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

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ESBL – PRODUCING ENTEROBACTERIACEAE – AN INCREASING PROBLEM AMONG PATIENTS WITH BACTEREMIA

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SUMMARY

Emergence and spread of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae present a challenge in antibiotic treatment of patients with bloodstream infections. The aim of this work was to analyze the incidence of ESBL-producing Enterobacteriaceae among patients with bacteremia 2003-2009 in a teaching multi-profile hospital and to evaluate the appropriateness of antibiotic therapy through antibiotic audits. Surveillance has shown that Escherichia coli strains occupy one of first 3 top positions in bacteremia: in 2003, 1 of 8 strains produced ESBL, while in 2008 25 % of 24 E. coli produced ESBL. In 2008 the relative rate of ESBL among Klebsiella pneumoniae reached 50 % of 12 cases, and 22 % of all 53 Enterobacteriaceae isolates. Enzymes of nine ESBL-producing strains isolated in 2003 showed pI of 8.2 and 8.4, gave positive PCR with SHV- and CTX-M specific primers and after sequencing were identified as SHV-12 and CTX-M-3 respectively. The audit of prescribed antibiotic therapy revealed that ~ 50 % of patients have received an appropriate therapy.
Rigorous measures should be undertaken in antibiotic policy (a limitation in use of (groups) of antibiotics, new guidelines for therapy (empiric therapy of sepsis), as well as in the control of infections, in order to contain the problem and improve the therapy.

Key-words: ESBL, sepsis, antibiotic audit

INTRODUCTION

Blood-stream infections are among the most serious worldwide. Escherichia coli and other Enterobacteriaceae strains account for up to half of pathogens in bacteremia (1). Now the continued emerging of Extended-spectrum beta-lactamase (ESBL)-producing strains represents a further challenge for the therapy of infections, due to the limited therapeutic options (6,7,12,13).

ESBL-producing Enterobacteriaceae have spread worldwide, incl. in Europe. Some countries of the Balkan peninsula are particularly burden by them, incl. Bulgaria (2,5,11).

The aim of this work was:

To evaluate the rate of ESBL producers among bacteremia pathogens at Medical Institute – Ministry of the Interior, during the recent years

To establish the type of ESBL, as well as the phenotype of antibiotic resistance

To evaluate the antimicrobial therapy prescribed

To draw recommendations for better management of the problem.

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MATERIALS AND METHODS

The Blood-culture method used was Bactec 9050, BD, USA and blood cultures were incubated in 7-day protocol. Routine subculture of bottles that became positive was cultured on blood agar and MacConkey agar plates. Biochemical identification of blood-culture pathogens was with API 20E, BioMerieux, France, and Crystal NE, Becton Dickinson, USA. Antimicrobial susceptibility testing was performed by the disk diffusion method (DDM) on Mueller - Hinton II agar (BD) with antibiotic disks (NCIPD, Sofia and BBL, BD) according to the CLSI 2005 guidelines.

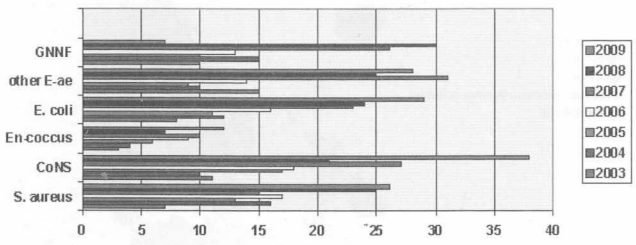
The study on ESBL production included:

- Double disk synergy (DDS) method by Jarlier V. et al. and the CLSI disk confirmatory method.
- Isoelectric focusing (IEF): Enzyme extracts of the strains were prepared with ultrasonic disintegration of bacterial cultures. IEF was performed by the procedure of Matthew et al .
- Bioassay was performed by a procedure of Bauernfeind et al to reveal the hydrolytic activity of the bands.
- Whole cell DNA preparations were used as template in specific PCRs to detect genes coding for b -lactamases of the TEM-, SHV-, OXA- or CTX-M-groups. PCR amplification products from representative strains obtained with oligonucleotides binding to the flanking region of the genes were subjected to automatic sequencing (ABI 3700, Applied Biosystems, Warrington, UK). The nucleotide and deduced amino acid sequences were analyzed and multiple alignments were performed with the Dnaman 4.11 Software (11). Analysis of the appropriateness of antibiotic therapy of patients was performed according to the current experts' recommendations (6,7,13).

RESULTS AND DISCUSSIONS

Epidemiology of the first top isolates in bacteremia at our institution during 2003 – 2009 is presented on Fig. 1. It is shown, that the proportions of Gram-positive and Gram-negative bacteria are similar. The most frequent isolates were respectively staphylococci and Esherichia coli.

Fig. 1. Epidemiological spectrum of bacteremia from 2003 to 2009: The number of patients with the following isolates



Legend to Fig. 1: CoNS, coagulase-negative staphylococci; En-coccus, Enterococcus spp; other E-ae, other Enterobacteriaceae; GNNF, Gram-negative non-fermenters

The most frequent etiologic agents of sepsis at MIMI correspond to the National data of bacteremia isolates (BulSTAR) (2,9).

The number of patients with bacteremia diagnosed with ESBL-producing Enterobacteriaceae is illustrated on Fig. 2.

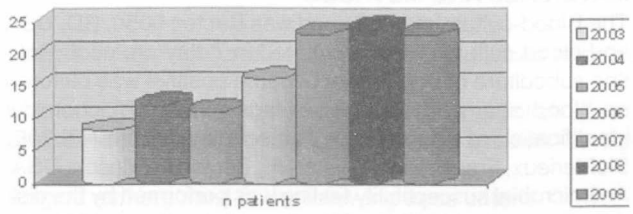
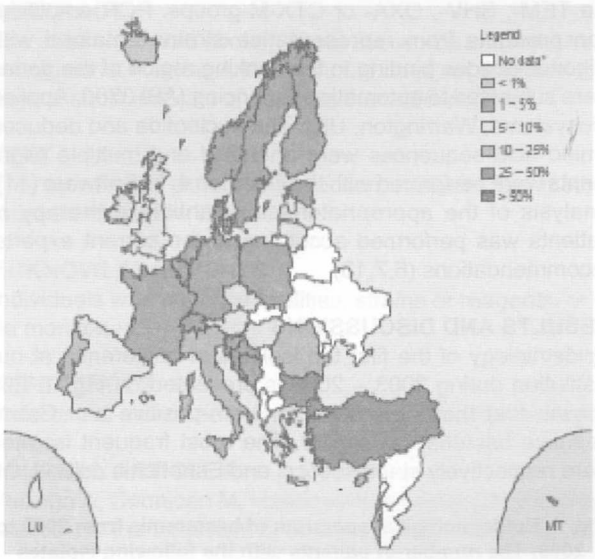


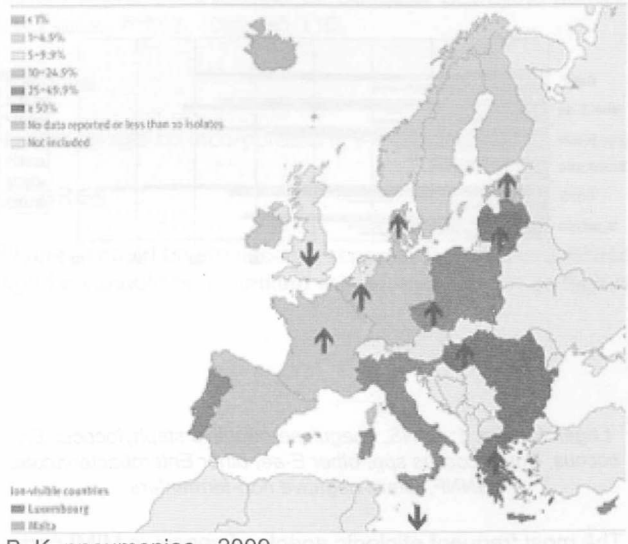
Fig.2. Dynamics of ESBL-producers from patients with bacteremia, 2003 – 2009

From 2003, when 8 patients had bacteremia due to ESBL-producing Enterobacteriaceae, till last tree years, their number increased triple.

Data about ESBL-producing Enterobacteriaceae has been traced both by the National programme BulSTAR (2,8) and the European, EARSS (TESSY) (5). Comparative data from these projects are presented on Fig. 3 and Fig. 4.



A. E. coli – 2008



B. K. pneumoniae - 2009

Fig. 3. Resistance to third generation cephalosporins in European countries (by EARSS) (5):

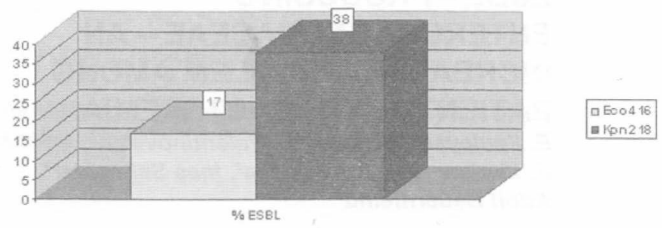


Fig. 4. National data from BulSTAR program about the frequency of ESBL-producers from blood-cultures in 2008 (2)

Legend: Eco, E. coli; Kpn, K. pneumoniae; 416, 218 – number of strains

The percentage, reported by EARSS may be higher because of the smaller number of isolates (a limited number of laboratories/hospitals participate in this project). Nevertheless, the number of cases with ESBL bacteremia is extremely high. Another problem is the multiple antibiotic resistance of ESBL-producing strains, due to multiple genes, gene cassettes, organized on a mobile genetic elements, most often transposons (4,12,15). The antimicrobial susceptibility of our isolates is summarized on Fig. 5 – Antibiotic susceptibility of ESBL producers from bacteremia in 2008.

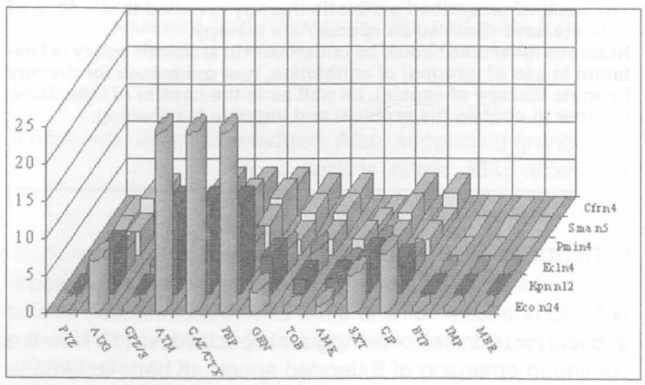
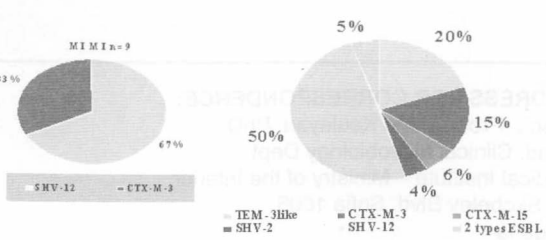


Fig. 5. Multiple antibiotic resistances of ESBL-producers – the example of 2008 (in 24 patients)

Legend: PTZ, piperacillin-tazobactam; AUG, amoxicillin-clavulanic acid; CPZ/S, cefoperazon-sulbactam; CAZ/CTX, ceftazidime/cefotaxime; FEP, cefepime; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; SXT, co-trimoxazole; CIP, ciprofloxacin; ETP, ertapenem; IMP, imipenem; MER, meropenem; Eco, E. coli, Kpn, K. pneumoniae; Ent, Enterobacter spp; Pmi, P. mirabilis; Sma, S. marcescens; Cfr; C. freundii

Some strains have been subjected to more detail evaluation: for them the type of ESBL has been determinated trough phenotypic and genotypic methods (11). The results obtained were summarized on Fig. 6.



