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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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CONTENTS

EFFECT OF A FUNGIAL Cu/Zn SUPEROXIDE DISMUTASE ON THE FUNCTIONS OF PHAGOCYTIC CELLS IN *YERSINIA ENTEROCOLITICA* PYV (+) INFECTED MICE

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SUMMARY

Effect of Cu/Zn superoxide dismutase (SOD) isolated from the fungal strain *Humicula lutea 103* (HLSOD) on the functions of phagocyte cells in *Yersinia enterocolitica pYV(+) (YE)* infected ICR mice and their survival rate was studied. Migration and killing activities of peritoneal macrophages, as well as phagocyte abilities of blood polymorphous-nuclear leukocytes (PMNs) and peritoneal macrophages were followed from days 3 to 21 post infection. Blood cultures and bacterial load of liver, spleen and small intestinies were examined too. A marked protective effect of HLSOD on the survivability of mice after lethal YE infection was established. The mortality percent (MP) in the control group of infected animals without treatment was 100% and strongly diminished (up to 17%) after treatment by 40U HLSOD. Acceleration of bacterial clearance from blood was another effect of HLSOD treatment. Augmented values of macrophage number in healthy and infected animals were established after HLSOD application with a maximum at day 15th. Phagocyte indexes of macrophages from healthy animals increased under the action of enzyme treatment. The phagocyte number was 2 times increased in both groups of the infected animals. Macrophage killing indexes against *S. aureus* 209 were also increased in both groups of infected animals with a maximum at day 21st post infection. HLSOD restored the suppressed peroxide formation and killing ability of macrophages from infected animals against *E. coli* O_{124}. HLSOD produced a marked stimulation effect on the migration ability of macrophages, better expressed in healthy animals. Additionally, HLSOD induced an increase of PMNs phagocyte indexes from the infected animals and the number of the *in vitro* engulfed *S. aureus* Smith cells was 1.5-2.0 times higher in both infected groups during the whole period of observation. The established protective effect of HLSOD on survivability of YE infected mice can be explained by the participation of this enzyme in the host oxidant-antioxidant balance, resulting in a restoration of the suppressed phagocyte, killing and migration abilities of peritoneal macrophages and blood PMNs of the infected mice.

Key words: *fungal superoxide dismutase, Yersinia enterocolitica infection, phagocyte cells*

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ABBREVIATIONS USED IN THIS PAPER:

HLSOD - Humicola lutea superoxide dismutase, YE- Yersinia enterocolitica, PYV - Yersinia virulence plasmid, PMNs - polymorphous nuclear leukocytes, PI - phagocytic index, KI - killing index, YOPs - Yersinia outer membrane proteins

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INTRODUCTION

Yersinia enterocolitica (YE) is a common cause of enterocolitis, gastroenteritis, mesenteric lymphadenitis, terminal ileitis and other suppurative infections (7, 13, 16). Virulent strains of YE harbor a 70kB virulence plasmid (pYV), which bears genes that code for production of several secreted proteins, called Yops (17, 27) including the outer membrane protein Yad A (Yersinia adhesin) (6). Yad A and several Yops are involved in the inhibition of phagocytosis and killing of YE by human granulocytes (10, 25, 30).

Yops are synthesized at 37°C and translocated into mammalian cells upon contact (23). One of these proteins, Yop H dephosphorylates multiple tyrosine-phosphorylated proteins in eukaryotic cells (5). This protein is involved also in inhibition of the uptake of *Yersinia pseudotuberculosis* by cultured mouse macrophages (11) and by epithelial cells (22). Investigations of Visser *et al.* (30) showed that a pYV (+) strain of YP inhibited receptor-dependent or N-formyl-methionin-leucine-phenyl-alanin (f-MLP) inducible O_2^- production by human granulocytes, but did not affect the receptor independent (phorbol-myristat-acetate inducible) O_2^- production by granulocytes mediated from YE. The inhibition of f-MLP inducible O_2^- production by granulocytes depends on the secretion of Yops, but not on the expression of Yad A (30).

The formation of O_2^- radicals is catalysed by NADPH-oxidase, a membrane-bound enzymatic complex, which converts O_2 into O_2^- (9). NADPH-oxidase can be activated by receptor-dependent mechanisms, such as opsonized bacteria, C_{5a}, f-MLP and immune complexes, as well as by receptor-independent mechanisms, including long chain unsaturated fatty acids and phorbol-myristat-acetate (PMA). Activation of NADPH oxidase in human granulocytes is accompanied by protein tyrosine phosphorylation, suggesting that NADPH oxidase can be switched on by tyrosine kinases (3).

It was found that YE translocates virulence YOPs into a wide range of eukaryotic cells including neurons and insect cells. Yop E, H and T are cytotoxic for the adherent type cells. Invasin and/or Yad A are required for translocation of Yop into non-phagocyte cells, but not for translocation in macrophages (8). Yop E is an important virulence factor which penetrate into mammalian cells via a plasmid-encoded type III system in Yersinia spp. Yop E promotes the disruption of actin filaments, cell rounding and blockage of phagocytosis (4).

The effector Yops disrupt cellular defense functions such as TNF- a , O_2^- production and phagocytosis and allow *Yersinia* to grow (2). Yops are active proteins, which can suppress eukariotic signal mechanism, for instance they can translocate the tyrosine phosphatase. Yop H dephosphorylates the focal adhesion proteins paxillin and 130C. The *Yersinia* effector Yop P is able to induce apoptosis in macrophages (2).

In the present work we aim to study the effect of Cu/Zn-superoxide dismutase isolated from *Humicula luthea* 103 fungial strain on the functions of phagocytic cells in mice infected by *Yersinia enterocolitica* 0:8 pYV (+).

MATHERIALS AND METHODS

ANIMALS

ICR mice (130), 8 weeks old, from both sexes and weighing 18-20 g were used for the experiments. The animals were obtained from the animal house of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia. They were grown up and fed at standard conditions accepted by the Bulgarian Veterinary Health Service.

Microorganisms

Yersinia enterocolitica strain *8081*,serotype O:8, pYV (+) (YE), was used for the induction of infection in mice. The strain was provided by Reference Laboratory, Institut Pasteur (Paris, France.). Bacteria were cultivated in Brain-heart infusion (BHI)(Difco) at 26°C for 18 h, than diluted 1:15 in fresh BHI supplemented by 0.02 M $MgCl_2$ and cultivated again for 4 h at 37°C. The bacterial cells were washed twice, suspended in saline and standardized by optical standard up to concentrations described elsewhere.

The referent strain *S. aureus* Smith was used for the phagocyte index tests of peritoneal macrophages and blood polymorphous-nuclear leukocytes (PMNs) of the experimental animals. The reference strains *S. aureus 209* and *E.coli* O_{124} K_{72} H_{30} were used to test microorganisms in the killing ability of peritoneal macrophages. All the 3 strains were maintained in the microbiological collection of the Department of Immunology, Bulgarian Academy of Sciences. For the present experiments the strains were cultivated on Bacto agar (Difco) (pH 7.2) at 37°C for 24 h. Bacterial cells were collected, washed and adjusted to the necessary concentrations in the respective tests.

Determination of lethal dose of YE for mice

The 50% lethal dose (LD_{50}) of YE for mice was determined according to the method of Reed and Muench (21). Four mice were injected from each dilution of bacterial suspensions. The pellets were resuspended in PBS in order to obtain a density 0.87 (at 650 nm) corresponding to log. CFU/ml=9.2.

Infections in mice

Sublethal and lethal YE infections were induced by i.p. inoculation of 1.056×10^5 CFU (LD_{50}) or 2.112×10^5 CFU (LD_{100}) respectively, per mice.

Humicola lutea Cu/Zn superoxide dismutase (HLSOD)

The fungal strain *Humicola lutea 103* from the mycological collection of the Institute of Microbiology, Sofia, was used as a source for SOD production. The method of cultivation of this strain, the enzyme extraction and purification procedures are described elsewhere (1). The purified enzyme corresponds to a Cu/Zn SOD, homodimeric glycoprotein with a molecular mass of approximately 31 700 Da (1).

Protective effect of HLSOD on the mortality of mice with YE lethal infection

HLSOD was injected in mice intra-peritoneally at doses of 20U, 30U or 40 U (groups 1, 2 and 3 respectively) two times in a week, one week prior the lethal infection and 3 weeks during the infection. The mortality percent (MP) was followed till the day 27 compared to the infected untreated controls. The dose with the best protective effect on the mortality of the lethally infected mice was used for the next experiments on the immune-protective action of HLSOD on mice with a sublethal YE infection.

Protective effect of HLSOD on mice with YE sublethal infection

Experimental animals were separated in 4 groups: group 1 - sublethally YE infected mice treated with 40 U HLSOD; group 2 - sublethally YE infected mice without treatment; group 3 - uninfected mice treated by 40U HLSOD; group 4- control- uninfected mice. Bacteriological and immunological parameters of the infected mice were examined at days 5th, 10th, 15th and 20th after infection.

Blood cultures

Blood samples obtained aseptically from the Plexus orbicularis of each animal, were inoculated in 5 ml volumes of BHI and cultivated at the above mentioned conditions. Afterwards the blood cultures were plated on Tryptic soy agar (TSA) (Difco) and cultivated again at 26°C for 24 h. Single colonies were tested for agglutination by rabbit polyclonal anti-YE antiserum (agglutination titer 1:16).

Bacterial clearance

Fragments from liver, spleen and small intestine (SI) of the infected animals were aseptically removed, weighed and homogenized separately in sterile saline. Than 0,1 volumes of serial 10 fold dilutions were plated on *Yersinia* selective medium (CIN agar, Oxoid). The plates were cultivated at 25°C for 48 h and colonies were confirmed by slide agglutination test with specific rabbit antiserum. The number of colonies were calculated for 1g weight.

Macrophage functional studies

Macrophage functions of mice with sublethal *Yersinia* infection, injected with the optimal protective dose of 40 U HLSOD per animal were followed.

a) Harvesting of peritoneal macrophages

Peritoneal macrophages were collected by washing procedure of peritoneal cavity by ice cold HBSS (pH 7.2) (10 ml per animal). After two washings the macrophage number was determined in a Burker-s camera. Afterwards the cells were adjusted to the necessary concentration for the following examinations.

b) In vitro phagocytosis by peritoneal macrophages

Macrophages in concentration $1x10^7$ cells ml^{-1} in RPMI 1640 medium (Sigma; Dieseldenhofen, Germany) without supplements were allowed to adhere on glass slides for 2 h at 37°C in humidified 5% CO_2 atmosphere. After washing with phosphate-buffered saline (PBS), fresh complete RPMI 1640 medium and suspension of killed *S. aureus Smith* cells was added to the macrophage layer (ratio 1:20 between macrophages and bacteria). The cells were allowed to interact 1 h under the same conditions, washed and stained according to the method of Pappenheim (19).

Phagocosis index (PI) (%) and phagocytic number (PN) were calculated according to the formula, proposed by Ossada *et al.* (18).

c) Killing effect of peritoneal macrophages

The killing effect of macrophages against the reference strains *S. aureus* 209 and *Escherichia coli* O_{124} were estimated according to the method of Visser *et al.* (29). Briefly, macrophages at concentration 1×10^6 ml^{-1}, suspended in RPMI 1640 medium, supplemented by 10% Fetal calf serum (Sigma; Dieselhofen, Germany), L-glutamine and antibiotics, were mixed with $5x10^7$ suspension of bacterial cells *(S. aureus* 209 or *E. coli* O_{124}) in saline. The ratio between macrophages and bacteria was 1:50, and the volume of samples was 0,5 ml. The cells were allowed to interact for 2 h or 24 h at 37°C in 5% CO_2 atmosphere. Bacterial cells and macrophages, at the same ratio, incubated at 4°C for 2 h were used as controls. After incubations the macrophage layers were washed and osmotically destructed by distilled water. The cell suspensions were then diluted 10-fold and cultivated on Bacto agar (Difco) at 37°C for 24 h. The number of colony forming units (CFU) from each sample was counted. The killing index (KI) was calculated as a ratio:

$$KI = \frac{\text{Initial CFU number (incubation at 4°C)}}{\text{CFU number after incubation at 37°C}}$$

Migration of peritoneal macrophages

Migration ability of peritoneal macrophages in complete RPMI 1640 medium was estimated by the method of Leu *et al.* (15). Macrophage suspension 6×10^7 ml^{-1} was introduced in glass capillaries covered by silicon (75/1 mm, for clinical laboratory use, DDR production). One side of the capillaries was stopped by bee vax. After centrifugation at 500 rpm for 5 min, the capillaries were cut at the levels of the visible macrophage cell pools and put into plastic plates containing complete RPMI 1640 medium. After cultivation at

37°C in 5% CO_2 thermostat the migration fields of macrophages were estimated by planimeter.

Hydrogen peroxide in peritoneal macrophages

Hydrogen peroxide (H_2O_2) production by peritoneal macrophages was determined according to the method of Pick and Mizel (20). Briefly, macrophage cells were collected from the peritoneal cavity, washed and suspended in Hanks' balanced salt solution (HBSS), containing 10% fetal bovine serum at a cell density of 4×10^6 ml^{-1}. Cell suspensions were distributed to the wells of a 96-well plate (Falcon) (4×10^5 per well). After incubation at 37°C, in 5% CO_2 thermostat for two hours the non-adherent cells were removed by washing with HBSS without phenol red. Reaction mixture was added (0.1 ml/well), containing 200 mg/ml phenol red and 50 mg/ml horseradish peroxidase type VI-A (Sigma) in HBSS without phenol red. After 45 min the reaction was stopped by addition of 10 ml/well 1N NaOH and the absorbanse was read at 620 nm on an ELISA reader (Organon Teknika). For calculations, a standard curve with H_2O_2 concentrations from 5 to 50 mM was used.

Phagocytic indices of blood PMNs

The phagocyte indices (PI) of blood PMNs of the experimental animals were estimated according to the method of Vulchanov (31). Briefly, 0.1 ml blood obtained from Plexus orbicularis was immediately mixed with 0.05 ml 2% sterile sodium citrate solution. Then 0.05 ml $2x10^6$ suspension of killed cells of *S. aureus* Smith was added and the cells were allowed to interact at 37°C for 1 h. Smears from each sample were prepared and stained according to the method of Pappenheim (1911). Phagocytic indices of PMNs were calculated by formula of Ossada *et al.*, (18).

Statistical analysis

Differences between the results of experimental groups were analyzed by the Student-s t-test. The data are presented as mean arithmetical values ±SD. $P<0.05$ was accepted statistically significant. All experiments were performed in triplicate.

RESULTS

It was found that the enzyme Cu/Zn SOD isolated from the fungal strain *Humicola lutea* produced a protective effect on the survivability of mice with a lethal *Yersinia enterocolitica O:8* infection. The most pronounced was the effect of 40U HL SOD, compared to the effects of the application of doses 20 U and 30 U HL SOD. The % mortality (MP) in the control group of the YE infected animals without treatment was 100% at day 5th, while the MP of the infected animals treated with 40 U of SOD was 17%. The mean survival time of animals in the group of mice treated with 40 U HL SOD was more than 27 days in 17% of animals. Mice from groups treated with lower doses HL SOD (20 U and 30 U) showed 100% mortality at day 17th of observation (Fig. 1). No mortality in the group of uninfected animals treated with HLSOD was observed (data not shown).

