidosis. 15 asymptomatic cases between 1992 and 2003 were evaluated by US detecting an average increase of the (QH) of 1,2 cm, with substantive changes in the type of image and in the serological response. 20% of the asymptomatics had been operated on before appearing the symptoms. The results are analysed in function of the age, of the propable individual susceptibility and of the echogenic image of the (QH), evaluating the pertinence of the current of the treatment.

Key words: hydatidosis, asymptomatic, ultrasonography (US)

INTRODUCTION

Hydatid disease is still endemic in various areas of the world. Non-complicated hydatid cysts are asymptomatic different time before the operation. The symptoms may be related to a toxic reaction due to the presence of the parasite or the local and mechanical effects depending on the location and nature of the cysts and the presence of complications (2, 4). Evidence has been found that an important percentage of the "non symptomatic carries" of the human hydatidosis (HH), with small cysts, stablish systems of agent\host balance that could make the application of surgical solutions and even medication unnecessary (1,5). We are presented the results of "non-symptomatic carriers" detected by echography in 1992 and evaluated in 2003.

MATERIAL AND METHODS

Between 1990 and 2003, 331 ultrasound surveys are made for detection of carriers of (QH) non symptomatic, diagnosing 47(14,2 %) carriers, with an average age of 34,8-f-DE 21,2 years. These cases presented images between 1.2 cm and 12cm. The cases detected were put into a database of human hydatidosis that receives information from the hospital network, laboratory and serologycal tests of the hydatidosis.

RESULTS

Of the 47 asymptomatic cases, 8(17,02%) could not be controled. Of the 39 cases from which information could be obtained, 11(28,21 %) had been operated on after the ultrasonographic diagnosis, without having presented symptoms. These carriers presented initial echographic images of 5x4, 5 cm, 4,5x5 cm, 8x5 cm, 6,5x7cm, and 3 cyst in one patient of 10x8, 4x5, 3x5 cm. In three of the cases, it was proven surgically that they were hyalines. Of the 32 remaining cases, a total of 8(25%) presented symptoms and operated on. These patients presented initial images of 6x5 cm, 10x8 cm, 5x6 cm, 7x5 cm, 9x6 cm, 1 lx8cm,Operative data confirmed to us that one was hyaline, three were soft\purulent and three with multiple cysts, hyalines, soft and broken. The average age of these patients was 28,9 $\pm$ DE 12,1 years. The average time of apparition of symptoms was 5,5 years DE 2,8. 24 (75%) of the 32 cases not derived from the early surgery, with average age of 30,5$\pm$ DE 12,5 years, did not present clinical symptoms compatible with hydatidosis 10 years after the initial echographic diagnosis (Table 1).

Table 1

Evolution	Number	%
With posterior symptoms	7	25%
Without posterior symptoms	32	75%
With echographic control	29	
With clinical control	10	
TOTAL	39	100%

29 cases were evaluated echographically and serologically, with 20 corresponding to the group of not operated on and 6 to the operated on (3 symptomatic and 3 non-symptomatic). In 10 of the cases not operated on (25,64%) modifications in the size were not produced in the period of time 1990-2003, in 6(15,39%) there was scarce growth (less than 2 cm) and in 2 cases - 4cm of growth was made. The average growth of the cysts in the 16 cases was 0,8cm in 13 years. Modifications were observed in the ecogenic images of the hydatid cysts, compatible with aging of the same, which is presented in Table 2.

Table 2

Type of cyst	1990-2003 Ultrasonography image	1990 N(%)	2003 N(%)
I	Hyaline	4(21,1%)	0(0%)
II	Hyaline with vesicules	2(11,5%)	1(15,8%)
III	Multiloculated	2(11,5%)	1(15,8%)
IV	With membrane	4(21,1%)	6(31.6%)
V	Heterogenic with or without partial calcification	5(26,3%)	8(42,1%)
VI	Solid with or without partial calcification	1(5.3%)	3(15,8%)
VII	Calcificated	1(5,3%)	0(0%)
Total		19(100)	19(100)

In four cases with surgical antecedents a new cyst was detected (size: 4x5 cm, 3x2 cm, 3x3 cm and 5x6 cm). If we consider exclusively those operated on for apparition of symptoms (7 and those not operated on but controlled echographieally (29) we find that in (74,4%) of the cases with hydatidosis, diagnosed by US evolved without substantive modifications of the size of the QH and in their clinical situation.

In relation to the serology, of the 20 cases studied, 10(50%) were ELISA and RPHA positivelly, and 5 (25%) are negative in to two tests. Of the cases not operated on, 7(50%) resulted ELISA positive. In the 19 cases, in 1990, 3 cases resulted positive to the ELISA and RPHA, with one corresponding to those not operated on.

DISSCUSSION

The uncomplicated hydatid cyst is asymptomatic for a long time. Ultrasonography is helpful for defining the international structure, number, and location of the cysts and the presence of complications. The specifity of ultrasonography is about 90%(2, 3). In 1981 Gharbi et al. Proposed a morphologic ckssification with five types (2), and many other clas-

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ABBREVIATIONS USED IN THIS PAPER:

Ultrasonography (US), human hydatidosis (HH), computed tomography (CT)

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sifications have since been proposed (3). The World Health Organization' (WHO) classification consist of six types and corresponds to a combination of three previously reported classifications (3, 5).

The articles for asymptomatic hydatidosis are limited numbers. In a few years it was habitual that the patients arrived in the doctor's office a hydatid cyst greatsize and many symptoms. Finding small cysts was so mething limited to autopsies. With the introduction of the serological surveys first of all and later the US, the field of the early detection amplified (1, 6). The hydatid cysts of this form, small and occasionally asymptomatic, began to be operated on producing a lessening of the lethabiess in the times of being in the hospital In this way, the frequency of the localization of hydatid cystsbegan demonstrate discordance with the results of the surveys paired up with US and radiology which indicated nuch more extensive relations (nine hydatid cysts for every pulmonary cyst). The observations found are equal that relationship between hepatic and pulmonary localization found in autopsies.

The findings in our study confirmed completely the previous observation, that at least 74% of the carriers of hydatid cysts established conditionsof agent\host balance, without cystic growth, with evidence of modifications in its contens and in its immunological response compatible with aging and without presentation of clinical symptoms. Some observation have shown a prevalence proportionaliy higher than the existing in the certain groups of the general population and a high rate of reinfection in patients operated on living in persist ently endemic areas, attributable to microphocus familiar of greater environmental contamination with Echinococcus granulosus or a greater susceptibility to the infection linked to immunitary aspects.

These aspects show the need to revise the criteria of treatment in human hydatidosis.In dealing with small, very non symptomatic cysts, the possibility of an echographic follow-up should be evaluated, to determine the need, opportunity and type of treatment in view of the knowledge of the natural history of the illness that the ultrasound contributed The echographic surveys could be used with the final of offering secure medical options to the new cases produced by mistakes in the program for diagnosis and treatmen of hydatidosis, in the form of echographic follow-up for determining the need of surgical treatment or eventually medical treatment.

The results of the present experience confirm that, besides their sensitivity, the ultrasound (US) in field surveys present an elevated speeifieness.

A standardized protocol for every patient with high levels of ELISA and RPHA employing ultrasonography or computed tomography (CT) with follow-up for at least 3 years is essential for the documentation of theurapeutic efficacy in this disease. They may occur many years later, however, and longer follow-up is recommended postoperatively when possible.

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