It was clearly shown, that the pathogens from bacteremia at MIMI produced mainly two type of ESBL, SHV-12, followed by CTX-M-3, while the isolates from the national study exhibited variety of enzymes/enzymes groups, and even some of them produced a combination of ESBLs.

The next question, especially when it concerns severe systemic infections, is how the patients were treated: if they received the most appropriate antibiotics and dosage regimens. We performed audits about the appropriateness of treatment. Some important examples are summarized in Table 1 and Table 2.

Table 1. Audit of antibiotic prescriptions in 2008

Patient	Age, sex	Ward	Diagnosis	M. species	R / S to antibiotics	AB therapy - Comment
ASM	88, F	MICU	Heart insufficiency	E. coli	R FQ, S AG, SXT	AUG – not appropriate
GAG	58, M	Urology - MICU	Stenocardia	E. coli	R AG, FQ	
BSM	72, M	Urology	Adenoma gl. prostatae	E. coli	S AMK, FQ, CPZ/S	GEN, SXT, SAM – I not appropriate, then CIP - tailored
AGT	63, M	MICU	Auricle flicker	E. coli	R FQ, SXT S AG, PTZ	Ex letalis
ABV	68, F	Internal diseases	Infarctus cerebri	K. pneumoniae	R GEN, TOB, SXT	
GDE	73, M	Urology	Adenoma gl. prostatae	K pneumoniae	R AG, FQ, SXT	Carbapenem - appropriate
JZD	64, M	Surgery	Intestinal impassability	K pneumoniae	R AMK, S SXT, GEN, FQ	GEN, CIP – not appropriate

Legend: MICU, medical intensive care unit; M. species, microbial species; AB therapy, antibiotic therapy; ex letalis, exitus letalis; M, male; F, female; S, susceptible; R, resistant; I – intermediate; FQ, fluoroquinolons; AG, aminoglycosides; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; AUG, amoxicillin-clavulanate; SAM, ampicillin-sulbactam; CPZ/S, cefoperazone-sulbactam; PTZ, piperacillin-tazobactam; CIP, ciprofloxacin; SXT, co-trimoxazole

Table 2. Audit of antibiotic prescriptions in 2009

Patient	Age, sex	Ward	Diagnosis	M. species	R / S to antibiotics	AB therapy -Comment
9144	40, M	SICU	Pancreatitis ac.	K. pneumoniae	R GEN, FQ, SXT, SAMK	CRO, then IMP
1163	77, M	Urology	Cavesicae urinariae	K. pneumoniae	R AG, FQ, SXT	AMK, CIP, with improvement
NIZ	50, M	Pulmology	Pleuro-pneumonia, abscess, sepsis	E. coli	S GEN, PTZ, CPZ/S	AMK, CLI, METRO – I not appropriate, then AMK, MER - tailored, ex let
VA	82, M	Urology	Adenoma gl. prostatae	K. pneumoniae	R FQ, SXT S AG, I PTZ CPZ/S	CIP – inappropr.; GEN – appropriate; improvement
GSV	78, F	Urology	Calculus renis sin	E. coli	R GEN, TOB, SXT, FQ	AMK, (Metro), IMP - appropr.; improvement
VS	80, F	SICU	Cholangio-hepatitis	K. pneumoniae	S AG, PTZ, CPZ/S; R FQ, SXT	CPZ/S, AMK, Metro – inappropr; ex let
VAS	64, F	SICU	St. p. resectio gl. pancreatis, shock, sepsis	K. pneumoniae, (S. aureus)	R FQ, SXT; S PTZ, CPZ/S; I AG	LZD, FLU – inappr. for ESBL; ex let

Legend: SICU, surgical intensive care unit; M. species, microbial species; AB therapy, antibiotic therapy; ex let, exitus letalis; M, male; F, female; S, susceptible; R, resistant; I – intermediate; FQ, fluoroquinolons; AG, aminoglycosides; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; AUG, amoxicillin-clavulanate; SAM, ampicillin-sulbactam; CLI, clindamycin; CRO, ceftriaxone; CPZ/S, cefoperazone-sulbactam; PTZ, piperacillin-tazobactam; IMP, imipenem; CIP, ciprofloxacin; SXT, co-trimoxazole; LZD, linezolid; metro, metronidazole; FLU, fluconazole

The examples showed that the initial antibiotic therapy was not appropriate for more than half of patients; in some of them the second regimen was tailored. Among 14 patients there were four deaths; however this was probably related to the severe patient's condition: respectively heart disease, severe pleuro-pneumonia with abscess formation, surgery for cholangio-hepatitis in an elder sick patient, and septic shock and pancreatitis.

The review of most recent literature demonstrated similar findings: majority of patients with ESBL-producing Enterobacteriaceae bacteremia had more severe outcome (in controlled studies 2-3-fold more deaths, higher length of hospital stay and higher cost of hospital care (10,14,16,17)). Although some authors, describing E. coli bacteremia did not find statistically significant difference in experimental and control groups (3) (mainly bacteremia, secondary to urinary tract infection), majority concluded about the great importance of this resistance mechanism for the outcome of sepsis (1,7,10,13,15). Most of the authors underline the importance of the early appropriate empiric therapy 1(4,16,17). As to the antibiotic choice, the carbapenems are always the preferred choice. A question arrises: should the patients with suspected enterobacterial bacteremia receive carbapenem as empiric therapy in settings with high relative rate of ESBL? Our opinion is - not (because this may be followed by severe multi-resistance and pan-drug resistance (7,15)), each case should be thoroughly evaluated and empiric therapy decided correspondently.

In conclusion, we should summarize:

During the last years there was a clear increase in number of ESBL producers (CTX-M and SHV-groups) among bacteremia isolates: E. coli, K. pneumoniae and other Enterobacteriaceae.

The consequences resulted in limited treatment options for severe systemic infections: presented mainly by the carbapenem antibiotics, despite possible in vitro susceptibility to other beta-lactams. The second choice could be aminoglycosides and fluoroquinolones, alone or in combination, only if susceptible in vitro (this is also a limited option because the majority of ESBL producers are multiply resistant).

Due to these constrains, majority of patients did not receive the most appropriate treatment (especially early appropriate empiric therapy).

The increasing rate of ESBL producers imposes two major interventions:

- More strict Infection control, organized at Hospital and National level, incl. written report for each new case, and its evaluation
-New guidelines for empiric therapy of bacteremia, especially, if settings where the rate of ESBL – producers is > 25 %.

Both screening for ESBL producers at admission to hospital and improved empiric coverage should be applied for the patients at risk.

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A part of this work was presented at the 6th Balkan Congress of Microbiology, 28-31 October 2009, Ohrid, Macedonia.

ANTIMICROBIAL ACTIVITY OF TOBRAMYCIN AGAINST RESPIRATORY CYSTIC FIBROSIS ISOLATES OF PSEUDOMONAS AERUGINOSA

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SUMMARY

Tobramycin solution for inhalation (TSI) is indicated for the management of cystic fibrosis (CF) patients aged six years and older colonized with Pseudomonas aeruginosa. It is not recommended as an alternative to intravenous antibiotics for the treatment of acute exacerbations of pulmonary disease. Inhaled administration of tobramycin assures high concentrations in CF lungs, improving the therapeutic ratio over that of parenteral tobramycin levels, particularly against P. aeruginosa. Conventional Clinical and Laboratory Standards Institute (CLSI) breakpoints only consider parenteral levels and do not take into account these high antimicrobial concentrations. Therefore, the Spanish Antibiogram Committee (The MENSURA Group) has defined specific breakpoint values for inhaled tobramycin when testing P. aeruginosa isolates from CF patients (susceptible, ≤64 µg/ml; and resistant, ≥128 µg/ml). In this study the antimicrobial activity of tobramycin against 40 respiratory CF P. aeruginosa isolates, prospectively collected from 25 patients, was determined by high range Etest strips (LIOFILCHEM, Italy). The MIC at which 50% of isolates were inhibited (MIC50) and MIC90 were 0.75 and 3 µg/ml, respectively. Applying MENSURA proposed breakpoints, 95% of the strains were categorized as susceptible to tobramycin. With CLSI breakpoints, susceptibility rate decreased to 92.5%. The tobramycin activity against non-mucoid P. aeruginosa was higher than that against mucoid isolates (MIC50=0.75 and MIC90=2 µg/ml vs. MIC50=1 and MIC90=4 µg/ml). Moreover, the isolates obtained from patients untreated with TSI were more susceptible to the drug than those from patients received a maintenance therapy with TSI (MIC50=0.75, MIC90=1.5 µg/ml and MIC50=1.5, MIC90=6 µg/ml, respectively). Duration of colonization with P. aeruginosa did not influence the tobramycin antimicrobial activity against the tested respiratory isolates. In conclusion, tobramycin revealed an excellent in vitro activity against the studied CF isolates. The antimicrobial activity was dependent on the isolate morphotype and pre-administration of TSI. Whenever TSI is considered for therapy, the CF P. aeruginosa strains categorized as intermediate or resistant to tobramycin according to the CLSI criteria should be recategorized by using the MENSURA interpretive criteria.

Keywords: Tobramycin, antimicrobial activity, Etest, P. aeruginosa, cystic fibrosis.

ABBREVIATIONS USED IN THIS PAPER: CF – cystic fibrosis, TSI – tobramycin solution for inhalation, MIC – minimal inhibitory concentration, MIC50 and MIC90 – MIC at which 50% and 90% of the strains are inhibited, respectively, CLSI – Clinical and Laboratory Standards Institute, FEV1 – forced expiratory volume in one second.