It was found that blood cultures isolated from both treated and untreated infected mice were positive in 50% until the day 7th of the observation. After day 14th until the end of investigation 25% positive blood cultures in HL SOD treated and infected group was found, when in the group of the infected mice without treatment positive blood cultures remained 50% until the day 14th. At the end of observation (day 21st) 25% blood cultures in both infected groups of animals were established (Table 1).

It was found that HLSOD treatment induced an increase of the peritoneal macrophage number both in healthy and infected animals with a maximum at day 15th (macrophages 20.2×10^6 and 16.3×10^6, healthy controls and infected being 13.2×10^6 and 9.0×10^6 respectively)(Fig. 2).

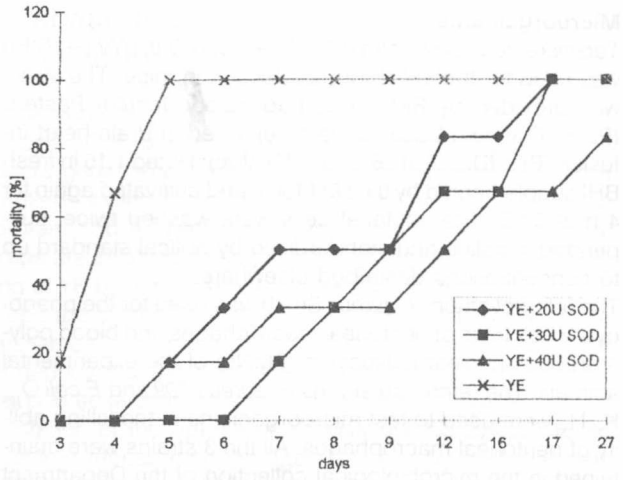

Figure 1.
Mortality (%) of *Yersinia enterocolitica O:8 pYV (+)* infected mice, treated by Cu/Zn superoxide dismutase, isolated from the fungial strain. *Humicula lutea 103.* Experimental groups:1 - YE infected mice treated by 20 U HLSOD, one week prior the infection (2 injections) and 2 weeks after the day of infection (2 injections weekly); 2 - YE infected mice treated by 30 U HL SOD by the scheme of the animals in the group 1; 3 - YE infected mice treated by 40 U HL SOD according to the scheme of the animals in group 1; 4 - healthy mice without treatments.

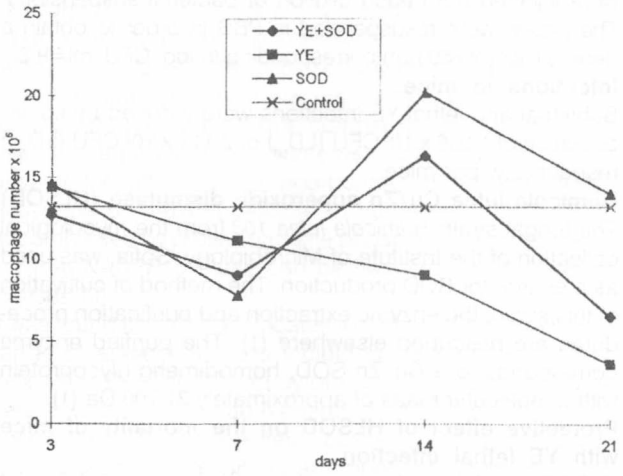

Figure 2.
Number of the peritoneal macrophages mice treated by 40 U HL SOD. Experimental groups:1- YE infected mice, treated by 40 U HL SOD one week prior the infection (2 injections), and two weeks after the day of infection (2 injections weekly); 2 - YE infected mice without treatment; 3 - healthy mice treated by 40 U HL SOD according to the scheme of the animals in group 1; 4 - healthy mice without treatments.

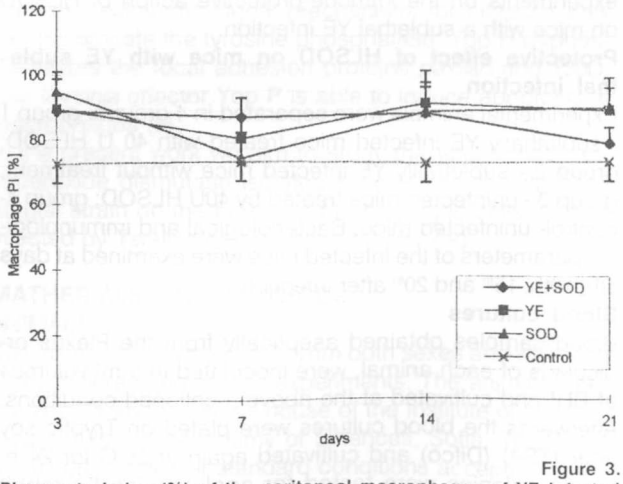

Figure 3.
Phagocyte index (%) of the peritoneal macrophages of YE infected mice, treated by HL SOD. Experimental groups: as in Figure 2.

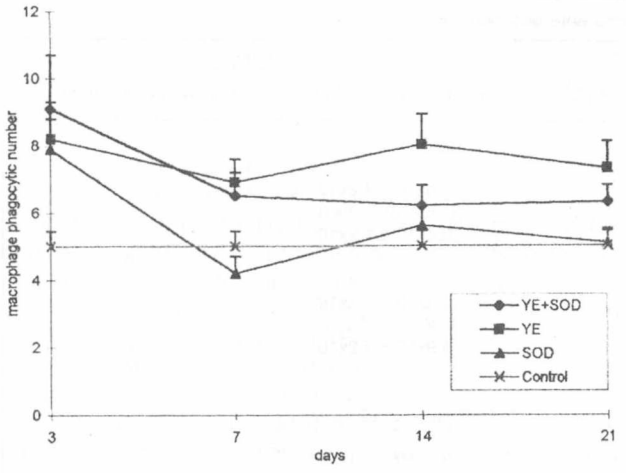

Figure 4.
Phagocyte number (bacterial cells per one macrophage) of peritoneal macrophages from YE infected mice, treated by HL SOD. Experimental groups: as in Figure 2.

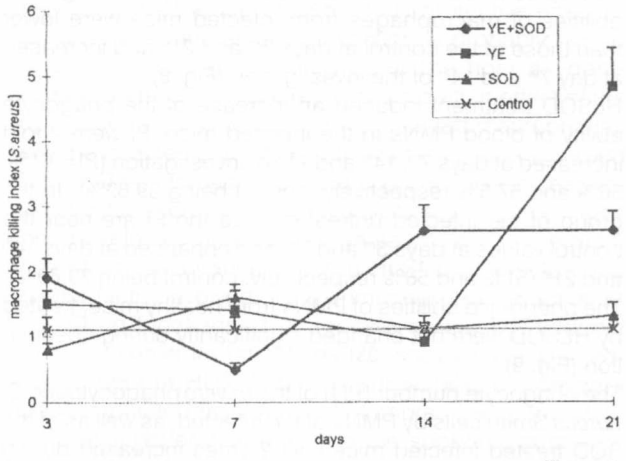

Figure 5.
Killing index against *S. aureus 209* of peritoneal macrophages from healthy and YE infected mice, treated by HL SOD. Experimental groups: as in Figure 2.

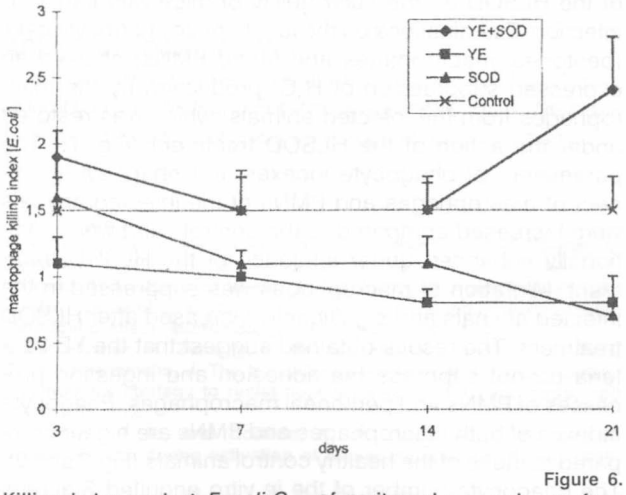

Figure 6.
Killing index against *E. coli* O_{124} of peritoneal macrophages from healthy and YE infected mice, treated by HL SOD. Experimental groups: as in Figure 2.

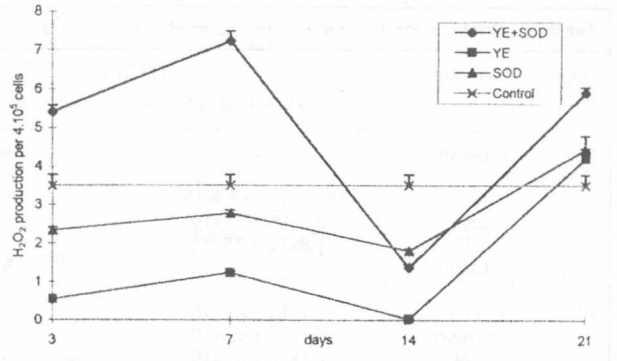

Figure 7.
Spontaneous H_2O_2 production (mM per $4x10^5$ cells) by peritoneal macrophages from YE infected mice, treated by HL SOD. Experimental groups: as in Figure 2.

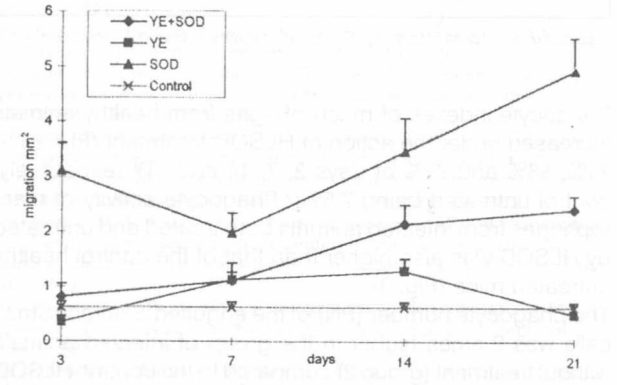

Figure 8.
Migration (mm^2) of peritoneal macrophages from YE infected mice treated by HL SOD. Experimental groups: as in Figure 2.

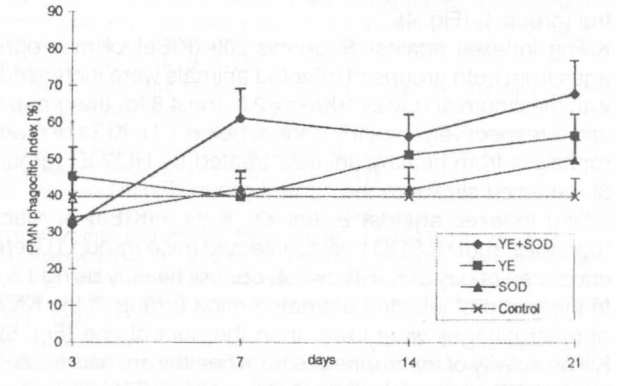

Figure 9.
Phagocyte index (%) of blood PMNs from YE infected mice, treated by HL SOD. Experimental groups: as in Figure 2.

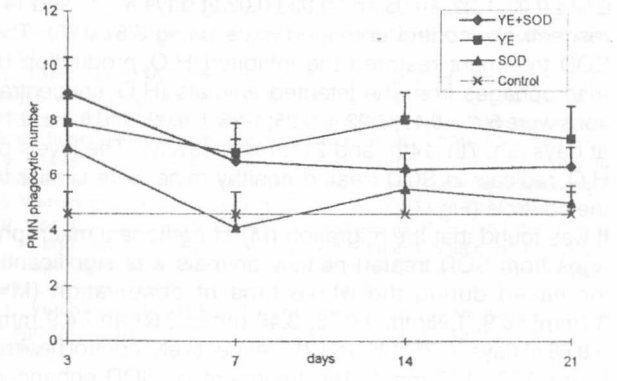

Figure 10.
Phagocyte number (engulfed bacterial cells per one PMN cell) of PMNs from YE infected mice. Experimental groups: as in Figure 2.

Table 1. Bacterial load of viscera and blood of mice infected with Yersinia enterocolitica 0:8 strain

Days p.i.	Viscera	GROUP 1		GROUP 2	
		Bacterial load(cfu/g tissue)	Positive hemoculture (%)	Bacterial load(cfu/g tissue)	Positive hemoculture (%)
2	blood	-	50	-	50
7	liver	$1.02x10^4 \pm 1.4x10^5$	-	$3.6x10^5 \pm 4.2x10^4$	-
	spleen	$5.2x10^5 \pm 3.6x10^4$	-	$1.06x10^5 \pm 1.8x10^4$	-
	SI	$1.8x10^2 \pm 2.5x10^1$	-	$9.4x10^1 \pm 1.2x10^1$	-
	blood	-	50	-	50
14	liver	$1.1x10^5 \pm 2.9x10^4$	-	$5.4x10^5 \pm 7.3x10^4$	-
	spleen	$6.7x10^5 \pm 8.3x10^4$	-	$7.06x10^5 \pm 1.1x10^5$	-
	SI	$4.5x10^4 \pm 6.1x10^3$	-	$3.9x10^5 \pm 5.2x10^4$	-
	blood	-	25	-	50
21	liver	$6.3x10^4 x 5.7x10^3$	-	$1.08x10^6 \pm 2.1x10^5$	-
	spleen	0	-	0	-
	SI	$1.3x10^5 \pm 2.5x10^4$	-	$3.3x10^5 \pm 4.4x10^4$	-
	blood	-	50	-	25

***Legend**: p.i. - post infection; SI - small intestine; Group 1 - infection + SOD; Group 2 - infection.*

Phagocyte indexes of macrophages from healthy animals increased under the action of HLSOD treatment (PI= 95%, 74%, 88% and 89% at days 3, 7, 14 and 21st respectively, control untreated being 7.5%). Phagocyte activity of macrophages from infected animals both treated and untreated by HLSOD was also higher than that of the control healthy untreated mice (Fig. 3).

The phagocyte number (PN) of the engulfed *S.aureus* Smith cells was 2 times higher in the group of infected animals without treatment (group 2) compared to the control. HLSOD treatment did not change the number of the engulfed bacterial cells (PN) in the macrophages of the infected animals (group 1). The PN of the macrophages from healthy animals treated by HLSOD (group 3) was similar to the control (group 4) (Fig. 4).

Killing indexes against *S. aureus* 209 (KISa) of macrophages from both groups of infected animals were increased, with maximum at day 21st (KISa=2.6 and 4.8 for the group 1 and 2 respectively; healthy control being 1.1). KISa of macrophages from healthy animals treated by HLSOD (group 3) remained similar to the control group (Fig. 5).