INTRODUCTION

Pseudomonas aeruginosa has been recognized as a major pathogen in cystic fibrosis (CF) (9, 13). By age 18 years approximately 80% of CF patients in the United States are chronically infected with P. aeruginosa in their respiratory tracts (7). Isolation of this organism from sputum samples is closely associated with progressive deterioration in lung function (losing an average 2% of the lung function per year) and mortality in adolescents and adults (23). P. aeruginosa in CF airways mainly grows in a biofilm disposition with an inherent resistance (14) that may be, at least in part, overcome with high antimicrobial concentrations similar to those reached with the inhaled form of administration.

The main reason for inhaled treatments in CF pulmonary infections is to achieve high antimicrobial concentrations in the lungs. Nevertheless, a decrease in the potential side effects of the long-term treatment schedules frequently used in these patients and a lower risk of organism resistance development has also been advocated (19, 27). Tobramycin solution for inhalation (TSI) is indicated as a maintenance therapy in primarily chronically colonized or P. aeruginosa-infected CF patients aged six years and older (1). It is not recommended as an alternative to intravenous antibiotics for the treatment of acute exacerbations of pulmonary disease (3). Intermittent administration of inhaled tobramycin in conjunction with standard therapy for CF significantly improves forced expiratory volume in one second (FEV1), decreases the density of P. aeruginosa in expectorated sputum and reduces the need for hospitalization and intravenous antipseudomonal antibiotics (20). The intermittent schedule of administration of TSI is a possible strategy that may contribute to preserve susceptibility among P. aeruginosa isolates (11, 20, 21).

Qualitative interpretative categories (susceptible, intermediate and resistant) used in conventional susceptibility testing procedures are generally adapted for parenteral and/or oral treatments and do not consider the high local antimicrobial concentrations obtained when inhaled therapy is applied. Considering both sputum and epithelial lining fluid concentrations (8, 22) and cumulative clinical experience (8, 11, 20, 21), the Spanish Antibiogram Committee (The MENSURA Group) has defined specific breakpoints for aerosolized tobramycin (susceptible, ≤64 µg/ml; and resistant, ≥128 µg/ml) when P. aeruginosa isolates from CF patients are tested (16, 17). These breakpoints are significantly higher than those currently recommended by the Clinical and Laboratory Standards Institute (CLSI) (susceptible, ≤4 µg/ml; intermediate, 6 µg/ml; and resistant, ≥8 µg/ml) (5), which do not take into account the specific pharmacology of inhaled antimicrobials. The aim of this study was to assess the in vitro antimicrobial activity of tobramycin against respiratory P. aeruginosa isolates obtained from chronically colonized or infected CF patients and to establish the differences in this activity in respect with isolate morphotype, preliminary administration of TSI and patient age.

MATERIALS AND METHODS

Patients and bacterial isolates

The study included a total of 40 P. aeruginosa isolates prospectively collected between December 2005 and December 2009 from sputum samples of 25 patients (15 females and 10 males) aged 6-27 years attending the CF center of Alexandrovska University Hospital. Some isolates were obtained at the same time during follow-up of one patient, but they belonged to different morphotypes on the basis of texture (mucoid or non-mucoid, rough or smooth edge, colony size, and pigmentation). The eligibility criteria regarding the isolate selection were: P. aeruginosa present in a sputum culture within the previous 12 months; age of ≥6 yrs; and mild-to-moderate lung disease (FEV1 percent predicted ≥25% and ≤75%) (20). Isolates of P. aeruginosa recovered from sputa of patients admitted with an acute exacerbation were excluded from the present study. Distribution of the strains in respect with morphotype, patient age and pre-treatment with TSI is shown in Table 1. Bacterial identification was performed using a BBL Enteric/Nonfermenter ID system (Becton Dickinson).

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Table 1. Distribution (in number) of the studied respiratory cystic fibrosis isolates of P. aeruginosa (n=40) in respect with different features.

Morphotype	Number of isolates	Preliminary administration of TSI	Number of isolates	Patient age	Number of isolates
Non-mucoid	19	No	21	6-10 yrs	9
Mucoid	21	Yes	19	11-15 yrs	17
				16-20 yrs	7
				Over 20 yrs	7

TSI – tobramycin solution for inhalation.

Table 2. MIC values of tobramycin against respiratory cystic fibrosis isolates of P. aeruginosa – clinical-laboratory data.

Patient No	Patient initials	Age (yrs)	Date of isolation	Lab. No	Morphotype	Administration of TSI*	MIC [μg/ml]	S/R MENSURA
1	I.K.	10	08.12.2005	3a	non-mucoid, small	No	0.75	S
		10	08.12.2005	3b	non-mucoid, large	No	1.00	S
2	Y.P.	10	07.12.2005	2	non-mucoid	No	0.75	S
3	T.K.	21	14.12.2005	4	mucoid	No	0.25	S
		22	02.04.2007	43	mucoid	No	0.75	S
4	V.Y.	14	15.12.2005	5b	non-mucoid	No	0.38	S
5	A.I.	11	09.02.2006	6	mucoid	Yes	1.50	S
6	C.G.	15	20.02.2006	7	non-mucoid	No	0.19	S
		16	13.02.2007	38	mucoid	Yes	0.38	S
7	T.T.	12	18.05.2006	15-I	non-mucoid, brown	Yes	0.75	S
		12	18.05.2006	15-II	non-mucoid, green	Yes	2.00	S
		13	28.03.2007	44	non-mucoid, green	Yes	2.00	S
8	A.A.	15	20.02.2006	9	non-mucoid	No	0.75	S
		16	19.02.2007	40	mucoid	Yes	2.00	S
9	R.B.	12	28.03.2007	42	non-mucoid	Yes	3.00	S
		13	13.05.2009	59a	mucoid	Yes	>1024	R
10	V.S.	10	27.04.2006	12	non-mucoid	No	0.75	S
11	M.M.	11	09.05.2006	13	non-mucoid	No	0.75	S
		14	16.10.2008	56	non-mucoid	Yes	6.00	S
		15	10.12.2009	65	mucoid	Yes	>1024	R
12	M.S.	13	09.05.2006	14	mucoid	No	1.00	S
		16	16.07.2009	63	mucoid	Yes	4.00	S
13	T.P.	14	05.12.2006	33	mucoid	Yes	0.25	S
14	T.B.	12	13.06.2006	18	non-mucoid	No	1.50	S
15	E.K.	12	19.06.2006	20	mucoid	No	0.50	S
16	N.Y.	25	27.06.2006	21	mucoid	No	1.00	S
17	O.D.	27	27.06.2006	22	mucoid	No	0.75	S
18	G.N.	25	27.06.2006	23	non-mucoid	No	<0.064	S
		27	05.03.2008	50	mucoid	Yes	3.00	S
19	G.M.	6	30.06.2006	24	non-mucoid	No	0.38	S
		8	13.11.2008	58	mucoid	Yes	0.38	S
20	V.M.	10	07.09.2006	28	non-mucoid	No	1.50	S
		11	05.03.2008	49b	mucoid	Yes	0.75	S
21	V.T.	16	19.12.2006	35	mucoid	Yes	1.00	S
		18	12.11.2008	56	mucoid	Yes	1.00	S
22	M.T.	17	21.02.2007	41	mucoid	No	0.25	S
23	Y.K.	20	28.01.2008	48	mucoid	No	2.00	S
		21	03.12.2009	64	mucoid	Yes	1.00	S
24	B.K.	6	30.05.2009	60	non-mucoid	Yes	0.38	S
25	R.M.	10	22.12.2009	66	non-mucoid	No	0.75	S

MIC – minimal inhibitory concentration, TSI – tobramycin solution for inhalation, S – susceptible, R – resistant.
* The patient is treated with inhaled tobramycin before the strain isolation.

CF exacerbation diagnostic criteria

An acute exacerbation was defined, as per the 1994 Cystic Fibrosis Foundation Microbiology Consensus Conference (6), as the presence of at least 3 of 11 new clinical findings including: increased cough, sputum production, fever, weight loss, school or work absenteeism, increased work of breathing, decreased exercise tolerance, or a deterioration in the chest exam, chest radiograph, FEV1, or hemoglobin saturation.

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of tobramycin were determined using high range (<0.064 – >1024 μg/ml) Etest strips (LIOFILCHEM, Italy) on Mueller-Hinton agar (Becton Dickinson) plates, according to the manufacturer's instructions. P. aeruginosa ATCC 27853 was used as a quality control strain. The strains were categorized considering the breakpoints proposed by The MENSURA Group for inhaled tobramycin (16, 17).

RESULTS AND DISCUSSION

Evaluation of the tobramycin activity against all studied CF strains of P. aeruginosa

The MICs values of tobramycin against the 40 studied CF P. aeruginosa isolates as well as clinical-laboratory data regarding the patients and strains are presented in Table 2.

The MIC at which 50% of isolates were inhibited (MIC50) and MIC90 were 0.75 and 3 µg/ml, respectively. Applying MENSURA proposed breakpoints, 95% of the strains were categorized as susceptible to tobramycin. With CLSI breakpoints, susceptibility rate decreased to 92.5% (Table 3). The strain with Lab. No. 56 isolated from M.M. (Table 1, patient No. 11) was inhibited at MIC of 6.00 µg/ml and was considered as intermediate according to CLSI-2007 breakpoints, but it was characterized as susceptible when MENSURA breakpoints were applied.

MIC – minimal inhibitory concentration; MIC50 and MIC90 – MICs at which 50% and 90% of the strains are inhibited, respectively.

There is some controversy in the literature about whether the mucoid phenotype of P. aeruginosa results in increased resistance. The concept of increased resistance in mucoid isolates is prevalent in the literature (10, 14) and in clinical microbiology lore. Mah et al. established that biofilms themselves are not simply a diffusion barrier to the antibiotics,

Table 3. MIC range, MIC50 and MIC90 of tobramycin determined by high-range E-tests strips against the studied respiratory cystic fibrosis isolates of P. aeruginosa.

			CLSI-2007 interpretation No (%) of strains:			MENSURA interpretation No (%) of strains:		
MIC range [µg/ml]	MIC50 [µg/ml]	MIK90 [µg/ml]	S ≤4 µg/ml	I 6 µg/ml	R ≥8 µg/ml	S ≤64 µg/ml	I NA	R ≥128 µg/ml
<0.064 - >1024	0.75	3.00	37 (92.5)	1 (2.5)	2 (5.0)	38 (95.0)	–	2 (5.0)

MICs – minimal inhibitory concentrations, MIC50 and MIC90 – MICs at which 50 % and 90 % of the isolates are inhibited, respectively, S – susceptible, I – intermediate, R – resistant, CLSI – Clinical and Laboratory Standards Institute; NA – not applicable.

It is well known that the Etest, a stable antimicrobial gradient method for MIC determination, ensures accurate results and is easy to perform (15). In the case of CF patient isolates, Etest methodology assures a timely evaluation of the problem of resistance within a group of patients for whom the need of an adequate therapeutic approach is essential, since they are frequently colonized with multiresistant P. aeruginosa strains for which few treatment options exist. The antimicrobial concentrations displayed in the tobramycin high-range Etest strips give a more precise reflection of the true levels reached in pulmonary secretions when this antibiotic is delivered by inhalation (8).