Killing indexes against *E. coli* $O_{124}K_{72}H_{30}$ (KIEc) of macrophages from HLSOD treated infected mice (group 1) were enhanced at day 21st (KIEc=2.4, control healthy being 1.5). In the group of infected untreated mice (group 2) the KIEc of macrophages were lower than the control one (Fig. 6). Killing activity of macrophages from healthy animals treated by HL SOD remained similar to the control (Fig. 6).

A suppression of H_2O_2 production by the macrophages of the infected animals up to day 15th of the investigation was observed (H_2O_2 concentrations in mM/ $4x10^5$ cells = 0.54 ± 0.02, 1.22 ± 0.05 and 0.02 ± 0.02 at days 5th, 7th and 14th respectively, control untreated mice being 3.5 ± 0.28). The SOD treatment restored the inhibited H_2O_2 production of macrophages from the infected animals (H_2O_2 concentrations were 5.4 ± 0.17; 7.22 ± 0.25; 1.36 ± 0.08, and 5.9 ± 0.14 at days 5th, 7th, 14th, and 21st respectively). The levels of H_2O_2 radicals in SOD treated healthy mice were similar to the controls (Fig. 7).

It was found that the migration (M) of peritoneal macrophages from SOD treated healthy animals was significantly increased during the whole time of observation (M= $3.1 mm^2 \pm 0.9$, $1.98 mm^2 \pm 0.35$, $3.46 mm^2 \pm 0.69$ and $4.9 mm^2 \pm 0.68$ at days 3h, 7th, 14th and 21st respectively, control healthy being $0.62 \pm 0.17 mm^2$). The treatment by SOD enhanced also the migration ability of macrophages from the YE infected animals with maximums at days 14th and 21st (M=2.14 $mm^2 \pm 0.35$ and 2.4 $mm^2 \pm 0.25$, respectively). The migration abilities of macrophages from infected mice were lower than those of the control at days 3rd and 21st and increased at day 7th and 14th of the investigation (Fig. 8).

HLSOD treatment induced an increase of the phagocyte ability of blood PMNs in the infected mice. PI were 2 fold increased at days 7th, 14th and 21st of investigation (PI= 61%, 56% and 67.5% respectively, control being 39.69%). In the group of the infected untreated mice the PI are near the control values at days 3rd and 7th, and enhanced at days 14th and 21st (51% and 56% respectively, control being 39.69%). The phagocyte abilities of PMNs from healthy mice, treated by HLSOD were not changed significantly during observation (Fig. 9).

The phagocyte number (PN) of the i*n vitro* phagocytosed *S. aureus* Smith cells by PMNs of the infected, as well as of the SOD treated infected mice was 2 times increased during observation. The PN of the healthy HLSOD treated mice was similar to the control (Fig. 10).

DISUSSION

The results of the investigation show the protective effect of the HLSOD on the survivability of mice with lethal YE infection. Examinations on the functions of phagocyte cells (peritoneal macrophages and blood PMNs) showed an expressed suppression of H_2O_2 production by the macrophages from the infected animals, which was restored under the action of the HLSOD treatment (Fig. 7). The parameters of phagocyte indexes and phagocyte numbers of macrophages and PMNs of the infected animals were increased compared to the controls and were additionally enhanced under influence of the HLSOD treatment. Migration of macrophages was suppressed in the infected animals and significantly increased after HLSOD treatment. The results obtained suggest that the YE bacteria do not suppress the adhesion and ingestion processes of PMNs and peritoneal macrophages. Phagocyte indexes of both macrophages and PMNs are higher compared to those of the healthy control animals (Fig.3 and 9). The phagocyte number of the in vitro engulfed S.aureus Smith bacteria by both classes of phagocyte cells is also higher compared with the control one. It is known that the most important anti-phagocyte factor suppressing bactericidal activity of phagocyte cells in the YE infected animals are the Yops proteins. These proteins suppress also the host receptor-dependent superoxide anion production by human granulocytes (30).

Pathogenic *Yersinia* export 14 Yop proteins into cytosol of eukariotic cells, by type III pathway. Some of them, named effector Yops, are targeted into macrophages, thereby preventing phagocytosis and allowing bacterial replication into lymphoid tissue. Untill now YopB and YopD were thought to promote the important of the effector Yops into eukariotic cytosol. Yop B and YopR are dispensable for the injection of toxins into eukariotic cells (14).

According to the investigations of Ruckdeschel *et al.* (26) interaction of YE with macrophages cell lines leads to macrophage cell death trough apoptosis. Our previous experiments showed that *in vitro* and *in vivo* interactions of rat peritoneal macrophages with YE did not induce apoptosis and bacterial cells can be eliminated by both mouse and rat peritoneal macrophages (12).

Mn-cofactored superoxide dismutase (SOD-A) is an important factor of the virulence of YE (24). Resistance of YE to exogenous oxygen radicals, produced by phagocytes involves the SOD-A. The important role of SOD-A for pathogenicity of YE could be also due to detoxification of endogenous metabolically produced oxigen radicals, which are encountered by YE during the invasion of the host (24).

The established in the present study protective effect of SOD on mice with lethal YE infection is probably due to the participation of the enzyme in the host oxydant-antioxydant balance. As a result a restoration effect of the suppressed H_2O_2 production by peritoneal macrophages from the infected and HLSOD treated mice was achieved (Fig. 7). The increased peroxide production is responsible for the increased killing activities of macrophages from the infected and HLSOD treated animals. The presence of polysaccharide chain in HLSOD molecule (1) is another factor involved in the immunoprotective action of HLSOD on functions of phagocyte cells in YE infected mice. Our earlier investigations (28) showed similarly protective effect of HLSOD on the survivability of hamsters with transplanted myeloid Graffi tumors. The preparation exhibited a restoration effect on the suppressed phagocyte activities of peritoneal macrophages and blood PMNs, as well as on the *in vitro* proliferation abilities of the spleen B-lymphocytes (28).

The obtained results suggest the possible use of anti-oxidant enzymes in the therapy of some infections caused by phagocytosis-resistant bacteria.

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COMPARISON OF THE SUSCEPTIBILITY TO 11 ANTIMICROBIAL AGENTS OF CLINICAL STRAINS BETA-HAEMOLYTIC GROUP A AND B STREPTOCOCCI ISOLATED FROM ADULTS AND REVIEW OF THE LITERATURE

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SUMMARY

Increasing antimicrobial resistance of group A and group B streptococci to some antibiotics has been observed during the last decade in Europe and worldwide. The aim of this study was to evaluate the susceptibility to antimicrobial agents of clinical strains group A and B streptococci isolated from adults and to compare our results to those from other parts of the world. Using the standard agar dilution method we studied the susceptibility to 11 antimicrobial agents of 156 S.pyogenes strains and 138 S.agalactiae strains isolated in 1999-2001. All isolates were susceptible to penicillin, cephalothin, cefuroxime and cefotaxime. The sensitivity to erythromycin in both groups was almost equal - about 96%, while clindamycin resistance (1,45%) was found only in group B streptococci. Resistance to chloramphenicol (3,62%), ciprofloxacin (1,45%), gentamicin (3,62%) and intermediate susceptibility to it (62,32%) was demonstrated again by group B streptococci. They also were resistant to tetracycline (92,76% against 9,62% for group A) and trimethoprim-sulfametoxazole (100% and 30,13% respectively). Among comparable studies concerning other countries our results demonstrate a bit lower or near the average values reported from Europe.

Key words: *group A streptococci, group B streptococci, antimicrobial susceptibility and resistance, MIC range.*

INTRODUCTION

Group A (GAS;S.pyogenes) and B (GBS;S.agalactiae) streptococci cause many illnesses and clinical syndromes in neonates, children and adults and the monitoring of their susceptibility to antimicrobial agents is very important.

Although the antimicrobial resistance among bacteria continues to increase and to be a clinical problem the beta-haemolytic streptococci remained remarkably susceptible to many antibiotics. Both group A and B streptococci are generally considered to be universally susceptible to the penicillins. As far as it is known there has never been isolated a single penicillin resistant group A streptococcal strain from a clinical source. Available data indicate that the minimal inhibitory concentrations (MICs) of penicillin for group A streptococci have not changed during the last 4 decades (15).

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ABBREVIATIONS USED IN THIS PAPER: GAS - Group A Streptococci, GBS - Group B Streptococci

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Although penicillin is the first choice for the treatment of pharyngeal and other infections due to those organisms, erythromycin (or someone of the newer macrolides) is the second-line drug of choice and is used in penicillin-hypersensitive patients (30). At the same time, the increased use of erythromycin and its derivatives has been related to the increased resistance of S.pyogenes and S.agalactiae to those antibiotics (6,11,13,17).

Addition of clindamycin to the therapy of serious streptococcal infections is recommended to inhibit protein synthesis and it is also a drug of choice for chronic, recurrent pharyngitis (30). Resistance to clindamycin, tetracyclines, aminoglycosides and sulfa drugs in this two streptococcal groups is also observed in many parts of the world and it becomes clinically important (12,15,16,17,29).

Many scientists considered that the antibiotic resistance rates varied with the geographic areas, as well as in different areas of the same country (6,11,15,30). Also there is an opinion that the susceptibility of the streptococci to antimicrobial agents depends on the age of the patients (15).

The aim of this study was to evaluate the susceptibility to antimicrobial agents of clinical strains group A and B beta-haemolytic streptococci isolated from adults and to compare our results to those from other parts of the world.

MATERIALS AND METHODS

Bacterial strains: The survey includes 156 beta-haemolytic group A and 138 beta-haemolityc group B streptococcal strains isolated from adults patients /over 16 years/ of Emergency Medical Institute "Pirogov"collected from January 1999 to June 2001. The isolates were identified on the basis of colony morphology, beta-haemolysis on blood agar, bacitracin susceptibility, commercial latex agglutination test (Oxoid, England) and confirmed by Api System bioMerieux, France. Most of the strains were stored at –70° C in Todd-Hewitt broth (DIFCO Laboratories, Detroit) with 15% glycerol until testing and some of them - at room temperature after lyophilization. The isolates were from different clinical sources: blood, pleural fluid, sputum, wound, throat, vaginal, urethral swabs, urine, sperm.

Control strains included: S.pneuomoniae ATCC 49619 and S.aureus ATCC 25923.

Antimicrobial substances: penicillin G, cephalothin, cefuroxime, cefotaxime, erythromycin, clindamycin, chloramphenicol, tetracycline, gentamicin, ciprofloxacin, trimethoprim/sulphamethoxazol (TMP-SMZ). From the macrolide group only erythromycin was tested since the susceptibility to it can be used to predict susceptibility to clarithromycin and azithromycin. All antimicrobial substances were supplied as standard reference powders with defined activity. Stock solutions were prepared according to the recommendations of NCCLS (22) and the drugs where incorporated into the agar medium in serial twofold concentrations.

Determination of antibiotic susceptibility: The antibiotic susceptibility of the strains was determined by agar dilution method with Mueller-Hinton medium (DIFCO Lab, Detroit, Mich.) supplemented with 5% defibrinated sheep blood as it is recommended by NCCLS (22). The final inocula of ~ $1x10^4$ to $5x10^4$ cfu was applied by means of a multipoint inoculator and plates were incubated overnight at 37°C in an atmosphere of 5%CO_2. The MIC of the antibiotic was defined as the lowest concentration which inhibited the visible growth of the organism. NCCLS (22) breakpoints were used for all antimicrobial agents. All control strains gave MICs within two dilution steps of target values.

RESULTS

The susceptibility (in percent and MIC range) to 11 antimicrobial agents of the group A and B streptococci isolated from adults is given in table 1 and table 2.

As it was expected almost all streptococcal strains (group A - 99,36%), group B - 96,38%) were sensitive to penicillin with one and the same MIC range (0,008 - 0,06 mg/L) but there were many isolates with intermediate susceptibility to penicillin in GBS (3,62%) with MIC range 0,25-2 mg/L as in GAS (0,64%) with MIC range (0,25-0,5mg/L). No resistance to penicillin was observed. The susceptibility to erythromycin in both groups was almost equal (about 96%) but the upper limit of MIC values for the sensitive strains was twofold dilutions higher (0,25mg/L) in GBS. The resistance to this antibiotic was 2,57% in GAS with MICs up to 4mg/L against 2,17% resistance in GBS with MICs - 2mg/L.

Group A streptococci as a whole were susceptible to clindamycin (MIC range 0,015-0,06mg/L) while 1,45% of GBS were resistant to it (MIC > = 2mg/L) and in addition in 2,17% of them intermediate sensitivity was observed. The two clindamycin resistant strains were resistant to erythromicin too. All S.pyogenes isolates expressed 100% susceptibility to cephalosporins (I, II and III generation) without a significant differences in their MIC values (0,008-0,06 mg/L), while in GBS the same sensitivity revealed only to cefotaxime (MIC range 0,008-0,06mg/L). A slightly higher MICs could be seen among cefuroxime sensitive (0,008-0,125mg/L) and cephalothin sensitive (0,015- 0,25mg/L) GBS strains. An intermediate susceptibility to cefuroxime (0,91%) and cephalothin (1,36%) was demonstrated only in this group. Chloramphenicol resistance was documented in 3,62% of group B streptococci while intermediate sensitivity was spread almost equally among the isolates of both groups. Although all streptococci tested had the same MIC range (0,5-64mg/L) for tetracycline there were significant differences in the susceptibility of the two groups. The prevalence of sensitive strains (88,46%) was well expressed in S.pyogenes while on the contrary 92,76% of GBS were resistant to this antibiotic.

The rates of susceptibility to gentamicin were nearly three times higher in group A (98,08%) than in GBS (36,96%). Characteristic for GBS was the availability of great amount of isolates (62,32%) with intermediate sensitivity.

TMP-SMZ MICs of S.pyogenes showed bimodal distribution – about three parts (69,87%) of the isolates were sensitive to this drug (MICs 0,06/1,2-2/38mg/L) while all GBS were resistant to it with very high MIC values (128/2432-256/4864mg/L). A resistance to ciprofloxacin was found only in two strains of GBS (1,45%).

DISCUSSION

Penicillin is the recommended drug of choice in the most cases of GAS pharyngitis in the prevention of acute rheumatic fever, in cases of GAS and GBS bacteremia and sepsis, deep tissue infections, osteomyelitis, meningitis, empyema due to this two groups streptococci. GAS are susceptible to penicillin independently of their isolation - neonates, adults, patients with invasive diseases or carriers.

The amount (in percent) and MIC values of the susceptible to penicillin GAS obtained in this study agree with those reported by Awand (2), Cornaglia (6), De Azavedo (7), Kaplan (15), Orden (23) and York (30). S.pyogenes continues to be sensitive to this drug with MIC range (0,008 – 0,012mg/L). In spite of the demonstration of penicillin tolerance by some S.pyogenes strains (12,24) which is considered to cause treatment failures in pharingitis, the consensus is that this phenomena does not have clinical relevance with respect to GAS (12,15,24).

As many authors report (3,8,9,15,16,17) GBS are also highly susceptible to penicillin (MIC 0,008-0,125mg/L), but intermediate sensitivity is more often met (2-8%) among those isolates (4,8,15). Our results confirm this fact – 3,62% GBS showed intermediate susceptibility (MIC 0,25-2mg/L).The penicillin tolerance is presented in GBS too (4,15) and in Spain it even reaches 17% (4), but as for S.pyogenes the clinical relevance is not convincing. In summary our data document the continued universal susceptibility of group A and B streptococci to penicillin which have not changed during the past four decades, therefore it remains the best antibiotic both for prophylaxis and treatment of infections caused by those microorganisms.

Despite of this fact bacteriologic failure occurs in up to 20% of the patients treated with penicillin, and half of those cases are also a clinical failure (24). Various theories have been offered to explain this phenomena: beta-lactamase-producing bacteria, bacterial interference, re-infection and penicillin tolerance (4,24).

However for patients sensitive to beta-lactam antibiotics and for the treatment of patients who failed penicillin therapy, macrolides are often the recommended drugs. Erythromycin has traditionally been regarded as an effective and very save drug for treatment of group A and B streptococcal infections, but his increased clinical use and the prescription of its macrolide derivates mostly for the upper respiratory tract infections has been associated with the increased resistance of GAS and GBS to this antibiotic (6,8,11,15,17,26). The incidence of erythromycin resistance in GAS still remains low in most part of the world - about 5%: Israel - 1% (29), Canada - 2,1% (7), USA - less than 5% (15), France - 6,2% (5), Chile - 7,2% (25). Widespread erythromycin resistance in S.pyogenes has so far been reported only in a limited number of countries - Germany 12,7% (2), Finland - 16-17% (26), Spain - 14,3% (23), 20%(11), Italy – 30-35% (6), Japan - 62% (21). With the 2,57% resistance to this antibiotic and 1,28% intermediate susceptibility our results confirm the conventional opinion about the generally low rates of erythromycin resistance (~ 5%) in this group.

York and co-workers (30) notified a statistically higher rate of erythromycin resistance (39%) among the strains GAS from serious infections compared to those from pharyngitis (13%) in San Francisco Country Hospital, but the cause of this resistance has not been determined. The authors had not identified an overuse of this drug in San Francisco but they supposed that the erythromycin resistance had an association with strains that were more invasive (30). The effect of different macrolides is well demonstrated in a Spanish multicentere study in 1998 where 23,5% of S.pyogenes isolates were resistant to erythromycin - a 14-membered ring macrolide and azithromycin - a 15-membered ring macrolide whereas only 1% of the isolates were resistant to miocamycin - a 16-membered ring macrolide (1).

The erythromycin resistance in GBS show also variations according to the geographic area. The resistance is notably high in Taiwan – 46% (13), France 18%,21,4% (10,19), USA - 8,5-32% (in different states (17), Canada - 18% (8), while Fernandez reports of 7,4% resistance (9) and Kaplan - 10% (15). The frequency of erythromycin resistance among our GBS is low (2,17%) and the same percent of the strains are with intermediate susceptibility.

As a whole both streptococcal groups studied demonstrated high susceptibility to erythromycin - over 96%. In fact the usage of this antibiotic in our country during the last two decades was limited because of its substitution with the modern cephalosporin groups. Also all new macrolides were not available in Bulgaria. Low resistance to erythromycin (only 3%) was also documented by scientists from Japan

Table 1. Susceptibility to 11 antimicrobial agents of 156 group A streptococcal strains

Antimicrobial agents	S (n/%) MIC range (mg/L)	I (n/%) MIC range (mg/L)	R (n/%) MIC range (mg/L)
Penicillin G	155 (99,36) 0,008-0,06	1(0,64) 0,25-0,5	—
Cephalothin	156 (100) 0,015-0,06	—	—
Cefuroxime	156 (100) 0,008-0,03	—	—
Cefotaxime	156 (100) 0,008-0,03	—	—
Erythromycin	150 (96,15) 0,008-0,06	2 (1,28) 0,5	4 (2,57) 1-4
Clindamycin	156 (100) 0.015-0.06	—	—
Gentamicin	153 (98,08) 1-4	3 (1,92) 8	—
Tetracycline	138 (88,46) 0,5-2	3 (1,92) 4	15 (9,62) 8-64
Chloramphenicol	153 (98,08) 0,25-4	3 (1,92) 8	—
Ciprofloxacin	156 (100) 0,125-0,5	—	—
Trimethoprim/ Sulfametoxazole	109 (69,87) 0,06/1,2-2/38	—	47 (30,13) 4/76-32/608

Note: *S - sensitive, I - intermediate, R - resistant*

for GBS isolated from pregnant women. There were no significant differences between the two studied periods (1985-1986 and 1999-2000) in the incidence of GBS resistance to 14 antibiotics (18). A statistically significant association was found between the local erythromycin consumption and the erythromycin resistance in GAS in Spain (11) and Finland (26) and this may be the explanation of the lower erythromycin resistance of our streptococcal strains. The routine determination of the macrolide resistance in group A and B streptococcal isolates on one hand and the confinement use of these antibiotics for prophylaxis and treatment of upper respiratory tract infections in outpatients on the other hand will preserve them for a successful applying to allergic to penicillin patients.

Some authors associated clindamycin resistance with the resistance to erythromycin (2,6,7). Our results support this thesis as the two clindamycin resistant GBS were resistant to erythromycin too. Data about clindamycin resistance in GAS and GBS show that it is more rarely met in comparison to erythromycin resistance. WHO collaborating center in Minnesota documents 5% resistance in S.pyogenes (15), in Italy it varies from 2,7% to 32,4% in different arias (6), in Germany - 11,3% (2), in Madrid - 1,4% (23), in Spain (multicenter study-1998) - only 0,8% (1). As for GBS a vast investigation including different institutes and centers in USA reports 12% rate of resistance; close to it is the resistance found in Canada - 8% (8), North Carolina - 9% (20); low is the resistance in Spain - 3,4% (9) and in Japan - 1% (18). The remarkably high sensitivity demonstrated by our isolates (100% in group A and 96,38% in group B) may be due to the fact that clindamycin is comparatively seldom used for the treatment of infections in Bulgaria. De Azavero et al (8) reported about a difference in the resistance to erythromicin and clindamycin in GBS isolated from neonatal blood cultures (8% and 4,5% respectively) and those isolated from vaginal swabs from adults (18% and 8%).

While our results do not indicate a notable differences in the susceptibility and MICs of S.pyogenes to the antibiotics from three cephalosporin generation studied (100% sensitivity and MICs - 0,008-0,06 mg/L), cefotaxime was the most effective against GBS. To our knowledge, there is no doubt that cefotaxime is the preferable cephalosporin antibiotic for infections caused by group A and B streptococci with the highest rates of sensitivity - about 100% (2,9,15,16,17,25). It is important to mention that so far it is not observed any clinically significant resistance to the cephalosporines (15). The susceptibility to ciprofloxacin, a guinolone, which use is more frequent than is recognized, is well expressed - over 95% in both streptococcal groups (2,15,23). In our strains only 1,45% from GBS show a resistance to ciprofloxacin. Nevertheless Kaplan and his colleagues did not consider it as first line antibiotic for therapy of group A streptococci (15). Although the notable chloramphenicol sensitivity (about 100%) GAS and GBS demonstrated in the studies all over the world (7,17,23,29). Its application should be limited because of its toxicity and lack of bactericidal activity.

The average resistance of S.pyogenes to tetracycline is about 10% (15,23) but in Israel it reaches 41% (29). 9,62% of GAS in this study are resistant to this drug and 1,92% have intermediate sensitivity. It is known that the frequency of tetracycline resistance in GBS is remarkably higher in comparison to GAS - 95,8% (17), 97,4% (9), >80% (12). The same tendency can be seen in our results - 92,76% of those isolates were resistant to it. This fact is very important because tetracycline is still used by some primary care physicians for prophylaxis and therapy of pharyngitis. In general GAS are susceptible to gentamicin with MIC range 1- 8mg/L (as our strains revealed too), while in GBS - a large cluster of strains (about 50%) are with intermediate sensitivity (9,16). Our GBS isolates from adults showed 62,38% intermediate resistance and 36,96% sensitivity.

The literature contains few and conflicting reports on the susceptibility of GAS and GBS to TMP-SMZ (14,27,29). Also high level resistance to TMP-SMZ (>1000-fold increase in MICs compared to MICs of sensitive strains) is observed among resistant strains, which more frequently cause a therapeutic failure than low level resistant. However, more clinical studies are needed to establish the role of both types of resistance to TMP-SMZ (14,27,29). So TMP-SMZ is not considered a drug for therapy of infections (pharyngitis, skin infections, pneumonia, meningitis, septicemia, etc) caused by GAS and GBS.

Table 2. Susceptibility to 12 antimicrobial agents of 138 group B streptococcal strains

Antimicrobial agents	S (n/%) MIC range (mg/L)	I (n/%) MIC range (mg/L)	R (n/%) MIC range (mg/L)
Penicillin G	133 (96,38) 0,008-0,06	5 (3,62) 0,2-2	— —
Cephalothin	0,015-0,25	1	—
Cefuroxime	0,008-0,125	1	—
Cefotaxime	138 (100) 0,00-0,06	—	—
Erythromycin	132 (95,66) 0,008-0,25	3 (2,17) 0,5	3 (2,17) 2
Clindamycin	133 (96,38) 0,015-0,125	3 (2,17) 0,5	2 (1,45) 2-4
Gentamicin	51 (36,96) 2-4	86 (62,38) 8	5 (3,62) > 32
Tetracycline	7 (5,07) 0,5-2	3 (2,17) 4	128 (92,76) 8-64
Chloramphenicol	131 (94,93) 1-4	2 (1,45) 8	5 (3,62) 6 - >32
Ciprofloxacin	136 (98,55) 0,125-0,5	— —	2 (1,45) 16 - >32
Trimethoprim/ Sulfametoxazole	—	—	138 (100) 128/2432-256/4864

Note: *S - sensitive, I - intermediate, R - resistant*

In conclusion the results of this study showed that beta-lactams are the drugs of choice for the treatment of Group A and B streptococcal infections in adults. Comparing the two groups, GBS were less susceptible to beta-lactam antibiotics than GAS. The development of resistance to erythromycin is a clinically important problem in the management of the infections caused by these microorganisms. It also emphasizes the need for routine determination of the antimicrobial resistance in GAS and GBS in our country.

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ISOLATION OF LEGIONELLAE FROM HOTEL WATER SUPPLIES IN SOFIA

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SUMMARY

Investigations for the presence of legionella bacteria,in hotel water supplies in Sofia were performed. For this purpose one hundred and twenty five samples of potable water were collected from eleven hotels. A total of eighty-seven strains were isolated and determinate as Legionella spp. using culture, biochemical, iminuno-fluorescent (DFA) and gas-liquid chromotography methods. The legionella concentration ranged between I0'-I0⁴ CFU/L. By the DFA technique the isolates were identified as Legionella pneiimophikt: Sg 1-2 strains, Sg 2- 5 strains. Sg3- 20 strains, Sg 4- 5 strains, Sg6-52 strains, Sg 8-2 strains. Sg 14- I strain. Twenty water samples from one of the hotels were detected for amoebae too. After cultivation and microscopic examinations eight strains of small free-living amoebae (FLA) were isolated, At the same hotel we traced out the water quality before and after decontamination by hyperchlorination. It was established that this procedure led to sharp decrease of the bacterial number or absence of culturable legionellae but after one-two months they appeared again. These data suggest that it is necessary to organize regular monitoring of hotel water supplies in Sofia for the presence and the concentration of legionellae.

Key words: *Legionella pneumophila, free-living amoebae, potable water, hotels*

INTRODUCTION

Monitoring for the presence of a pathogen in the environment was initially implemented for the determination of potable water quality with bacterial counts. The monitoring for the presence of fecal contamination in the potable water has been one of the major achievements of public health programs. In recent years, it was found that other pathogens are also present in potable water supplies and that some of them are responsible for severe and even life threatening infections. Legionellae are among these bacteria.

Legionellae first commanded attention in 1976-77, when L. pneumophila was identified as ethiological agent in a massive outbreak of pneumonia in a hotel in Philadelphia [1,2]. The intensive survey on the problems of legionellosis found out that inhalation of" contaminated with legionellae aerosols is the basic mechanism for infection [3,4,5]. The role of protozoa, especially free-living amoebae (FLA) for the persistence and multiplication of legionellae in the hostile aquatic environment has been confirmed by many investigators [6,7.8,9.10]. In this respect mart made hot and cold water supplies are of a great health importance. There have been reported many epidemic and also sporadic cases of legionellosis (Legionnaires' disease, Pontiac fever) associated with hotels, leisure complexes and cruise ships [1,10.11.12,13,14} which designate the significance of this problem for tourists business.

The aim of the present study is to shed a light on the presence of legionella bacteria in the plumbing systems of big hotels in Sofia.

MATERIALS AND METHODS

Hotels and water samples. One hundred and twenty five samples of potable water were collected from eleven hotels of residence in Sofia. The frequency of examination for ten hotels was one to ten samples and in one hotel (X) - five samples monthly for one year. For each sample an amount of 200 ml water was collected in a sterile glass container mostly from showerheads and also from taps and tanks.

Processing for isolation of legionellae. An amount of 200mL from the sample was centrifuged at 5000 rpm for 30 ruin. The supernatant was removed, leaving approximately 1% of the original volume in which the sediment was resuspended. A volume of 0.1 ml from the suspended deposit was seeded on Petri dish with Buffered Charcoal Yeast Extract agar- BCYE agar (GIBCO) with PAV selective supplemenls. .spread with a glass spreading rod and after that a second plate with BCYE agar was inoculated with the same glass spreading rod. For 25 samples we used additional 2 plates with BCYE agar (OXOID) and MWY selective supplements which were inoculated by the same manner. All seedings were incubated at 37°C in a humidified atmosphere containing 2.5% CO; for 14 days and were examined dailv from the third day. Colonies that morphologically resembled legionellae were liable to further identification using culture methods, DFA technique (using antisera for 37 species) and gas liquid chromatography analysis of the fatty acid profile. Processing for isolation of FLA, 0.05mL from the bottom of the glass container were inoculated in the center of a plate with non-nutrient agar (NNA) seeded in advance with inactivated (at 56°C) Emerobacter aerogenes. Cultivation was carried out at 25°C for 7 days and regular observation on stereo microscope. FLA containing pieces NNA were transfer on bi-phasic media (NNA, Page's amoeba saline and inactivated E. aerogenes) for further observation by phase contrast microscopy.

RESULTS

A total of 125 water samples from 11 hotels in Sofia were investigated for the presence of legioneltae. From them 41 samples were positive [tabl.1]. The number of legionella colonies on a plate varied from 1 to 42 colonies on BCYE agar (GIBCO) and from I to 120 colonies (or more in some cases- data not shown) on BCYE agar (OXOID) [fig. 1]. The concentration of legionellae in the water samples was mostly between 10^2-10^4 ci U/L. From the positive samples a total of 87 strains was isolated and underwent further identification. They showed typical for legionellae culture (morphology of the colonies, absence of growth on 5% blood agar) and biochemical characteristics, as well as production of brown pigment on FG agar.