The percentage of tobramycin susceptible isolates (95%) was identical to that of the entire collection comprising recently isolated CF P. aeruginosa from a University Hospital in Madrid (Spain) (17) when MENSURA criteria were adopted. The resistance rate of our CF isolates according to CLSI breakpoints (MICs ≥8 µg/ml) was similar to that of English CF patient isolates of P. aeruginosa obtained from 17 UK hospitals in 2000 (5% and 10.1%, respectively) (18).

Comparative activity of tobramycin against mucoid and non-mucoid CF P. aeruginosa strains

MIC distributions of tobramycin against non-mucoid and mucoid strains included in the study are presented in Fig. 1. As it is shown, MICs were generally low, but the in vitro antimicrobial activity of tobramycin (evaluated by MIC50 and MIC90) was slightly higher against non-mucoid CF isolates of P. aeruginosa.

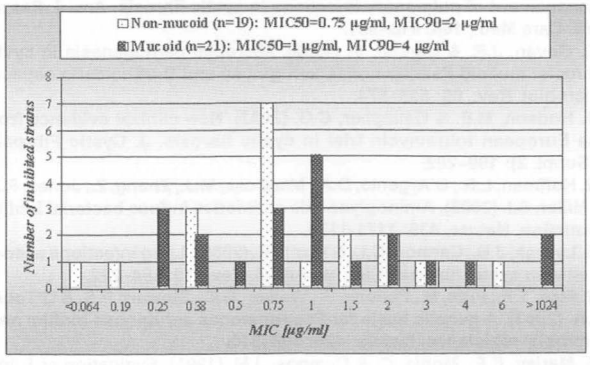

Fig. 1. MIC distributions of tobramycin against different morphotypes of cystic fibrosis P. aeruginosa strains.

but rather that bacteria within these microbial communities employ distinct mechanisms to resist the action of antimicrobial agents (14). They reported a genetic determinant (ndvB locus) of the high-level resistance in biofilm-forming P. aeruginosa. This locus is required for the synthesis of periplasmic glucans which interact physically with tobramycin and may prevent antibiotics from reaching their sites of action by sequestering these antimicrobials in the periplasm. Recently, Zhang & Mah identified a novel efflux pump in P. aeruginosa that is important for biofilm-specific resistance to a subset of antibiotics including tobramycin (28). Although traditionally mucoid P. aeruginosa isolates have higher resistance, there are enough scientific reports describing more susceptible CF mucoid P. aeruginosa strains compared with non-mucoid strains (4, 25, 26).

Relationship between the tobramycin activity and preliminary administration of inhaled tobramycin

MIC distributions of tobramycin against strains isolated from CF patients untreated with TSI and those isolated after administration of TSI are shown in Fig. 2.

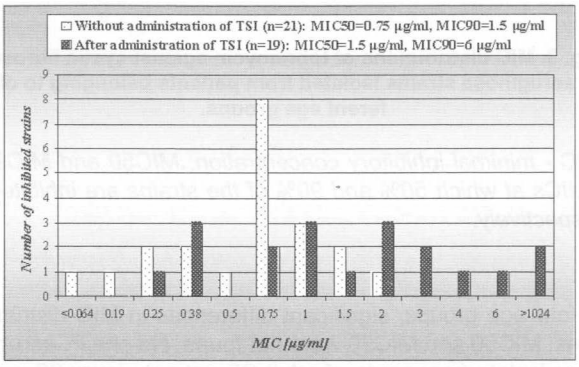

Fig. 2. MIC distributions of tobramycin against cystic fibrosis P. aeruginosa strains isolated from untreated and treated with TSI patients.

MIC – minimal inhibitory concentration; MIC50 and MIC90 – MICs at which 50% and 90% of the strains are inhibited, respectively; TSI – tobramycin solution for inhalation.

The in vitro antimicrobial activity of tobramycin (evaluated by MIC50 and MIC90) was higher against P. aeruginosa strains obtained from CF patients untreated with TSI than that against strains isolated from treated (intermittent regimein of inhaled tobramycin, i.e. 28 days on drug, followed by 28 days off) CF patients. In a study using aerosolized tobramycin in CF patients, a trend towards more resistant strains was seen in the tobramycin-treated group compared with the placebo-treated patients (2). The tobramycin MIC50 for all P. aeruginosa isolates was 1 µg/ml at all time points for both the tobramycin and the placebo groups. In the tobramycin group, the MIC90 increased from 8 µg/ml (week 0) to 16 µg/ml (weeks 20 and 24). In the placebo group, the tobramycin MIC90 decreased from 8 µg/ml (weeks 0 and 20) to 4 µg/ml (week 24).

An important finding in our study was the conversion of non-mucoid P. aeruginosa strains to mucoid strains in seven CF patients received TSI during a period of 1-2 years (Table 1, patient numbers: 6, 8, 9, 11, 18, 19 and 20). Recently, Hoffman et al. showed that subinhibitory concentrations of aminoglycosides induce biofilm formation in P. aeruginosa (12). They identified a gene, designated as aminoglycoside response regulator (arr), which was essential for this induction and contributed to biofilm-specific aminoglycocide resistance. Their results demonstrated that biofilm formation can be a specific, defensive reaction to the presence of antibiotics, and indicated the molecular basis of this response.

Relationship between the tobramycin activity and duration of P. aeruginosa colonization

MIC distributions of tobramycin against strains isolated from CF patients belonging to different age groups are presented in Fig. 3.

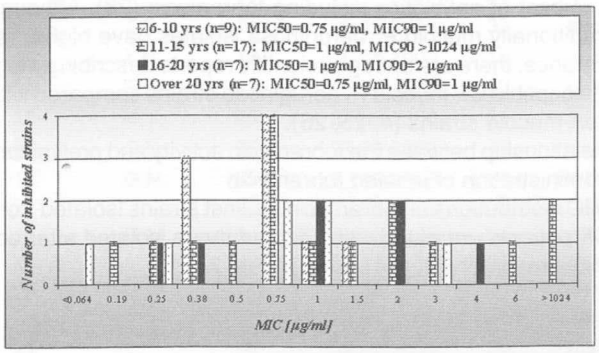

Fig. 3. MIC distributions of tobramycin against cystic fibrosis P. aeruginosa strains isolated from patients belonging to different age groups.

MIC - minimal inhibitory concentration; MIC50 and MIC90 - MICs at which 50% and 90% of the strains are inhibited, respectively.

In four age groups, significant differences in MIC distributions, MIC50 and MIC90 were not found. No one P. aeruginosa isolate from sputa of adult CF patients (over 20 yrs), with long-term colonization, was inhibited at MIC>3 µg/ml. In comparison, 35.8% of the studied P. aeruginosa strains isolated from 57 adult CF patients at Freiburg University Hospital during 1998-1999 were inhibited at MICs of tobramycin >4 µg/ml (non-susceptible strains according to CLSI-2007 breakpoints) (24). The main cause for unusually high susceptibility to tobramycin among our CF P. aeruginosa isolates obtained from adult patients was the antibiotic policy in Bulgaria (until recently, difficulties in the supply of inhaled therapy with tobramycin, because of the high price and the necessity of prolonged use of the drug).

CONCLUSIONS

Tobramycin revealed an excellent in vitro activity against the tested respiratory CF isolates of P. aeruginosa. The antimicrobial activity was dependent on the isolate morphotype and pre-administration of TSI, but there was no relationship between patient age (i.e. duration of colonization) and resistance of the isolates recovered from sputa. The non-mucoid strains and those isolated from patients untreated with aerosolized tobramycin were inhibited at lower MICs (MIC50=0.75 µg/ml; MIC90≤2 µg/ml) than the strains with mucoid morphotype and recovered from patients treated with the drug (MIC50≤1.5 µg/ml; MIC90≤6µg/ml). Whenever TSI is considered for therapy, the CF P. aeruginosa strains categorized as intermediate or resistant to this antibiotic according to the CLSI criteria should be recategorized by using the MENSURA interpretive criteria for the inhaled tobramycin formulation. CLSI criteria should still be applied when intravenous tobramycin is prescribed in CF patients, particularly during the course of exacerbations.

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PROSTHETIC VALVE ENDOCARDITIS CAUSED BY CANDIDA RUGOSA

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SUMMARY

Here we described the first case in world practice of prosthetic valve endocarditis caused by Candida rugosa. Due to blocked prosthetic valve, operation required valve replacement. From the valve, a huge amount of Candida rugosa was isolated and etiological treatment was conducted. Nine months later, the valve was again affected and replaced and new C. rugosa strain was isolated.

Seven species of the genus Candida are well known opportunistic pathogens in humans and many others are described as pathogens in individual cases or short series of cases Evidence of the clinical significance of a rare species, should be conclusive, because some published identification of atypical yeasts are not very clear and are unconfirmed by international reference laboratories and the strains have not confirmation for clinical importance. Therefore, it is not surprising when some of these yeasts are occasional sources of infection in severely immunocompromised hosts.

Here we described the first case in world practice of prosthetic valve endocarditis caused by Candida rugosa (1). Male 39 years old (N.I.P.,) underwent heart surgery 18 years ago in Germany endoprosthesis of the mitral valve. Until two weeks ago he felt in good health when a sharp deterioration in hemodynamics unconscious emergency hospitalization on occasion blocking prosthetic valve. Operation required valve replacement because it sprouted with micelles and clotting. From the valve, a huge amount of Candida rugosa was isolated. Serological tests to determine strain resistance were carried out. Antibodies in patient's sera to antigens of Candida albicans were not confirmed by indirect immunofluorescence reaction (1: 80, IgA and IgM). However, the immune response to antigens of Candida rugosa was a total of 1: 640, IgA lacking, and IgM 1: 2. (Study done on 14/07/1994). After the new endoprosthesis, longer course of antibiotics was held, initially with Amphotericin B 0,8 mg / kg / day for 28 days, but due to anemia and increasing urinary nitrogen treatment was changed to Fluconazole 400 mg / day for three months. Nine months after surgery, the patient was in excellent health when suddenly a pain appeared from the back of the thigh, judged by the physician as a manifestation of disc hernia. After a week, the patient reported increasing pain, which required hospitalization and examination by a vascular surgeon. Peripheral artery surgery performed thrombectomiya and elongated microscopic hyphen and yeast culture in thrombus proved Candida rugosa. Echocardiography visualized valvular vegetation. Operational replacement of the affected valve was made. On the second week of therapy with Amphotericin B, the antibiotic was stopped, due to intolerance. Continuous treatment with Fluconazole was started. At the beginning of therapy MIC of Fluconazole was 2 mg /L. Two months later, back surgery was done due to another and sprouting vegetation on the valve. A month later, C. rugosa strain was isolated and the strain showed minimum inhibitory concentration 64 mg / L. This change of drug resistance during treatment caused therapeutic difficulties.