Species and serogroup identification was carried out by the DFA lest. All isolates stained very well with work dilution of the antisera, demonstrating bright yellow-green fluorescence of the periphery and relatively darker center of the cells. All they were rod shaped with length polymorphism in some cases. We found out that the isolates were from seven serogroups of L. pneumophila as follows: L.p, Sg6- 52 strains. L.p. Sg3- 20, L.p. Sg2- 5. L.p. Sg4- 5, L.p, Sgl-2, I./j.Sg8-2.L/J.Sgl4-l [tabl.21.

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ABBREVIATIONS USED IN THIS PAPER:
FLA - free living amoebae

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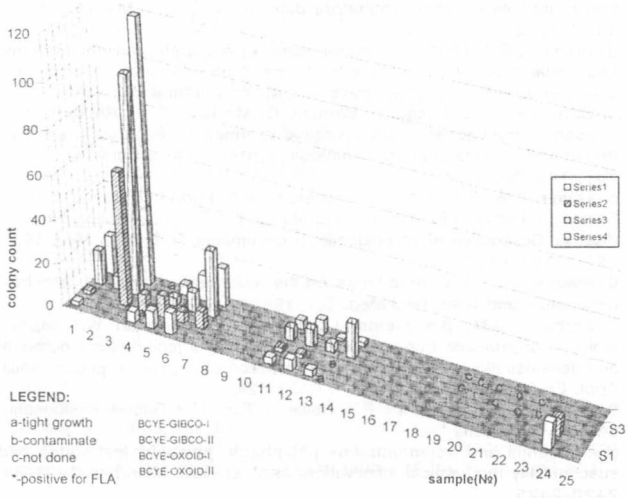

Figure 1.
Presence of Legionellae and FLA in water samples from hotel "X"

The gas-liquid chromatography of the isolated strains (compaired to the corresponding referent strains) revealed predomination of branched-chain fatty acids with big amount of $iC_{16:0}$ and $iC_{17:0}$ which are typical for genus Legionella. [fig.2].

Because of the big number of colonies in sample N3 [fig.l] we performed parallel serotyping of ten colonies ,wilh anti-sera for thirteen Sg of L. pneumophila. Eight colonies react with work dilution of fluorescent antibodies for L.p. Sg6 [fig.31 and two colonies - for L.p. Sg4 [fig.4].

In the investigation of 20 water samples from hotel "X" we were able to isolate FLA in eight of them [fig.1]. Their identification is forthcoming but some preliminary data (presence of typical acanthapodia) gave grounds to assume that most of them are from Acanthamoeba spp, [fig.5). Six samples positive for FLA were positive for legionellae two.

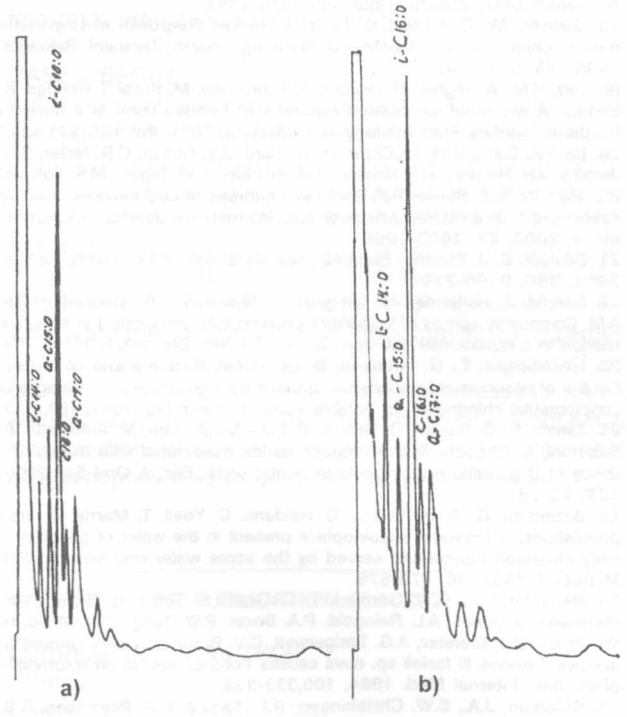

Figure 2.
Gas liquid chromatography of fatty acids of L. pneumophilla Sg 6:
a) referent strain Chicago-2 (ATCC 33215)
b) strain SH-VIII from water sample № 3

In the same hotel ("X") the presence of Legionella spp. was traced out monthly for one year and legionellae were found to be common isolates. Because of this a mechanical cleaning with following hyperchlorination of the plumbing system was performed. It was established that this procedure led to sharp decrease of the bacterial number or absence of culturable legionellae but after one-two months they ippeared.

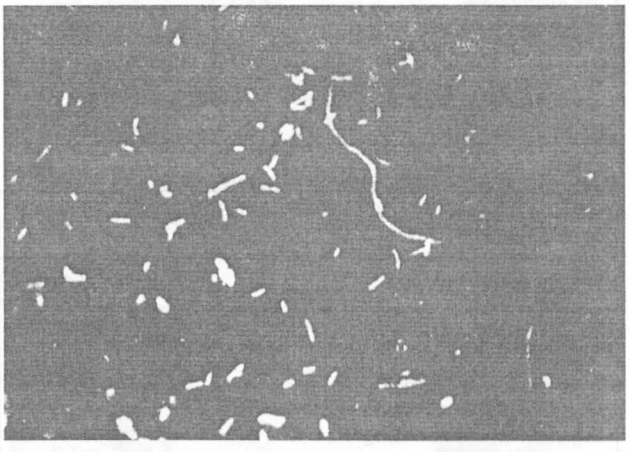

Figure 3.
L. pneumophila Sg 6 strain SH VIII isolated from sample N 3 DFA staining

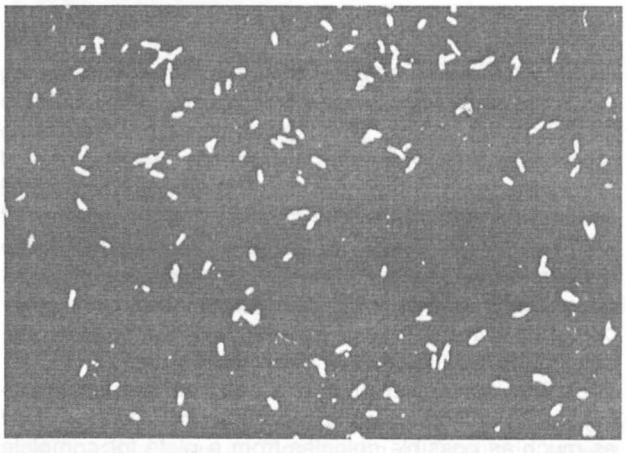

Figure 4.
L. pneumophila Sg 3 strain SH 340d isolated from sample N 3 DFA staining

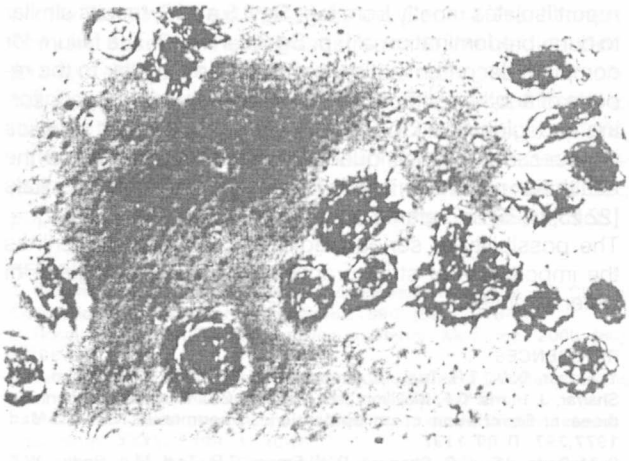

Figure 5.
FLA isolated from sample N 3. Presence of typical acanthapodia. Phase contrast microscopy

Table 1. Presence of legionellae in water samples from eleven hotels

Hotels	Investigated samples	Positive Samples
I	2	-
II	3	-
III	1	-
IV	5	-
V	5	1
VI	3	-
VII	3	2
VIII	5	2
IX	3	-
X	85	35
XI	10	1
Total	**125**	**41**

Table 2. Results from the identification of 87 legionella strains by the dfa method

Species and serogroup	Number of strains
Legionella pneumophila Sg 1	2
Legionella pneumophila Sg 2	5
Legionella pneumophila Sg 3	20
Legionella pneumophila Sg 4	5
Legionella pneumophila Sg 6	52
Legionella pneumophila Sg 8	2
Legionella pneumophila Sg 14	1
Total	**87**

DISCUSSION

In the investigation of water samples from the plumbing system of eleven hotels in Sofia legionellae were found to be a common isolates. When the investigations were done monthly with five samples per month the isolation of legionellae was more successful. The results from parallel seeding on selective media showed that the number of colonies on BCYE agar (OXOID) was more than twice greater compare to these from the same water sample but grown on BCYE agar (GIBCO). These findings are in accordance with the data of Fallown and Rowbotham concerning the isolation of Lmicdadei from water [II].

The mixed contamination in one water sample is interesting finding which shows the necessity for detailed study of as much as possible colonies from a plate for complete picture of the Legionella population in a plumbing system. The presence of FLA and legionellae in 6 (from 20) samples confirm that FLA are basic hosts of legioncIla bacteria in the environment, found by another authors [15,16,17]. They report isolates mostly from L.p. Sg 3.5 and 6, results similar to ours- predomination of L.p. Sg 6,3,4 and 2. The failure for complete decontamination is observation similar to the reports of another authors [18], It shows that regular monitoring for legionellae (and FLA) in man made water supplies is necessary for their quantity control and to reduce the health risk not only in hotels [19,20,21] but also in hospitals [22,23,24,25] and plants [26,27].

The possibility of serious legionella infection underlines the importance of strict practices to maintain hygiene of water supplies.

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CASE OF DIROFILARIASIS WITH MALE BRAEST LOCALIZATION CAUSED BY DIROFILARIA REPENS

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SUMMARY

The rare case of dirofilariasis with male breast localization of Dirofilaria repens has been described. The parasitological diagnosis has been set up after diagnostically and therapeutically extirpation and histopathological examination of the tissue material.

Key words: *dirofilariasis, pathohistology, Dirofilaria repens.*

INTRODUCTION

Sporadic cases due to Dirofilaria repens have been rarely diagnosed in Bulgarian population. The invasions of nematode Dirofilaria repens that predominate in South Europe are mainly described (2). The females are usually longer than males and reach a length of 15 cm. The parasite's body is threadlike with diameter 0, 5 mm. Dirofilaria repens is distinguished with longitudinal ridges cuticle, in contradistinction from Dirofilaria immitis, which cuticle is smooth (1). The human is accidental unspecific host and he is not source of infection, because in him the parasite doesn't develop to sexual maturity. The main final host is dog, the cat is possible too, and the transmissible vector is different types of mosquitoes (3). The parasite is localized in the subcutaneous tissues over all parts of the human body where microphilaria is inoculated by the mosquitoes (4).
In half of the all clinical cases Dirofilaria repens is located in the conjunctive of the eye (5).
In this report we are going to describe briefly one case with rare male breast localization by Dirofilaria repens.

CASE REPORT

A male patient, 28-y-old, during the present summer, has noticed a nodule in his left breast near the areola area. On this place is determined a painless, nodular tumor at the size 2 cm. With diagnostic and terapeftic aim an operative removal of the tumor has been carried out. The initial patient's diagnosis was: Obs. Fibroadenoma glandulae mammae sinistri. But histological examination of the extirpated "in toto" tissue material showed unexpected result. The tissue material was fixed in 10 % formalin and carried by the routine histological method. The microscopic appearance of the nodule is a granuloma abscess with a well-defined fibrous wall and necrotic center, containing many sections of parasites Dirofilaria repens. Note the mass of eosinophils and polymorphonuclear cells against the worm's cuticle making the morphology rather vague (Fig.1). High magnification of cross section of Dirofilaria repens shows the thick layer cuticle, the internal thickening and the longitudinal ridges (Fig.2).

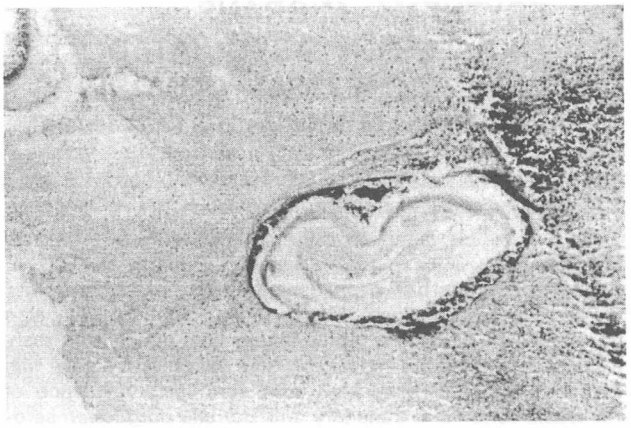

Figure 1.
Dirofilaria repens hematoxylin and eosin stain. Low power view of granuloma with necrotic center, containing of parasite and a lot of polymorphonuclear cells (40 x 2,5)

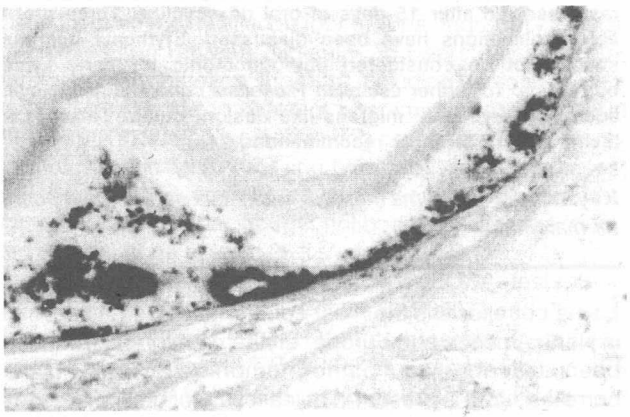

Figure 2.
Dirofilaria repens - high magnification showing the thick layer cuticle, the internal thickening and the longitudinal ridges(10 x 2,5)

DISCUSSION

The described pathologic-histological finding shows only one possible unusual localization of Dirofilaria repens, because the parasite usually is found subcutaneous or subconjunctive. In that case the exact parasitological diagnosis has become possible after histological examination of the tissue material. Usually etiological diagnosis of dilofilariasis is not set initially by the General Practitioners. The rare cases of the diseases reach to the Medical Doctors with different specialties, who are surprised of parasitical diagnosis. It is necessary for General Practitioners to foresee the disease in the differential diagnostically plans. In the last years the cases of dirofilariasis in humans are more often. Probably because of the uncontrollable dog population- the final host and the source of the infection.