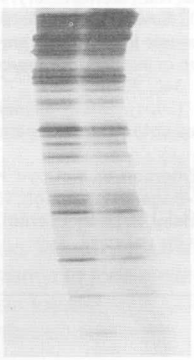

Fig1

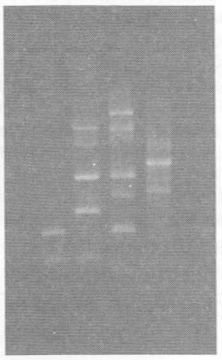

Fig2

Both strains of C. rugosa, isolated at baseline (1) and after acquisition of resistance to terminal (2) from a patient with endocarditis, marked by two genetic method AFLP (left) and RAPD (right). Considered more sensitive method showed no differences in the strains, while RAPD-PCR observed significant differences, probably accidental primer in the area of a mutation leading to resistance to Fluconazole, is the reason for the differences.

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SEROPREVALENCE OF POLIOVIRUS ANTIBODY IN BOURGAS REGION, BULGARIA

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SUMMARY

The last three cases of poliomyelitis in Bulgaria occurred in 2001 after 9-years period of zero-reported cases as a result of wild poliovirus importation from Indian subcontinent. In 1959 oral polio vaccine (OPV) was introduced for routine use for all eligible infants and children. In 2007, OPV was replaced by trivalent inactivated polio vaccine (IPV). The overall vaccination coverage using administrative method sustains regularly high - 94.24% (86.08-98.17). In order to estimate the herd immunity against polioviruses on a district level 250 hospitalized patients were tested for antibody presence for polioviruses types 1, 2 and 3 using microneutralization assays. The overall antibody prevalence for polioviruses types 1, 2 and 3 was 90.00%, 93.60% and 80.4% respectively. Increasing of antibody prevalence for all three types polioviruses by age was detected which revealed the necessity of national seroprevalence study for identifying seroprevalence of poliovirus antibody in population, immunization gaps and unprotected groups as well.

INTRODUCTION

Information about polio incidence in Bulgaria is available from 1927, but detailed data has been collected since 1940 (Figure 1). A total of 4586 cases included 76 deaths were registered in 1940-2008. The incidence peaked in 1957 with 1065 affected people resulting into implementation of trivalent oral polio vaccine (OPV) in 1959 with 3 doses for infants and boosters at 2 and 7 years [1]. The last three cases of poliomyelitis in Bulgaria occurred in 2001 after 9-years period of zero reported cases as a result of wild poliovirus importation from Indian subcontinent [2,3]. In 2002 Bulgaria is certified as a polio-free country.

The immunization schedule consisting of 6 doses OPV has been slightly changed several times according to age and time intervals for vaccine administration. In 2007, OPV was replaced by trivalent inactivated polio vaccine (IPV).

The overall vaccination coverage using administrative method sustains regularly high. For the last 15 years, the average coverage with three doses polio vaccine is 95.54% (93.64-97.22) and with completed scheme – 94.24% (86.08-98.17).

The aim of this study is to estimate the herd immunity on a district level. Since the poliomyelitis outbreak 2001 started among unvaccinated children in Bourgas, the herd immunity in this district was investigated.

METHODS

Blood samples were drawn from 250 randomly selected patients in several hospitals in Bourgas region.

Antibody titers to types 1, 2 and 3 poliovirus were determined at the National Enterovirus Laboratory, Sofia using microneutralization assays as recommended by WHO [4]. The assigned serum antibody titer was the highest dilution of serum that protected 50% of the cultures against 100 TCID50 of the challenge virus. Antibody titers were expressed as reciprocals of the dilution. Sera in which the neutralization titer was less than 8 were considered negative. Poliovirus vaccine strains Sabin 1, Sabin 2 and Sabin 3 were used as references.

Additional information only about age was gathered.

The data were entered in Excel and analyzed by EpiDataAnalysis.

RESULTS

The overall antibody prevalence for polioviruses types 1, 2 and 3 was 90.00%, 93.60% and 80.4% respectively.

Antibody prevalence for type 1 was highest in people > 30 years (100%) that determined them as fully protected but lowest in children up to 9 years (0-4 y -79.07%, 5-9 y - 85.71%). For poliovirus type 2 titers were highest in people of 40s (100%) and lowest in children 5-9 years – 82.14%.

Data for type 3 showed highest percentage of seroconversion in people over 40 years (100%) and lowest prevalence in teenagers and young people (73.85% and 76.56%). (Table 1).

Five children (12 m, 24 m, 5 y, 9 y, 16 y) were negative for all three types' polioviruses.

Taking into account the relevant vaccine status of studied group tailored to the national immunization schedule the highest antibody prevalence for poliovirus types 1 and 3 was found in persons with natural acquired immunity (born before 1959) following by vaccinated with live attenuated vaccine. For poliovirus type 2, people with OPV were protected in 94.12% compared to 87.5% vaccinated with IPV and non-vaccinated as well. (Table 2).

DISCUSSION

The highest level of antibody (> 90% seropositive persons) was established against poliovirus type 2 in almost all age groups (except 5-9 years and >50 years).

For poliovirus type 1, more than 90% seroprevalence were observed in age group 10-19 years and in people over 30 years. For poliovirus type 3, presence of > 90% seropositive persons were found only in people over 40 years.

The lowest rates for seroconversion were registered for persons born in 2007-2008 (vaccinated with IPV) taking into consideration the number of vaccine doses received due to completed months of age. Other studies had been estimated antibody prevalence to IPV varied from 67% to 99% against type 1, 65% to 99% against type 2, and 91% to 100% against type 3 [6].

Increasing of antibody prevalence for all three types polioviruses by age was detected in our study which highlighted long-lasting persistence of serum antibody after natural infection with poliovirus in people born before 1959 [5,7]. We should be aware that seroconversion rates to polio could be influenced by OPV mass vaccination implemented for almost 50 years in Bulgaria [6].

Our study shows inadequate seroconversion for polioviruses in infants and children which defines the necessity of national seroprevalence study for identifying immunization gaps and unprotected groups in population and immunization effectiveness as well.

There are two limitations that need to be acknowledged and addressed regarding the present study - small size of studied population, which is not reflects the real age distribution, and lack of immunization data.

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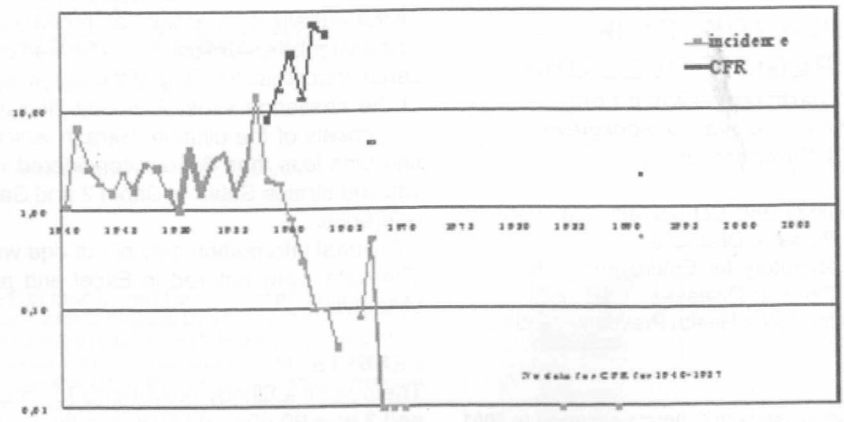

Figure 1. Poliomyelitis in Bulgaria, 1940-2008

Table 1. Antibody prevalence by age groups

		Poliovirus type 1		Poliovirus type 2		Poliovirus type 3	
Age group	N tested	%	95% CI	%	95% CI	%	95% CI
0-4	43	79.07	66.91-91.23	93.02	85.41-100.63	81.4	69.76-93.03
5-9	28	85.71	72.75-98.67	82.14	67.96-96.33	89.28	77.83-100.74
10-19	65	93.85	88.00-99.69	96.92	92.73-101.12	73.85	63.16-84.53
20-29	64	87.50	79.39-95.60	96.87	92.61-101.13	76.56	66.18-86.94
30-39	36	100		91.67	82.64-100.69	83.33	71.16-95.51
40-49	6	100		100		100	
> 50	8	100		87.5	64.58-110.42	100	
Total	250	90.00	86.28-93.72	93.60	90.57-96.63	80.4	75.48-85.32

Table 2. Immunity and immunization status

	Vaccinated with IPV (n=16)		Vaccinated with OPV (n=221)		Non vaccinated (>50 years) (n=8)	
Poliovirus	Immune %	Non-Immune %	Immune %	Non-Immune %	Immune %	Non-Immune %
Type 1	75.00	25.00	90.71	9.29	100.00	-
Type 2	87.5	12.5	94.12	5.88	87.5	12.5
Type 3	68.75	31.25	80.10	19.90	100.00	-

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UPON SOME EPIDEMIOLOGICAL, CLINICAL AND LABORATORY CHARACTERISTICS OF THE OUTBREAK OF MEASLES IN THE REGION OF PLOVDIV, FEBRUARY-JUNE 2010

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Introduction: Measles as a highly infectious disease that can result in serious complications returned in Bulgaria. Launched in 2009 a major outbreak covered the majority of the population in the Roma districts of Plovdiv and the region. The reasons for this vary from high-risk populations unimmunized for cultural or religious beliefs to low knowledge of the means of transmission and severity of measles. Objectivity required accepting the fact that infectious diseases and responsibility of them challenge the health system to increase the immunization – coverage up to 95%.
Material and methods: During February and July, incl. 2121 cases of measles were registered and 1969 of them were hospitalized in the University Clinic of Infectious Diseases, town of Plovdiv. Laboratory tests were conducted on the standard methodology; virus and serological parameters were investigated in Regional Public Health Institute. The data were statistically processed with SPSS 14 analysis system, using parametric methods in Gaussian distribution and nonparametric when needed. As a significant difference interval was accepted p <0.05, guaranteeing 95% confidence.
Results and discussion: The highest incidence of measles was reported during April and May, 34.3% of hospitalized patients. Mainly medium – heavy forms of disease were observed. 65% of treated were children between 1 to 18 years. Measles complicated with pneumonia was found in 504 patients – 25.6%. Pronounced respiratory failure and need of oxygen therapy had 59 fellows. Antibiotics received all complicated cases. X – Ray control was achieved in 74.3 % of lung – affected. We observed complications of the nervous system in 7 patients, aged 8 months to 52 years. Measles, complicated with meningitis – two cases, viral encephalitis – 4 and one 8 years old boy with meningomyelitis.
Conclusion: Outbreak of measles in Plovdiv and the region in 2010 once again put reasonable challenges of organizational, financial, legal and social - legal aspect to epidemiologists and infectious diseases in particular and the healthcare system in the country as a whole. The neurological complications were rare – in analyzing study 0.36% with benign ending.