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ERYTHEMA MIGRANS - LIKE LESION ASSOCIATED WITH RICKETTSIA CONORI

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SUMMARY

Erythema migrans has been described as "pathognomonic" for early Lyme borreliosis. Mediterranean spotted fever, also transmitted by ticks, is a febrile illness with generalized rash, although few cases are "spotless". Here we present a case with Erythema migrans-Hke lesion with serological evidence of *Rickettsia conori* and transitory anti *Borrelia burgdorferi* seropositivity. A 58-year-old woman was admitted to the hospital in March 2000 with a 2-day history of low-grade fever and rash on her popliteal fossa. An engorged tick was removed from the same place, later identified as *Demacentor marginatus.* The rash resolved after 15 days of oral doxycycline. Three probable explanations have been discussed. Erythema migrans should not be considered pathognomonic for early Lyme borreliosis. To further establish *Rickettsia conori* as aethiologic agent of Erythema migrans-like lesion culture and PCR technique are strongly recommended.

Key words: *Erythema migrans, Rickettsia conori, Dermacentor marginatus*

Lyme borreliosis is the most common tick-borne infection in North America and Europe. Erythema migrans (EM) has been described as "pathognomonic" for early Lyme borreliosis and is present in at least 90% of objective cases of Lyme disease Although once considered classic finding for Lyme disease, EM may be confused with other rashes identical in appearance. These include a hypersensitivity reaction to tick or other insect bites and a recently described rash linked to the bite of an *Amblyomma* tick. This infection likely is caused by another, as yet uncultivable *Borrelia* (2). Mediterranean spotted fever, also transmitted by ticks, is a febrile illness with generalized rash, although few cases are "spotless" (1). The aim of this study is to present a case with Erythema migrans-like lesion with serological evidence of *Rickettsia conori.*

Case report: In March 2000 a 65-year old woman was admitted to the hospital with a 2-day history of low-grade fever and localized rash. Twelve days earlier she removed a tick from his popliteal fossa; subsequently a circular red rash developed at the site of the tick attachment. The tick was kept and later it was identified as *Dermacentor marginatus,* a species found across Europe. At presentation the woman was afebrile and her physical examination was unremarkable apart from a fading erythematous circular rash in his popliteal fossa measuring 25/30 cm. Laboratory tests were within normal range. A diagnosis of Lyme borreliosis was suggested and treatment with oral doxycycline 100 mg twice daily for 15 days was prescribed. She recovered uneventfully and remained well 6 months later. The acute-phase serum sample, taken on day sixth, was positive by immunoenzyme assay (DAKO) for IgG antibodies to *Borrelia burgdorferi* but the convalescent serum sample taken 20 days later was negative. The patient seroconverted against *Rickettsia conori* by imunofluorescence assay at a liter of 1:80.

Discussion: Our patient presented with fever and an erythematous rash around the site of a tick bite that resembled EM, and she had serological evidence of infection with *R. conorii.* It might be that the case is Mediterranean "spotless " fever with a cutaneous reaction to the bite. However the 6-day incubation period makes this unlikely, although rarely, delayed reaction sometimes occur.

Another explanation would be a dual infection with *Rickeitsia conori* and *Borrelia burgdorferi,* which to our knowledge has not been previously described. Yet transitory anti *Borrelia burgdorferi* IgG seroposhivity is difficult to explain, as IgM and IgG responses to *Borrelia burgdorferi* not rarely may persist for years even after successful treatment.

Finally, it is probable, thai there may be an unidentified rickettsial tick-borne pathogen, causing the clinical picture in our case, that cross reacts with *Rickettsia conorii.* Recently two new rickettsia species, pathogenic for humans, have been described in Europe - *Rickettsia helvetica* and *Rickettsia slovaca* (3,5). Interestingly, they have been reported in patients with no rash, which is the characteristic clinical feature of other rickettsial diseases. The main symptoms of infection with *Rickettsia slovaca* are lymphadenopathy and inoculation skin lesion, varied from crusted scalp lesion (eschar) to EM-like lesion (5). What's more, cases with *Rickeftsia slovaca* occurr during early spring mainly in March and April and are associated with *Dermacentor marginatus* tick, as was the case with our patient.

Our case highlights difficulties that might come out when evaluating patient with EM-like lesion in early spring. To further establish *Rickettsia conori* as aethiologic agent of Erythema migrans-like lesion culture and PCR technique are strongly recommended.

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ABBREVIATIONS USED IN THIS PAPER:
EM - Erythema migrans

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APPLICATION OF RUBELLA IgG TOTAL ANTIBODY AVIDITY MEASUREMENT IN THE DIAGNOSIS OF RUBELLA INFECTION

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SUMMARY

OBJECTIVE: The measurement of total IgG antibody avidity in human sera is a useful technique in confirming the differentiation of an infection. MATERIALS AND METHODS: Sera samples (n=79) from rubella immune patients (n=54),classified in four groups: I (group)-including cases (n=12) with clinically and serologically confirmed primary infection; II(group)-including persons (n=25) with MMR vaccine in different post-immunization period; III(group) - including women during pregnancy and women in vitro inseminate (n=15) and IV(group) - including cases (n=2) with rubella embriopathy (CRI) were investigated. ELISA test (commercial kit, firm Bio-Rad) - Platelia Rubella IgG was used. 8 molar urea in wash buffer as a protein denaturing agent was used too. RESULTS: Low avidity - antibody index (AI) below 30% (cut-off value) in a range between 10.80% to 25.54% was proved among cases with primary infection, immunized persons in the first three months following vaccination and in one of the rubella embryopathy cases. High avidity - antibody index (AI) above 30% was proved in convalescent sera (previous infection and immunization) - 68.90% (mean value). CONCLUSION: The priorities of the IgG total antibody avidity measurement test as good diagnostical marker were discussed.

Key words: *IgG total antibody - avidity measurement, urea index (ratio), primary infection, reinfection, rubella embryopathy*

Primary rubella infection is usually diagnosed by the detection of specific IgM antibodies in addition to the clinical information. When the blood sample is taken during the first one to three weeks after onset of symptoms the result must be confirmed in paired sera by routine hemagglutination inhibition test (HIT), radial hemolysis test (RHT) and enzyme-immunoassay (EIA) test for detection of specific IgG antibodies.

Sometimes the diagnosis may be difficult to interpret by these sensitive methods,for example in cases when a specific IgM antibodies can persist longer than 8 to 12 weeks and it is difficult to decide if a low positive IgM results is due to recent rubella infection or is caused by rubella reinfection. The last is observed following previous vaccination and is mostly detected following rubella contact.

The HI antibodies rise and often reacted at very high titer with or without presence of specific IgM antibodies.

The avidity or affinity of IgG is initially low after primary antigenic chalenge, and it matures slowly withi weeks and months (3 , 9).The conventional affinity assays of basic immunology for example the globulin precipitation tests are not suitable for the measurement of the polyclonal antibody responses. Decade before, novel techniques have been developed for the serodiagnosis of rubella infection which measure the avidity of specific IgG. These tests are based on the finding that the avidity of antibodies for their antigenic target increase with time,reflecting maturation of the immune response. In the quantitative test,urea and an other chemical substances (diethylamine, thyocianate, guanidine-hydrochloride) is used as a protein denaturing agent (7) to elute IgG from immobilised rubella virus antigen. This results in almost complete dissociation of low-avidity IgG in conditions where high-avidity IgG antibodies mostly remain antigen-bound (6).

Here we examine the ability of the IgG avidity assay,under routine diagnostic laboratory conditions by using a collection of sera previously examined for different evidence of rubella immunity status.

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ABBREVIATIONS USED IN THIS PAPER:

HIT - hemagglutination inhibition test, ELISA test - enzyme-linked immunoassay test, MMR vaccine - measles mumps rubella vaccine CRI - congenital rubella infection, AI - antibody index

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MATERIALS AND METHODS

1. Materials: Sera samples (n=79) from rubella immune patients (n=54),classified in four groups: I (group) - including cases (n=12) with clinically and serologically confirmed primary infection; II(group),including persons (n=25) with MMR vaccine in different post-immunization period; III(group) - including women during pregnancy and women in vitro inseminate (n=15) and IV(group) - including cases (n=2) with rubella embriopathy (CRI) were investigated.The concentration of specific IgG antibodies in the urea studied sera must be at least 25 IU/ml.

2. Methods: standart HI test, indirect ELISA tests, Bio-Rad, (Platelia Rubella IgG - Lot.Ch.B.:1G0073 and Platelia Rubella IgM - Lot.Ch.B.:1H0064,commercial kits) were used. 8 molar urea in wash buffer as a protein denaturing agent was used too. Each test included controls for low and high avidity antibodies (pool from acute and convalescent sera).In case of equivocal result,one more serum was tested.

RESULTS AND DISCUSSION

The maturation of specific IgG antibodies after clinically and laboratory confirmed rubella infection was followed in 34 sera from 12 patients *(the I-st group)*. Sera samples were taken in the first to six and above months after oncet of symptoms. Initially,all sera were tested with standart HI test,indirect ELISA IgG and IgM tests for presence of antibody activity. Than a standart ELISA IgG test by urea-index (ratio) procedure was applied. An avidity index is calculated in per cent for each specimen as follows:optical density (OD)-assay with urea /optical density (OD)-assay without urea x 100. The results are shown in Table 1.

After the first week and up to the fourth week following rubella the IgG ratios increased steadily and were in range 5% and 36%.

The development of IgG ratios in 31 sera from 25 vaccinees *(the II-nd group)* was also studied. In 11 sera from 7 persons,age 1 - 2 years we have observed post-vaccination rubella infection (the sera were taken from 17 days to one month) with IgG ratios below 30% in range between 18.50% and 27.10%. In all seven sera IgM antibodies were also positive. In 20 sera taken between 8 months and 10 years post-vaccination the ratios increased to 47% - 77%.

The investigated sera from the *III-rd group* show IgG ratios in range between 32% and 79%. Three of them had serological data probably for re-infection with very high HI titer-above 1:512 but no evidence for IgM antibodies. It worth to remark that antibody-index of the present IgG antibodies(OD of the sample before urea treatment was 2.199) in one serum was only 22% (28 years after rubella infection).

Table 1. Serological investigation and development of IgG antibody-avidity index in 12 cases with recent primary rubella infection

Patient No	Sera	Oncet of symptoms (day)	HI test	ELSA IgM test	ELISA IgG test IU/ml	Ratio (AI) %
1	I	3	256	+	< 15	-
	II	15	1024	+	60	18.50
	III	240	64	-	200	73.30
2	I	2	16	+	< 15	-
	II	12	64	+	120	25
	III	210	16	-	190	51
3	I	2	< 8	-	< 15	-
	II	10	256	+	90	26
	III	210	64	-	150	62
4	I	1	< 8	-	< 15	-
	II	7	256	+	130	24.30
	III	180	32	-	230	56
5	I	3	32	+	< 15	-
	II	30	128	-	160	24
	III	170	128	-	290	64
6	I	5	1024	+	200	5
	II	27	1024	+	205	32
	III	210	128	-	216	67
7	I	4	256	+	80	15
	II	30	256	+	105	25
	III	240	128	-	250	65
8	I	8	128	+	178	14
	II	29	128	+	170	34
	III	210	32	-	150	64
9	I	9	512	+	140	25
	II	20	256	+	145	30
	III	180	64	-	175	57
10	I	17	128	+	108	21
	II	36	256	+	178	36
	III	240	128	-	252	59
11	I	32	32	+	187	31
	II	200	16	-	230	88
12	I	2	8	+	<15	-
	II	23	1024	+	55	35.50

Our data are in accordance with Hedman et al. (6) and Enders et al. (4) who have discussed this diagnostical method.

We have studied only two cases *(the IV-th group)* with rubella embryopathy (CRI) whose mother had been infected in early pregnancy. Information on the maturation of avidity of specific IgG after intrauterine infection is not available,however. Low avidity of specific IgG antibodies (ratios - 5%,15%,16%, 25% and 29.50%) was demonstrated in consecutive sera (20 days,6 months,one year,1 1/2 years and two years of age) from one of these infant. All sera with exception of the last one (2 years after birth) gave positive Ig M values. The present HI rubella antibodies in birth's sera were:baby's - 1:128 and mother's - 1:256. The next sera from the child show results:1:32/64,!:32/64,1:64 and 1:32 as well as a specific IgG - 350IU/ml,180 IU/ml,180 IU/ml,200 IU/ml and 150 IU/ml. The mother's IgG antibody-avidity was 61.50% at birth. The child has emphatic clinical manifestations:low birth weight, hepatosplenomegaly, microcephaly, rubelliform rash, rubella pneumonitis, inguinal hernia and mental retardation.

The second child had a thrombocytopenic purpura (Thr,32000-38000) only as a clinical manifestation after delivery. The present HI rubella antibodies at birth were:baby's serum-1:128,mother's serum 1:128;specific IgG of baby's serum were 109 IU/ml and spesific IgM - positive. Spesific IgG urea-ratio was 58%.The mother's antibody-avidity was 56.80% at birth.

The two mothers have been had a rash in the first trimester of their pregnancy,estimated as allergical. We have studied the other four sera (IgM antibodies negative) from children at birth or shortly after delivery (14,25 and 28 days and 6 months) as controls. They have also maternal antibodies and the urea-ratios were 15%, 11%, 48.30% and 75.40% respectivly. Our data are in accordance with Thomas et al. (8) who have studied the persistence of specific IgG and IgM antibodies and immuno-pathological problems in CRI. Maturation of the immune response is abnormally slow in congenital infection with regard to both isotype switching and the development of high-avidity IgG (8).

A difference in avidity in sera from mother and child at,or shortly after delivery would be diagnostic of an early infection in utero. These results may also be of relevance in immunopathology observed in CRI. The last may result from the formation in certain tissues of antigen-antibody complexes containing low avidity antibody that are not readily cleared by the immune system (2, 8). Rubella spesific immune complexes,not observed in sera from cases of remote rubella,have been demonstred in sera from CRI cases,although the avidity of the specific IgG antibodies was not ascertained (1, 8).

In conclusion, it is necessary a large number investigations with sera in different stage of rubella infection. The results show that the IgG total antibody avidity measurement have priorities as useful marker in the diagnosis of rubella infection.

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CLINICAL EXPERIENCE IN THE TREATMENT OF FLU PATIENTS WITH OSELTAMIVIR DURING THE INTERPANDEMIC PERIOD

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SUMMARY

The influenza epidemics are very important health and economics issues. That explain why defining the methods for proper treatment and prophylaxis of influenza has become so important over the past few years. The study included people with present influenza symptoms throughout the last influenza season / December/ 2001-April/2002 / confirmed by IIF, ELISA-IgM, isolation of the virus and with clear clinical symptoms. The goal of the study was on the basis randomized, double-blind study to evaluate the therapeutic effects of oselatamivir concerning the duration of the illness and symptoms. The study shows that the therapy with oselatinivir resulted in reduction of symptoms in febrile as well as in non febrile patients, smaller administration of symptomatic therapy, less complications as a secondary bacterial infections, lower administration of antibiotics. The drug oselatamivir is very well tolerated and only 5,9% of the treated patients noted intolerance with vomiting. From the present study the authors conclude that treatment of influenza with oselatamivir causes significant shortening of illness period as well as of clinical expression and symptoms of influenza.