Keywords: Measles, meningitis, immunization.

INTRODUCTION:

Measles as a highly infectious disease that can result in serious complications returned in Bulgaria. Launched in 2009 a major outbreak covered the majority of the population in the Roma districts of Plovdiv and the region. The reasons for this vary from high-risk populations unimmunized for cultural or religious beliefs to low knowledge of the means of transmission and severity of measles. Objectivity required accepting the fact that infectious diseases and responsibility of them challenge the health system to increase the immunization – coverage up to 95%.

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AIM:

The aim of the study is to analyze the epidemiological, clinical and laboratory characteristics of the patients with measles, treated in the Clinic of Infectious Diseases – University Hospital, Plovdiv during the first six months of 2010.

MATERIAL AND METHODS:

During February and July, incl. 2121 cases of measles were registered and 1969 of them were hospitalized in the University Clinic of Infectious Diseases, town of Plovdiv. Laboratory tests were conducted on the standard methodology; virus and serological parameters were investigated in Regional Public Health Institute. The data were statistically processed with SPSS 14 analysis system, using parametric methods in Gaussian distribution and nonparametric when needed. As a significant difference interval was accepted p <0.05, guaranteeing 95% confidence.

RESULTS AND DISCUSSION:

1. From hospitalized 1969 cases 51.5% were mail. The distribution of the patients by months from January to June 2010 shows the dynamic of the outbreak. It began relatively smoothly with few cases during January and February (respectively 1.4% and 3.6% of all cases). They increased essentially in March (12.7%) and reached their maximum in April (34.3%) and May (29.3%). In June a great decrease of the measles-cases was notified (18.6%) and subsidence of the outbreak. During the following days (up to the middle of August) a few cases were registered, a total of 41 /diagram 1/.

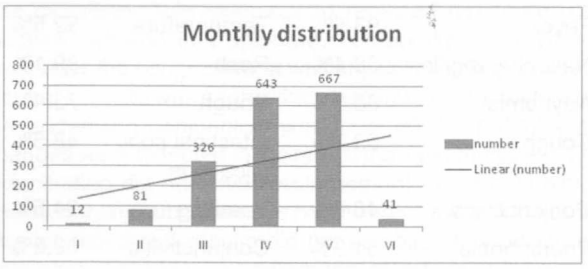

Diagram 1

2. The analysis of the age distribution (diagram 2) disclosed that the number of patients is the highest among children aged 13 months to 12 years (51%). This is the age group which includes the period from the vaccination with the first dose MMR (at 13 months) to the administration of the second dose (at 12 years). It was followed by the cases of infants aged 4 weeks to 13 months (27%). The diseased from the elder age groups were less, respectively: 13-18 years (11%), 19-35 years (9%), >35 years (3%) /2/.

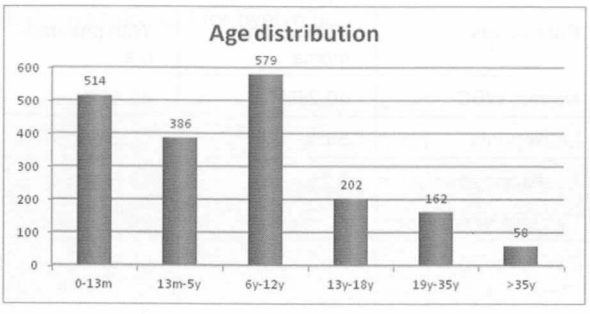

Diagram 2

3. The age distribution by months (diagram 3) did not show deviation from the age distribution of the case-patients for the whole period of the outbreak. There was no shifting of incidence of cases from one group to another /1, 3/.

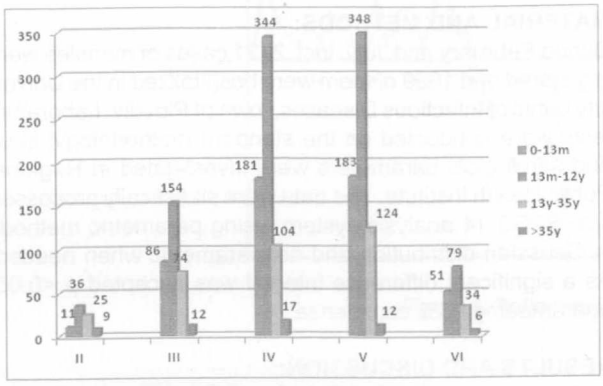

Diagram 3
The age distribution by months

More of the cases (65%) were epidemiologically linked to another patient with measles and in 35% the initial case was not discovered /3, 9/.

4. Incubation period for measles is an average of 9 days. *Clinical symptoms in the catarrhal period: Average hospital stay is 6.5 days:*

Fever	93.5%	Temperature	92.5%
Nasopharyngitis	88.4%	Rash	99.1%
Adynamia	68.3%	Cough	73.4%
Cough	59.2%	Intestinal complications	48.5%
Conjunctivitis	48.6%	Vomiting	24.5%
Photophobia	51.3%	Conjunctivitis	69.3%
Vomiting	38.5%	Pneumonia	25.6%

For 2/3 of the cases (64%) isolation was timely – on the first day after rash eruption or even during the last days of the catarrhal period. Cases of late isolation were not few in number (36%) – they were isolated on the second or third day after rash eruption /7,8/. The main reason for the delay was tardy seeking of medical attention and in some of the cases – delay in the transfer of patients from other clinics.

5. Laboratory investigations.

Parameters	Without pneumonia	With pneumonia
Normal WBC	40.25%	80.40%
Leukopenia	58.%	13.6%
Leukocytosis	1.2%	13.6%
Lymphocytosis	38.5%	26.3%
ESR over 20 mm/h	21.3%	66.3%

Carried our research do not differ significantly from those in the open literature. This also applies to other manifested signs of the disease /11, 12/.

6. Measles, complicated by pneumonia is found in 504 patients /25.6%/. Marked respiratory failure and need for oxygen is required in 59 patients. Antibiotic therapy is conducted in all cases of complicated measles. X-ray control was achieved in 14.3% of lung injury.
What distinguishes the epidemic of measles in the Plovdiv region is the frequency of neurological complications /1, 2/. In seven patients were observed lesions of the nervous system - four patients with encephalitis symptoms, incl. a woman with cerebelitis, two viral meningitis, meningomielitis - a 3 years old child.

CONCLUSION:
Outbreak of measles in Plovdiv and the region in 2010 covered more than 2,000 people, mostly Roma and incidence reached 270% ooo.
The main cause of the disease are gaps in prophylactics, which imposes a significant increase in immunization coverage among the Roma population.
Neurological complications are rare, with a dramatic move, but with a benign outcome.

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SUBCONJUNCTIVAL DIROFILARIASIS CAUSED BY D. REPENS: A CASE REPORT

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Abstract

Dirofilariasis caused by D. repens is a natural-focal zoonosis. Dogs are the main dead-end host and reservoir of the parasite, and mosquitoes of the Aedes, Anopheles и Culex families are vectors. Humans are non-specific, accidental host and not a source of infection. Canine dirofilariasis is found in the southern and eastern parts of Europe. Isolated cases of human dirofilariasis in Bulgaria have been reported during the last decades. We report a case of subconjunctival localization of dirofilariasis. A lithe helminth of cylindrical shape, 14 cm in length was extracted from the subconjunctival space. The histological examination of the helminth revealed a cuticle with longitudinal ridges with transverse striations. The size of the parasite, the typical localization and the morphology of the tegument made us assume that the case described was one of ophthalmic dirofilariasis caused by D. repens.

Key words: D. repens, subconjunctival dirofilariasis

Dirofilarioses are natural endemic zoonoses. The causative agents - dirofilariae are thread-like, dioecious helminths of the Dirofilaria species, classified in the Filariidae family. They can parasitize in the heart, blood vessels and subcutaneous tissues in carnivorous animals, dogs being the major dead-end host and reservoir for the parasite. Mosquitoes of the Aedes, Anopheles и Culex families are intermediate hosts and vectors (2, 5, 11).

The adult parasites release numerous microfilariae, which invade the peripheral vessels of the infected mammal. When a mosquito bites the animal, it ingests micofalariae. The mosquito serves as an intermediate host for 10-16 days, during which period the microfilarial larvae undergo stages in their development to become infective. When the mosquito bites other animals, it injects the larvae into their blood.

Humans are infected by chance, and non-specific hosts for the parasite. They are not a source of infection (2, 5, 11).

There are about 40 species of dirofilariae identified, though only several of these have been found to infect humans. Of most importamce for the clinical practice are the Dirofilaria immitis and Dirofilaria repens species, parasitizing predominantly on dogs, cats, jackals, foxes and other dead-end hosts. The invasion of Dirofilaria repens is the most common dirofilariasis worldwide, affecting both animals and humans (10). It is a dioecious, thread-like nematode. The female worms are about 10-15 cm in length, while the male ones are much shorter, measuring 5-7 cm. The adult worms reach 1-2 mm in diameter (1,11). The cuticle is with longitudinal ridges with transverse striations. This is a characteristic feature of the D. repens, which makes it possible to distinguish it from D. immitis (20). D. Repens is found in the subcutaneous connective tissue in animals, while in humans it is found in the subcutaneous and submucosal tissues and the eye (13).

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The first case of human dirofilariasis was reported by Addario in 1885 in Milan, Italy. The parasite was found in the conjunctiva, and was called Filaria conjunctivae. Later it was established that the parasite described was of the Dirofilaria repens species (10). Canine dirofilariasis is mostly spread in the southern parts of Europe, South and Central Asia. Over the last decades, 800 cases of infection in humans have been reported for these areas (13).

The first case of human eye dirofilariasis was reported by Prof. K. Pashev in 1928 (4). In 1961, Manev and Bachvarov reported another case of eye dirofilariasis. The parasite was extracted from an eyelid and identified as Dirofilaria repens (3). Since the 1990s, isolated cases of dirofilarioses in humans have been reported in the country, and described by Vuchev et al. (1, 2, 7, 18, 19).

The case presented here was diagnosed in the Pleven region.

Case report

A female patient, aged 73, resident of Glava village, Pordim municipality, was bitten by an insect in the left of the face in mid-August. A day later, a swelling formed on the site of the bite, red and itchy. The swelling spread and affected the skin around her left eye. The patient was seen by a general practitioner who prescribed Allergosan for topical application, and the symptoms quickly subsided.

Two months later, itching appeared in the medial part of the left eye, accompanied by a periorbital swelling. The patient reported feeling the presence of a foreign body in the eye, and described it as "dust in the eye". There was no history of tearing, deterioration in vision, skin allergic reactions or high temperature. The patient was referred to an ophthalmologist. The biomicroscopic examination revealed moderate conjunctival injection and epiphora. Temporally to the limbus and under the conjunctiva, a whitish, slow-moving, coiled in a spiral parasite was seen, which became more active when the underlying conjunctiva was touched. No pathological changes were detected in the rest of the eye structures. The examination did not reveal enlargement of regional lymph nodes, nor any subcutaneous nodules anywhere in the body. The full blood test results did not establish any deviations from normal ranges. The differential blood count did not reveal eosinophilia. Blood smears, stained after Romanovski-Gimsa, were examined three times for microfilaremia, and no microfilariae were detected.

Under local anesthesia with drops of Alcaine, a linear 3-4 mm incision in the conjunctiva was made, right across from the parasite. A sterile hooked needle was used to catch a coil of the worm and pull it out of the subconjunctival space. After the operation, the patient was treated with antibiotics and corticosteroids. All symptoms disappeared several days after removal of the helminth.

The cuticle of the helminth extracted was whitish; the body was cylindrical in shape, 12-14 cm in length and about one millimeter in diameter. (Fig.1)

When the parasite was placed in physiological serum, motor activity was present for twelve hours.

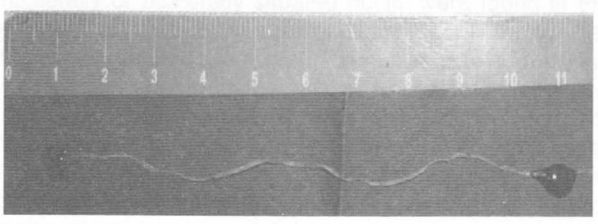

Figure 1. The adult D. Repens, extracted from the subconjunctival sac.

The histologic microscopic examination ascertained a helminth with longitudinal ridges with transverse striations. (Fig.2)

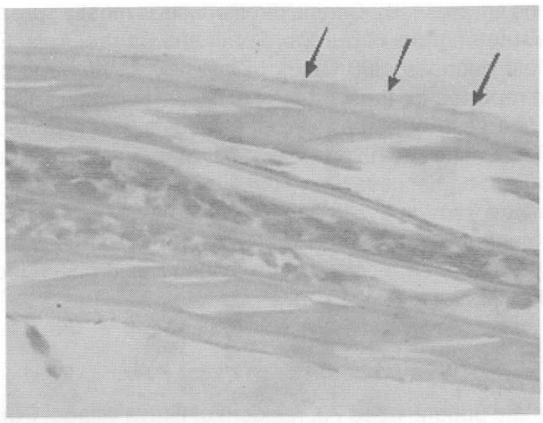

Figure 2. Transverse striations on the cuticular surface.

The size of the parasite and the morphology of the tegument suggest eye dirofilariasis caused by D. repens. Eye localization of D. repens in humans is one of the most often reported in literature. In the Russian Federation, eye localizations account for 45% of all cases of dirofilariasis reported (6). The parasite has been detected in anatomical structures of the eye such as a tumor-like nodule in the soft tissue around the eye, in the eyelid under the conjunctiva, in the vitreous humor, in the retrobulbar space of the orbit, etc. (8, 9, 10, 12, 13, 14, 15, 16, 18). The most common complaints associated with eye dirofilariasis include local symptoms like swelling, pain, hyperemia, itching, feeling the presence of a foreign body. The parasite is located in a subcutaneous node around the eye, or can be seen under the conjunctiva (9, 10, 16, 18). General bodily reactions are likely, such as temperature rise, urticarial rash, fatigue, etc. (13, 15). Complications in eye localization might be detachment of the retina, discoloration of the vitreous humor, glaucoma, uveitis, episcleritis and deteriorated vision (10). In case of subconjunctival localization, the diagnosis is based on direct observation and extraction of the parasite. Making the diagnosis is impeded in cases of subcutaneous localization, which necessitates surgical extirpation of a node, followed by histological examination. In such cases the body of a round helminth is found, situated in the center of an infected ridge of neurtophils and eosinophils, epithelioid cells, histiocytes, and polynuclear giant cells. (12). In the majority of cases, diagnostic procedures are also therapeutic. The removal of the parasite results in full recovery: the infection of humans is incidental and the human organism cannot serve as a dead-end host and a reservoir for D. repens.

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LIGHT MICROSCOPY DISTINCTIVE MORPHOLOGICAL FEATURES OF THE VACUOLE FORM OF BLASTOCYSTIS HOMINIS

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Abstract

Light microscopy of fresh stool samples most often helps detect the vacuolar form of Blastocystis hominis, which is at the basis of diagnosing blastocystosis. Computer processing and analysis were used to carry out a detailed study on 150 vacuolar forms in samples of 50 randomly selected patients. The morphological differences were assessed base on internal structure, the shape and size of the parasite cell, the number and location of nuclei, and the presence of other structures detectable by light microscopy. The morphological features found could be considered as species characteristics of B. hominis, applicable in differentiating it from other intestinal protozoa in human stool samples.

Key words: Blastocystis hominis, vacuolar form

Blastocystis hominis is a unicellular intestinal parasite, widely spread in animals and humans. It was discovered by Alexeieff in 1911, and classified as a protozoon by Zierdt and collaborates in 1967 (6).

Light and electron microscopy investigations have demonstrated several distinct forms of B. hominis, found in samples of bowel contents: vacuolar, granular, amoeboid and cystic (2, 3, 5, 6, 8, 9). The diversity of forms depends on the medium in which they are detected (faeces or culture), as well as on the conditions for storage and development (3, 6). There is no scientific evidence how these factors contribute to the prevalence of one form or another. In routine diagnostics procedures in parasitology, using light microscopy techniques to investigate stool samples, the vacuolar form is most commonly detected, and is an important clue to make the diagnosis of blastocystosis (6, 7, 8).

The aim of this study was to investigate the morphological and structural characteristics of the vacuolar form of B. hominis, most commonly isolated in patients from the Pleven region.

MATERIALS AND METHODS.

Fresh stool samples, collected in chemically clean and sterile vials were investigated. The investigations were carried out using a light microscope OptiTech (magnification 400x), on samples in native preparation stained with Lugol's solution. The diagnosis of blastocystosis was made on the basis of detection of various forms of B. hominis. The form detected in most of the cases was the vacuolar form. A detailed morphological study with computer processing and image analysis was performed on 150 vacuolar forms, collected from 50 randomly selected patients with a variety of clinical symptoms. A system of computer microphotometry was used, consisting of the following components: a light microscope OLYMPUS BX 40, a digital video camera - OLYMPUS 5050 ZOOM - 5 MGpx, software for computer microphotometry (image analysis- Image tool - v. 3.0.). The morphological differences were assessed on the basis of internal structure, shape and size of the parasite cell, the number and location of nuclei, and the presence of other structures visible with a light microscope.

RESULTS.

The vacuolar forms of the cells of B. hominis detected and then investigated were round or oval in shape, with a clearly discernible cell membrane, cytoplasm and vacuole, and had one or more nuclei. (Fig. 1)

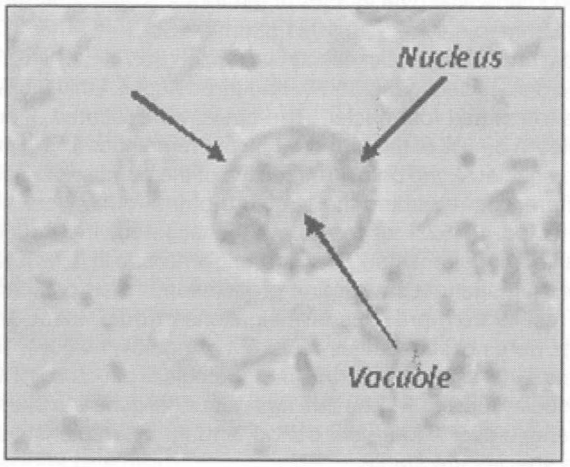

Figure 1. Vacuolar form of B. hominis in a stool sample preparation dyed with

Lugol's solution (magnification 400x)
Original microphoto (I. Angelov)

The average vacuole was large, occupying about 90% of the volume of the parasite cell, with an average diameter of 4.82 µm. In preparations dyed with Lugol's solution, its density varied, visibly empty or finely granular, which suggests a transition to a granular form.

The vacuole was encircled by a thin band of cytoplasm, thicker at one or both oppositely located poles of the parasite. The mean width of the band was 1.3
µm, while at the wide thickened portion of the band the width was 2.5 µm. In some cases, a portion of the cytoplasm was found invaginating the vacuole, or projecting to the outside, thus making the parasitic cell irregular in shape. The thickness in that portion was well expressed, which could be attributed to clustering of cytoplasmic material. One to four unilaterally or bilaterally situated nuclei were found in the cytoplasm of the vacuolar form, and the predominant number of forms possessed two nuclei. The nuclei were surrounded by a nuclear membrane, though it was not well differentiated in all cases. Their average size was 0.87-1.2 µm. The rest of the cell organelles were not visible under a light microscope. Some of the morphological characteristics of 150 vacuolar forms in preparations of stool samples collected from 50 randomly selected carriers of B.hominis and dyed with Lugol's solutions are shown in Table 1. The morphology of vacuolar forms was analysed after computer microphotometry.

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Table 1. Summarized data on morphological characteristics of vacuolar form of B. hominis

Number of nuclei	Number of parasites		Size of parasite (μm)	Parasite form				Location of nuclei			
				circular		oval		unipolar		bipolar	
	No	%	x±SD	No	%	No	%	No	%	No	%
1	33	22,0	7,8±1,3	28	30,1	5	8,8	32	52,5	0	0
2	76	50,7	9,0±1,3	54	58,1	22	38,6	24	39,4	53	59,5
3	31	20,7	9,8±1,8	9	9,7	22	38,6	4	6,5	27	30,4
4	10	6,6	11,2±1,4	2	2,1	8	14,0	1	1,6	9	10,1
Total	150	100	9,1±1,6	93	62,0	57	38,0	61	40,7	89	59,3

The table illustrates well-expressed differences in shapes and sizes of vacuolar forms. The shape was round in 62% of the forms, the size of vacuoles ranging between 5.34 μm and 12.57 μm, mean size 8.37±1.26 μm (x±SD). The majority of these were 8.1 μm to 8.9 μm in size.

The shape was oval in 38% of the forms, sizing 7.04 μm to 14.82μm, mean size 10.19±1.58μm (x±SD).

Most of the forms possessed two nuclei (50.7%), while 22% had one nucleus, and 20.7% had three nuclei. Only 6.6% of the forms had four nuclei. The nuclei were located at both poles in 59.3% of the forms, while the remainder 40.7% had their nuclei at one pole, irrespective of the number of nuclei.

Differences regarding the structure of the forms were also detected. In 52% of the forms investigated, the vacuole was homogeneous and transparent, whereas in 48% it was non-homogeneous, with fine granulation. When compared to results from previous investigations of ours, these findings were not substantially different. (Angelov, I. 2008). This makes us assume that the morphological structure of the vacuolar form of B. hominis has not undergone substantial changes over a four-year period, and the characteristics of the species are stable.