Key words: *influenza epidemics, oseltamivir, influenza virus A and B*

In regions having a temperate climate, influenza epidemics occur annually. They occur predominately during the winter months, and are usually associated with increased mortality among the higher risk population (namely, the elderly and persons with chronic diseases). Likewise, the general population's morbidity rate also increases, very dramatically in some years. The effect of influenza epidemics is here attested by significant work or school absenteeism, and decreased level of worker productivity. These effects are prolonged, in many cases, as symptoms of the illness can last for up to seven days. For illustration, in 1997 influenza and pneumonia were ranked sixth among the leading causes of mortality in USA. Over the past 25 years, an average of 20,000 Americans a year have died from these disease. It is estimated that the annual economic loss due to influenza stands at $3-5 billion. These factors explain why defining the methods for proper treatment and prophylaxis of influenza has become so important over the past few years. While vaccination remains the standard form of prophylaxis, an increasing number of studies have begun to investigate the prophylactic potential of certain chemotherapeutics. Despite everything the illness continues to appear, especially among the children and young adults, the groups with the highest risk for morbidity. Antiviral agents remain a complementary treatment, and in no way substitute for extensive and efficient vaccine programs. Antiviral agents administered early during the infection can reduce the duration of the illness and lessen the degree of symptom expression (intensiveness, severity), and with all that also to reduce the general impact of influenza.

The first antiviral agents used to treat influenza infections were amantadine and rimantadine, but their use was limited only to a few regions worldwide. With time, questions were raised regarding their true role in the treatment of influenza, their side effects, and most importantly, their potential in selection of resistant viral strains.

Previously, a consensus had been reached regarding the need for prompt treatment of influenza. The rationale was that even the most uncomplicated bout of influenza is a long-lasting illness which significantly limits daily activities. However, this consensus changed after several additional considerations were taken into account. The risk of side effects, especially during the amantadine therapy, the possibility for appearance of resistant types, and the need for reassurance that the illness treated is in fact caused by the Influenza A virus. The efficiency of these drugs is documented only against influenza virus type A. Therefore, their administration becomes insufficient in years when mixed influenza epidemics occur. Thus the need for an efficient antiviral agent with broader activity spectra and with better characteristics regarding the safety profile. First in the new class of antiviral agents which actively inhibit the influenza-neuraminidase, the enzyme which is essential for viral replication in vitro, was zanamavir. Its efficiency, proven in vitro on experiment model with animals and young adults, covers both influenza A and B infections. The clinical effectiveness of locally administered (through oral inhalation) zanamavir against natural influenza infection in humans has been documented in large placebo-controlled studies. But the very use of the inhaler for its application means that patients need to be taught how to use an inhaler. In patients with asthma or chronic obstructive pulmonary disease (COPD), Zanamavir can induce bronchospasm and reduced pulmonary function- such patients, therefore, should avoid using zanamavir. In addition, zanamavir has certain, albeit rare, side effects; most prominent are irritation of the nose and throat, coughing and headaches. Unlike zanamavir, the newer neuraminidase inhibitor, oseltamivir, is administered orally (75 mg tablets) and is a "prodrug". This means it must be converted, with the activity of hepatic esterase, into its active form, oseltamivir carboxilate. Oseltamivir carboxilate is not further metabolized and as such is completely eliminated through renal excretion. Its only registered side effects are nausea and vomiting, which can be lessened by taking the drug together with food. Compared with other antiviral agents, this drug has a very small potential for selection of resistant strains/variants of the virus. With all of these considerations in mind, we sought to define the clinical efficiency and safety of oseltamivir on the general population, with our own material. Furthermore, this study involved persons at high risk for developing complications associated with influenza infection. This randomized, double-blind study evaluated the therapeutic effects concerning the duration of the illness and symptoms relief in function of defining the indicators for the severity of the illness.

MATERIAL AND METHODS

This study included people aged 18 or older with present influenza symptoms, of a duration of 48 hours or less throughout the last influenza season (December/2001-April/2002). Primarily, these were patients treated during the last two months of the season. Influenza symptoms are defined by fever, chills and the presence of two or more of the following: myalgia, headache, cough or sore throat. The probability of proving influenza infection in the patients that were included was high, considering the fact that this study was undertaken during the period in which there was high percent of 85% of serologically proven (by the IIF method) influenza cases. In addition, this study also included patients over 65 years of age, as well as patients with chronic diseases (cardiovascular, respiratory, endocrinal, diseases of the metabolism, excluding those patients having an unstable disease); this category of patients is marked as a high risk group for developing complications or more severe or prolonged forms of the illness. In the study, oseltamivir was administered in an oral dosage of 2x75 mg/daily, for a period of 5 days. The study was completed with successive gathering of the planned 50 (20 with, and 30 without oseltamivir) patients, based on the principle of coincidence and according to their daily arrival at the Clinic for Infectious Diseases in Skopje. We randomly analyzed the patients in a 1:1/1:2 relation to those patients who did not

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ABBREVIATIONS USED IN THIS PAPER:
COPD - chronic obstructive pulmonary disease

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take Oseltamivir. All of them were outpatients and they were given a questionnaire in which, on a twice daily basis, morning and evening, over the next ten days, they marked their temperature and the severity of symptoms (clinical manifestation). The clinical manifestation of symptoms we marked on a 6 degree scale, on which 0 corresponds with a total absence of symptoms, and 5 with the highest intensity of presence of symptoms. On the same questionnaire the patients noted any sleeping disorders, their capability for completing daily normal activities, and their overall health condition. The further analysis of the study included only patients with confirmed influenza infection (IIF, ELISA-IgM, isolation of the virus from samples taken from the upper respiratory tract, on the very first day the patients arrived at the Clinic). Of 41 patients (82%, 41/50), it was found later that 17 (85%, 17/20) had taken oseltamivir, and 24 (80%, 24/30) had not. Our primary clinical goal was to determine the duration of the illness until any lessening could be noted in clinically significant symptoms, defined with the absence of chills, fever of lower than 37.5°C, and a mark "0" (absence) or "1" (mild) for any other major symptoms (for example headache, mialgia, sore throat or cough) remaining during the next 24 hours. The time needed for relief of symptoms was determined on the basis on half day from starting of the treatment (day one), with the morning parameters from day one of the treatment that correspond with "0" day. The time period from the morning measurements until the evening ones represents half day. In addition, we sought to determine the middle score of symptoms, sleep disruption, duration of symptoms before normalization of daily activities, as well as the usage of acetaminophen and cough-relieving drugs. The degree of clinical expression of the five symptoms (chills, headache, mialgia, cough, and sore throat) was summarized for each patient by calculating the middle score of symptoms, used as a measure for the overall severity of clinical symptoms. The middle score of the symptoms is determined with varians analysis.

RESULTS AND DISCUSSION

In the group of patients treated with oseltamivir, the final point, alleviation of major symptoms, was achieved around the fourth day. In the group of patients who were not treated with oseltamivir, alleviation occurred only on the sixth day. This difference was more obvious in patients who arrived at the Clinic and began their treatment during the first 24 hours of symptoms appearance, (for 2-3 days). The difference was also most notable among those patients who were febrile or belonged to the high risk group at the time when put under therapy. In patients who had their treatment started within 24 hours of symptomatic appearance, therapy with oseltamivir shortened the illness period to 0.5-1 day. Therapy with oseltamivir resulted in a reduction of symptomatic duration in febrile, as well as non febrile patients (at the time of inclusion in the study), though the effects were more evident in the former group. The middle duration of symptoms in the high risk group of patients was longer compared with the entire treated group (6.56 contrary to 6.0 days), which documents the trend for faster relief of symptoms in patients with high risk. Therapy with oseltamivir also resulted with smaller administration of symptomatic therapy, e.g. the same was associated with reduction of the usage of acetaminophen and antitusics. During the first three days, administration of acetaminophen was decreased for 38%, and for cough-relieving drugs 29%. In this study we also analyzed the incidence of secondary diseases (otitis media, pneumonia, etc), as well as the incidence of hospitalizations. Complications were rare in the group treated with oselatmivir, meaning, a reduction of patients with otitis media as a complication has been achieved from 12.5% (3/24) to 5.9% (1/17) as well as with pneumonia from 20.8% (5/24) to 11.8% (92/17), and generally speaking with all this a dramatic reduction of hospitalizations has been managed, from 29.2% (7/24) to 11.8% (2/17). Administration of antibiotics was significantly lower in the group of patients treated with oseltamivir. Patients treated with oseltamivir tolerated the medicine very well, and only in one patient is noted intolerance with vomiting (5.9%). It took many years for consensus on use of amantadine and rimantadine to be reached in North America. This was partly due to the fact that the studies, especially those involving treatment, were small and differed in design, making assessment of the precise therapeutic benefit difficult. The development of neuraminidase inhibitors provides an alternative antiviral therapy for the management of influenza infection. This agents have convincing efficacy against influenza caused by different types of influenza viruses, as is shown in many studies. In contrast to older antiviral drugs, studies with the neuraminidase inhibitors have used the same well-defined end point and similar design. The benefits of them appear to be greatest where the illness is most severe, as would be expected from the natural history of influnza infection, and when treatment is given early. There are also benefits on patient quality of life and functioning. The results of all studies with oseltamivir indicate that it is well tolerated, with a reported side-effect profile comparable to placebo. In this study this was evident in both otherwise healthy persons and in those at high risk of complications. The fact that oseltamivir can be used to treat all strains of influenza A and B viruses and that resistance has not been detected during acute therapy, also suggests that it has definite advantages over the older antiviral agents. From the presented results we can conclude that treatment of influenza with oseltamivir causes significant shortening of illness period as well as of clinical expression and symptoms of ifluenza. Thus, there seems to be a clear role for oseltamivir in the treatment of type A and B influenza infections.

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INTRADERMAL TESTS WITH ALLERGENS *S. AUREUS* AND *C. ALBICANS* IN PATIENTS WITH ATOPIC ECZEMA/ DERMATITIS SYNDROME

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Atopic eczema/dermatitis syndrome (AEDS) - is a chronic allergic skin disease. There is increasing evidence that infectious agents are important triggering factors in the pathogenesis of AEDS [23].

S. aureus has been studied as possible trigger factor. It is well known fact that the skin of 70-90% patients with AEDS is colonized with *S. aureus* in extremely high density (10^7 colony - forming units/cm^2) [3,20]. In contrast only 5-10% of healthy individuals without any skin diseases are carriers *S. aureus* in low density of colonization [2,27]. *S. aureus* is known as the producer of proteins with superantigenic properties: staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C (SEC), toxic shock syndrome toxin - 1 (TSST-1), staphylococcal exfoliative toxin (SET). At first, they can bind to the MHC II molecules of monocytes and dendritic cells and release a number of proinflammatory molecules such as interleukin-1 (IL-1) and tumor necrosis factor - alpha; at the second, they can stimulate the proliferation of T-cells with Vв chain expression [11,15,18]. Moreover staphylococcal enterotoxins are considered as a new group of allergens. Specific IgE-antibodies to SEA, SEB, SEC and TSST-1 have been detected in sera from 57% of patients [4,16,17,26]. Basophils of the patients with AEDS were found to release histamine upon incubation with these toxins [9].

The relationship between the severity of AEDS and the density of *S. aureus* colonization in the skin was revealed. The moderate and severe courses of AEDS are characterized by the high density of staphylococcal colonization. Recently a number of studies have published that the severity of AEDS depends on presence of staphylococcal enterotoxins in the skin secreting by *S. aureus* [1,3].

The lipophilic yeast *Malassezia spp* is not only part of the normal human skin flora but also an opportunistic pathogen. Various recent reports have discussed *Malassezia spp* as a pathogenic factor in AEDS [7,22]. *C. albicans,* unlike *Malassezia spp,* does not normally colonize the skin, but rather the gastrointestinal tract and the nasopharyngx. A pathogenic role for allergy of *C. albicans* has for long been proposed, particularly in asthma and rhinitis, and a high rate of positive immediate skin test to *C. albicans* has been observed in atopic patients [24]. Allergenic cross reactivity between *Malassezia spp* and *C. albicans* has been investigated and found to be significant [6,12,14].

The allergenic properties of *Malassezia spp* and *C. albicans* are well known and few recombinant allergens have been obtained and characterized [28]. The allergens have been recognized as mannoproteins. It was shown that predominantly sensitization to *Malassezia spp* has been occurred in patients with head and neck skin lesions [19]. *C. albican* as allergen, together with other antigenes (PPD, tetanic toxin, trichophytin), is as the marker of cell immunity and reveals delayed type hypersensitivity reactions *in vivo* [5,8].

The aim of the study was to investigate immediate and delayed type skin reactivity by means of intradermal testing by allergens from *S. aureus* and C. *albicans* in patients with AEDS.

MATERIALS AND METHODS

Patients and diagnostic criteria. 28 patients from 14 till 40 years (age - 23,9±4,6 years) with different severity of AEDS were observed. The following participants were recruited: AEDS was diagnosed according the criteria Hanifin and Rajka [10] and the revised nomenclature for allergy was used [13]. The severity of AEDS was scored according SCORAD system [25]. The severity of AEDS was mild in 16 patients, moderate - in 12, severe - in 7 cases. All patients did not receive antihistaminic, cytostatic and corticosteroid drugs for 2 weeks before testing.

28 healthy nonatopic people in the age of from 15 till 38 years (mean - 18,1 years) made the control group.

The local ethics committee approved the protocol and we obtained written informed consent from all patients.

Intradermal tests with allergens. Allergenic extracts of *S. aureus* and *C. albicans* provided by the National Center of Infectious and Parasitic Diseases (Sofia, Bulgaria) were used. Testing was performed by means of intradermal injections of the 0,05 ml allergen solution in skin of an internal surface of a forearm. The estimation of immediate reaction was carried out after 20 minutes and delayed reactions after 72 hours. The following parameters were taking into account: the size of wheal in 20 minutes and the size of papula in 72 hours. Measuring of the papula/whealÿs sizes after 20 minutes 24, 48 and 72 hours was provided to estimate the dynamics of the skin reactivity in patients with AEDS.

Statistics: P - value of less than 0,05 was considered to be statistically significant. *Chi* - squared tests and t - tests were used for categorical and continuous variables, respectively. The statistical package spss 9,0 for Windows was used for the analysis.