DISCUSSION.

The investigation of the morphological diversity of any parasite species allows for establishing and describing its morphological forms. In addition, this facilitates the diagnostic process.

Our study presents a description and comparison of vacuolar forms of B.hominis from patients with blastocystosis, aiming at establishing typical morphological features of the species in a population of a concrete region (Pleven region).

As demonstrated in both the literature (2, 6, 10) and the study, the vacuolar form of the parasite is the morphological form most commonly found. This form serves as a major clue in making the diagnosis of blastocystosis using light microscopy to examine the stool samples.

The light microscopy investigations carried out on the morphological characteristics of the vacuolar form of B. hominis and the conclusion made on these characteristics demonstrated a marked morphological diversity of the parasite: round or oval shape, size ranging from 5.34 μm to 14.82 μm, and presence of one to four nuclei, located at one, or both poles. The results are in agreement with the data on the morphology of the parasite, reported by other authors (2, 3, 4, 6, 7, 10).

On the basis of the results from the study, which illustrate the specific morphology of the vacuolar form, two assumptions could be made. It is possible that the differences found manifest a certain stage in the biological evolution of the vacuolar form into one of the three other morphological forms of the parasite – granular, amoeboid or cystic. On the other hand, there is a chance that these morphological differences of the vacuolar form are due to the strain morphological features of the B. hominis species.

The morphological features identified are important for routine clinical practice, and can be regarded as characteristic of the B.homimis species. They can be useful in the process of differentiating the parasite from other protozoa found in human stool samples.

Conclusions:

The vacuolar form of B.homimis is the one most commonly detected in fresh human stool samples under a light microscope, and is a major clue in diagnosing blastocystosis.

The round form of the parasitic cell is predominant (62%), with a size of 5.34 μm – 12.57 μm, mean size 8.37±1.26 μm (x±SD).

The cytoplasm is band-like, of varying density, thickness and contours, encircles the vacuole and contains one to four nuclei.

The vacuolar forms with two nuclei are the most common (50.7% of the vacuolar forms studied), the two nuclei located at opposite ends.

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ANALYZIS AND EVALUATION OF SOMATIC ANTIGEN PRECIPITATED WITH 40 % SATURATED AMMONIUM SULFATE FROM TOXOPLASMA GONDII

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SUMMARY:
The aim of the present study was Toxoplasma somatic antigen, precipitated with ammonium sulphate (40 % saturation) and analyzed by dot-ELISA-IgG, ELISA-IgG and WB. In dot-ELISA and ELISA sensitivity and specificity were determined to be 96 % and 98 % in the first method and 100 % in second one, respectively. In WB obtained results showed a very complex antigen profile. Antigen yielded in WB several bands with molecular masses between 23-103 kDa. High recognized antibodies were from bands with MM 87, 50, 48, 44 and 35 kDa in IgG-WB and 35 kDa in IgM/IgA-WB.

Key words: Toxoplasma gondii, somatic antigen, ammonium sulfate precipitation, ELISA, dot-ELISA, Western blot.

INTRODUCTION:

Toxoplasma gondii is an obligate intracellular protozoan parasite. Epidemiological studies indicate that 20 % to 90 % of adult population worldwide have been in contact with the parasite. In immunocompetent individuals the infection is usually subclinical. Primary infection in pregnant women may cause severe damage to the fetus and reactivation of a chronic infection can cause some complications in immunocompromised patients (5). The laboratory diagnosis of Toxoplasma infection is usually based on the presence of specific antibodies in the serum samples of infected patients. More problems in serological tests are related the antigens that used in these tests. Most of the studies have used total lysate antigens, membrane antigens or excretory-secretory antigens. The aim of the present study was to perform new soluble antigen fractionated by precipitation with ammonium sulfate solution (40% saturation) and to determine its immunochemical reactivity for detection of Toxoplasma specific antibodies in human serum.

MATERIALS AND METHODS:

Antigens preparation:

For antigen preparation tachyzoites of the RH strain were harvested from peritoneal cavities of previously infected BALB/c mice. Tachyzoites were purified by centrifugation in PBS solution and then disrupted by sonication. The supernatant was used as the source of soluble antigen which was fractionated by precipitation with ammonium sulfate solution (40% saturated) according to Yammamoto (8). After precipitation the pellet was dissolved in PBS and dialysed against PBS for 2 days at 40C to remove the ammonium sulfate residue. Protein concentration of antigen preparation was determined by measuring the optical density at wavelength 280 nm following Warburg`s and Christan`s method (7). The antigen contained 1mg protein /ml.

Sera samples:

Serum samples were obtained from infected adults during chronic or acute phase of infection. Sera from patients with no detectable T. gondii specific antibodies and no history of toxoplasmosis were used as negative controls. To study IgG reactivity with somatic antigen precipitated with ammonium sulfate in dot-ELISA were used 45 chronic-phase sera and 40 negative sera, 34 of them (17 positive and 17 negative) were used in ELISA. To study IgG, IgM and IgA reactivity by immunoblotting were used 10 acute-phase sera, 10 chronic phase sera and 10 negative sera. All sera have been tested previously by ELISA (BioRad Laboratories).

ELISA:

Indirect ELISA was performed according Voller (10). Polystirene strips (Nunc) were coated with soluble antigen diluted 1:500 in carbonate buffer (pH 9,6). The sera dilution was 1:50 and the dilution of the antihuman IgG horseradish peroxidate conjugate (BulBio-NCIPD) was 1:2000. The substrate was orthophenyl-diamine (OPD). The optical density was measured by ELISA reader at 492 nm.

Dot-ELISA:

The assay was carried out on nitrocellulose membranes (0,45 µm, Millipore) spotted with 1µl samples of antigen according to Zdravkova, Karaslavova (1). The sera diluted 1:50 and the antihuman IgG horseradish peroxidate conjugate (BulBio-NCIPD) diluted 1:300 in TBS/BSA/0,05% Tween 20. The positive reactions were determined by the appearance of clearly defined dark dots with diaminobenzidine (DAB) substrate (Sigma).

SDS-PAGE and Western Blot (WB):

Electroforesis was performed in 4% stacking gel and 12 % separating gel under reducing conditions according to the method of Laemmli (3). The gels were transferred to nitrocellulose paper as described by Towbin et al. (6). The dilution of serum was 1:50 in TBS/ 0,5% skimmed milk and the dilution of the antihuman IgG/IgM IgA horseradish conjugate (BulBio-NCIPD) was 1:200. DAB (Sigma) was used as a substrate. Apparent molecular masses of tested Toxoplasma antigen were estimated by using broad-range marker (BioRad Laboratories).

RESULTS AND DISCUSSION:

Western blot was performed to study immunoreactive components, reacting with antibodies from human sera. The immunoblot assay was revealed many bands. Sera from all patients reacted with antigens in the range of 23-103 kDa (table 1). The most of antigens displaying molecular masses over 50 kDa (excepting 87 kDa) were not analyzed, because were observed also in negative sera (although slight reaction in these sera) and we considered that they were nonspecific bands. IgG toxoplasma antibodies reacted with 19 polypeptides with molecular masses between 23-103 kDa. In IgG-blot the bands of 50 kDa, 48 kDa and 35 kDa were detected using all postitive IgG human sera, antigens of 87 and 44 kDa was also with high recognition, whereas 46, 41, 38, 36, 32, 27, 25 and 23 kDa were not consistently observed. In contrast to the antigenic patterns by IgG toxoplasma antibodies a very different pattern with few antigenic components was observed by IgM and IgA toxoplasma antibodies. The restricted number of antigenic components reacting with IgM and IgA toxoplasma antibodies has been observed by other authors (4). In IgM and IgA blots the highest frequency

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recognition was for 35 kDa antigen.

Table 1. Immunoreactivity of somatic antigen, precipitated with ammonium sulfate (40% saturation) by Western blot. Antigen fractions in kDa which revealed in serum samples

Serum samples	103	87	79	73	64	56	54	50	48	46	44	41	38	36	35	30	27	25	23
+IgG (n=10)	10	9	9	10	7	10	10	10	10	5	9	3	6	4	10	4	4	2	2
-IgG (n=10)	10	1	6	9	1	9	5	-	-	-	-	2	-	-	-	-	-	-	-
+IgM (n=10)	9	-	4	9	2	9	-	-	1	2	2	-	1	1	8	1	1	-	-
-IgM (n=10)	10	-	-	4	1	9	-	-	-	-	-	-	-	-	-	-	-	-	-
+IgA (n=10)	10	-	2	10	-	5	-	-	2	4	2	-	-	-	10	-	1	1	2
-IgA (n=10)	8	-	-	3	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-

Protein fractions with MM over 50 (excepting 87 kDa) were very slight positive in WB when tested by negative sera. This gives as reason to test the precipitated somatic antigen in other methods - dot-ELISA and ELISA with human chronic-phase and negative sera. The obtained results were showed on table 2. In dot-ELISA was observed false-negative reactions in two patients with latent toxoplasmosis. False-positive reaction was observed only in one negative human serum. In ELISA the extinction value showed positive results for all of the positive sera and the method showed 100% sensitivity and specificity using this antigen.

Table 2. Immunoreactivity of somatic antigen, precipitated with ammonium sulfate (40% saturation) by dot-ELISA and ELISA.

Meth-ods	+IgG №positive results/total +IgG serum samples	sensi-tivity %	-IgG №negative results/ total -IgG serum samples	specifi-city %
Dot-ELISA	43/45	96	39/40	98
ELISA	17/17	100	13/13	100

There are no studies in the literature about somatic antigen fractionated with ammonium sulfate. But has few publications about exo-antigens, fractionated by the same manner and authors obtained good results (2, 8). They reported that peritoneal fluid of mice, infected with T.gondii RH strain reacted with serum samples from toxoplasmosis patients and showed that fraction precipitated with 30-40 % ammonium sulfate saturation contains most of the antigens of potential use in immunodiagnosis. According our previous data the somatic antigen in IgG blot only the bands of 103 kDa, and 35 kDa have highest recognition (9). In this study 103 kDa antigen was shown also in negative sera, although exhibiting slight reaction. Probably this fraction consisted two bands with closely molecular masses.

The present study indicate that fractionated somatic antigen by precipitation with ammonium sulfate (40% saturated) may be used as an antigen for detection of Toxoplasma specific antibodies and may be valuable for the serodiagnosis of toxoplasmosis. Due to high frequency recognition of 87, 50, 48, 44 kDa protein by IgG antibodies, we consider these proteins a good marker for chronic form of infection. The presence of antibodies to 35 kDa protein in chronic as well as in the acute patients point to this protein as a marker for every persons that have been in contact with T. gondii. Further research is needed to confirm if these proteins can be considered as diagnostic markers for toxoplasmosis.

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