RESULTS

Intradermal testing with allergen *S. aureus* and *C. albicans* was provided in 28 patients with AEDS and in 28 non atopic healthy persons. There were not registered side effects during intradermal testing by allergens *S. aureus* and *C. albicans.*

The results of intradermal testing with allergen *S. aureus* with an estimation of the sizes of skin reactions after 20 minutes are presented on fig.1. Apparently the sizes of wheal in the group of patients with AEDS have made on the average 3,9 mm, against 1,8 mm in a control group, i.e. in 2 times more. However there was no statistically significant correlation between these data in both groups (P=0,5). At the same time, the wheal after introduction of allergen *S. aureus* was absent in 15 patients with AEDS and in 19 - from group of comparison. Thus immediate reactions to *S. aureus* are revealed in 46,4 % of atopic patients and in 32,1 % of nonatopic healthy people. The severity of AEDS (according to SCORAD) in the patients with positive immediate re-

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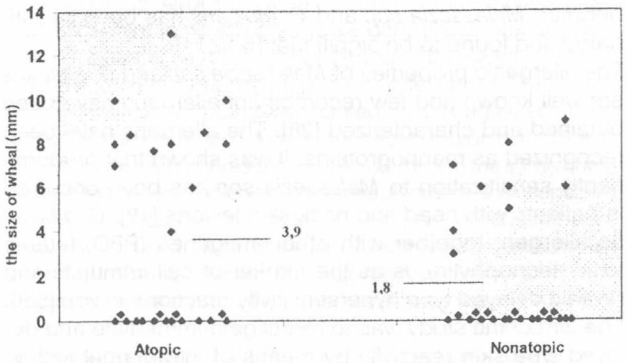

Figure 1.
Immediate hypersensitivity type reactions at intradermal testing with allergen *S. aureus* in atopic and nonatopic patients

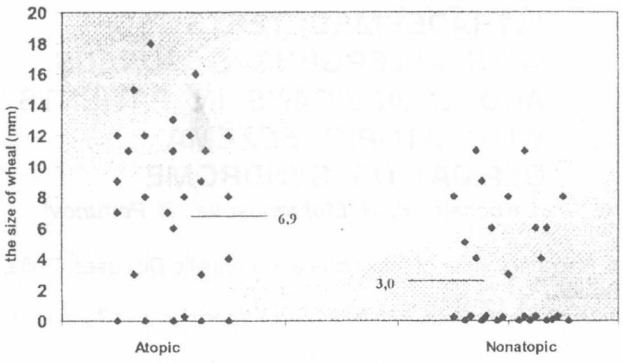

Figure 2.
Immediate hypersensitivity type reactions at intradermal testing with allergen *C. albicans* in atopic and nonatopic patients

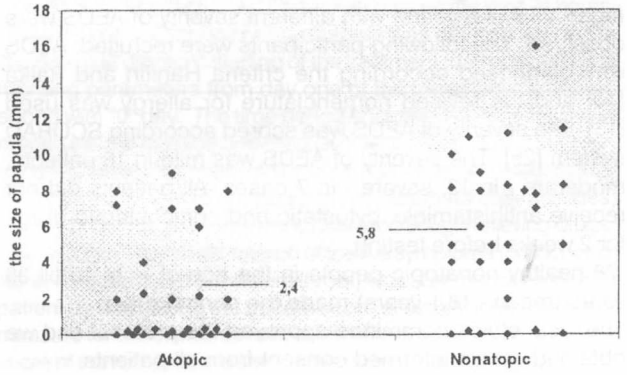

Figure 3.
Delayed hypersensitivity type reaction at intradermal testing with allergen *C. albicans* in atopic and nonatopic patients

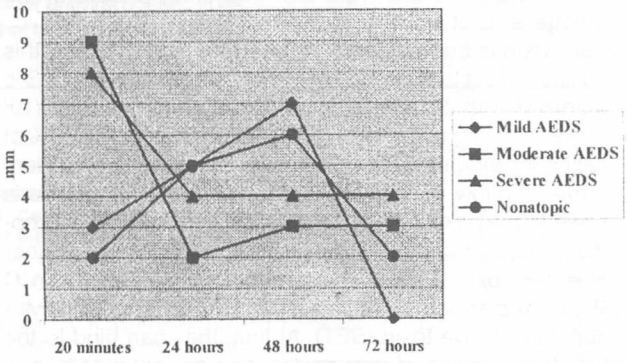

Figure 4.
Dinamics of medians' sizes of wheal/papula at intradermal testing with allergen *C. albicans* in patients with various severity of AD

actions to *S. aureus* was regarded as moderate and severe in 8 of 28 (28,6 %) patients.

The comparative analysis of papula's sizes detecting delayed type reactivity after testing *S. aureus* has not revealed significant differences between these parameters in both groups (0,8 mm and 1,0 mm, accordingly).

The results reflecting immediate reactions to allergen *C. albicans* are shown on pic.2. There was absence of any reactions in 7 patients with AEDS against of 15 nonatopic persons after intradermal introduction of allergen *C. albicans*. Frequency of positive immediate reactions to allergen *C. albicans* in group with AEDS has made 75 % against 46,4 % in a control group. The average size of wheal in patients with AEDS has made 6,9 mm and 3,0 mm in nonatopic people. These data differed statistically significant according to *chi* - test, but P=0,5.

The results demonstrating delayed reactions to yeasts essentially differed from those in testing with allergen *S. aureus*. Obvious oppression of cellular infiltration in place of intradermal injections of allergen *C. albicans* in patients with AEDS is observed in fig.3. The skin reaction to *C. albicans* in 22 (78,6 %) patients with AEDS either was absent or did not exceed 5 mm against 10 (35,7 %) in nonatopic persons. Accordingly the mean size of papula also significantly differed in 2 times in both groups (in patients with AD - 5,8 mm, in patients from control group - 2,4 mm, P=0,01). Absence of delayed type reactions to *C. albicans* was observed in the same 8 patients with moderate and severe AEDS and positive immediate reactions to *S. aureus* that were revealed earlier.

The intradermal testing by allergen *C. albicans* was provided for investigation of the correlation between wheal/papula size and severity of the disease in 28 patients with various severity of AEDS. The estimation of skin reactivity was carried out after with 20 minutes, 24, 48 and 72 hours.

Dynamics of medians' sizes of wheal/papula at intradermal testing with allergen *C. albicans* in patients with mild, moderate, severe AEDS and in nonatopic persons is shown on fig. 4. The lines of changes of skin reactions' sizes practically coincide in patients with mild AEDS and nonatopic people. The threefold increase of the wheal's sizes reflecting immediate skin reactivity on the background of decrease of delayed skin reactivity is marked in patients with moderate and severe AEDS.

DISCUSSION

The AEDS is multifactorial disease, in pathogenesis of which both infectious and noninfectious agents are participating. It is necessary to establish individual spectrum of sensitized factors for adequate therapy and elimination measures.

The publications of Leung and coauthors proved that the staphylococcal superantigens influence on the severity of the course of AEDS [16,17,18]. But using of such expensive methods of diagnostics in clinical practice is unacceptable.

In contrast the results of the present and published before papers [21] in studying of immediate and delayed reactions to infectious agents *S. aureus* and C. *albicans* allow using intradermal testing to verifying a sensitization of pa-

tients to infectious allergens and an objective estimation of severity of AEDS. It is obvious that the deepest infringements of immune answer are characteristic in patients with positive immediate reactions to *S. aureus* and *C. albicans*. Positive immediate reactions and the absence of delayed reactions to allergens *S. aureus* and *C. albicans* in a control group testify, most likely, the presence of infection and decrease of cellular immunity [5,8].

The data to be obtained suggest that the changes of skin reactivity to *S. aureus* and *C. albicans*, the increase of the frequency and the sizes of immediate reactions and the decrease of the sizes of delayed reactions are typical features in patients with AEDS. Besides these changes are especially characteristic for severe AEDS.

The use of intradermal test with allergens *S. aureus* and *C. albicans* can serve as the objective marker of the severity of AEDS and prognostic factor of chronization of AEDS.

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SEASONAL DYNAMIC OF MITE ALLERGENS EXPOSURE (DER 1, DER 2) AND HOUSE DUST MITES IN MOSCOW'S APARTMENTS

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SUMMARY

It is well known that the number of pyroglyphid mites and, therefore, exposure to mite allergens varies during the year. Seasonal dynamic of mites has its specific geographical features, which plays a causal role in the development of bronchial hyperreactivity in patients with asthma. In this connection the monitoring of house dust mites and mite allergen levels in patients' homes should be considered as the strategy for the treatment of bronchial asthma. Our aim was the study of the seasonal dynamic house dust mites and mite allergens exposure (Der 1, Der 2) in some asthma patients' apartments in Moscow. In 1994-1996 the number of pyroglyphid mites and exposition to mite allergens of groups 1 and 2 were monitored in the apartments of asthma patients sensitized to house dust mites. The study was carried out in 5 apartments, random chosen as models, by means of the acarological analysis and ELISA with the use of monoclonal antibodies to mite allergens. The data obtained in this study made it possible to determine the periods of rises and falls in the concentration of mite allergens in the dust of apartments in Moscow. The seasonal dynamics of exposition to mite allergens of groups 1 and 2 had a regional character. One period of a rise in the concentration of mite allergens (>2 μg/g of dust), common to all model apartments under study in Moscow, was established: the end of August -the beginning of October. Ail other periods of rises in the concentration of allergens were specific for each individual apartment. Thus, the prognostication of the periods of rises in the concentration of allergens in a patient's environment proved to be rather difficult. Therefore, prior to the prescription of specific immunotherapy to an individual patient exposition to mite allergens should be established and, with such concentration exceeding 2 μg/g of dust (100 mites/g of dust), the intensive sanitary and hygienic treatment of the apartment should be carried out.

Key words: *mite allergens exposure, house dust mites, seasonal dynamic*

Epidemiological studies, carried out all over the world, indicate the presence of dose-dependent relationship between sensitization and exposition to house-dust mite allergens (Der 1, Der 2) (1-3). It is well known that the number of house dust mites and, therefore, exposure to mite allergens varies during the year. Seasonal dynamic of mites has its specific geographical features, which plays a causal role in the development of bronchial hyperreactivity in patients with asthma. In this connection the monitoring of house dust mites and mite allergen levels in patients' homes should be considered as the strategy for the treatment of bronchial asthma (8). Our aim was the study of the seasonal dynamic house dust mites and exposure to mite allergens (Der 1, Der 2) in some asthma patients' apartments in Moscow.

MATERIAL AND METHODS

The study was carried out in 5 Moscow apartments, inhabited by asthma patients with pronounced sensitization to house dust mites and random chosen as models, during the period of 1994-1996. The apartments were located on the lower stories (1-3) of multistory houses with central heating. The houses were built of bricks or concrete blocks. The area of the apartments varied from 30 to 65 sq. m, the number of inhabitants was 3-4 persons. The apartments were furnished with standard modern furniture.

The dust was collected from beds and bedding, by means of a household vacuum cleaner with the use of textile filters. The mites were detected by means of a binocular microscope (model МБС-3) in the water suspension of dust. The mite preparations were obtained with the use of 40% lactic acid. Sifted house dust samples were extracted in ammonium bicarbonate buffer solution (pH 8.2) (w/v 1:40) for 24 hours. After filtration the supernatant was stored at a temperature of - 20°C.

The detection of Der p 1, Der p 2, Der f 1, Der f 2 was carried out by biotin-avidin ELISA method of Chapman M.D., described earlier (9).

RESULTS

In apartments Nos. 1-3 the presence of D. farinae population was established.

Apartment No 1. The number of mites varied from 20 to 1267 mites/g of dust. The concentration of allergens in the dust was 1,0-21.0 μg/gof dust and 0.1-20.0 μg/g of dust for Der f 1 and Der f 2 respectively. The number of mites below 100 mites/g of dust was registered from February to the end of April and in November 1995. A considerable rise in the number of mites (exceeding 500 mites/g of dust) was observed from the end of November to December 1994 and in September 1995. The mite allergens level exceeding 10 u.g/g of dust was registered at the end of December 1994, the end of February and in September 1995 for Der f 1, at the end of November 1994 and the end of September 1995 for Der f 2 (Fig. 1A).

Apartment No 2. The number of D. farinae varied from 1 to 319 mites/g of dust. The concentration of Der f 1 and Der f 2 was 0.02-17.0 μg/g of dust and 0.02-11.0 μg/g of dust respectively. Out of 15 months of observation, during 11 months the number of mites was below 100 mites/g of dust. Four peaks in the number of D. farinae were noted: the beginning of May, the end of June, the beginning of October 1995 and the beginning of January 1996. Still the number of mites never reached 500 mites/g of dust during the whole term of observation. The level of Der f 1 exceeded 2 μg/g of dust at the end of June and the beginning of January 1996. During the remaining two periods of rises in the number of mites the concentration of allergens exceeded 10 μg/g of dust (at the beginning of May and October). At the same time the concentration of Der f 2 was equal and exceeded 2 μg/g of dust during 3 periods (the end of June, the beginning of October 1995 and the beginning of January 1996), and only at the beginning of May 1995 it exceeded 10 μg/g of dust (Fig. 1B).

Apartment No 3. The number of D.farinae varied from 20 to 1320 mites/g of dust. The mite allergens concentration varied from 0.5 to 84.0 μg/g of dust for Der f 1 and from 0.5 to 30.0 μg/g of dust for Der f 2. The periods when the number of mites was below 100 mite/g of dust were registered in April and December 1995, as well as in February-March 1996. At all other periods the number of mites never fell below 600 mites/g of dust. Changes in mite allergens exposure, both Der f 1 and Der f 2, showed practically complete correlation with the number of mites (Fig. 1C).

In apartments Nos. 4 and 5 the presence of coexisting D. pteronyssinus and D. farinae populations was established.

Apartment No 4. The number of D. pteronyssinus and D. farinae varied from 10 to 690 mites/g of dust and from 15 to 380 mites/g of dust respectively. At the same time the maximum number of D pteronyssinus was almost twice greater

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than those of D. farinae. Still the average number of D. farinae exceeded that of D. pteronyssinus and reached 225 mites/g of dust in comparison with 159 mites/g of dust. The concentration of allergens varied, respectively, from 0.1 to 9.0 μg/g of dust and from 0.1 to 10.0 μg/g of dust for Der p 1 and Der p 2, from 1.7 to 10.3 μg/g of dust and from 0.3 to 8.0 μg/g of dust for Der f 1 and Der f2. For D. pteronyssinus population only one peak of rise in its number was noted in June 1995 when the number of mites exceeded 500 mites/g of dust and exposure to Der p 1 and Der p 2 was 9.0 and 10.O μg/g of dust respectively. This peak was preceded by the period of rise, April-May 1995, when the number of mites exceeded 100 mites/g of dust and the concentration of allergens exceeded 2 μg/g of dust. Such period of rise took place also in September 1995. At all other periods of observation the number of mites was below 100 mites/g of dust, and the concentration of allergens was below 2 μg/g of dust (Fig. 2A).

The peak of density of D.farinae occurred at the end of May and September when the levels allergens Der f 1 exceeded 10 μg/g of dust, though the density of these mites did not reach 500 mites/g of dust. Only in March 1995 the number of mites was below 100 mites/g of dust with exposure to Der p 2 exceeding 2 μg/g of dust (Fig. 2B).

Apartment No 5. The number of D. pteronyssinus and D. farinae varied from 5 to 250 mites/g of dust and from 5 to 90 mites/g of dust respectively. At the same time the maximum number of D. pteronyssinus was almost 3 times and their average number was 2 times greater than those of D. farinae. The number of mites in this apartment did not reach 500 mites/g of dust during the whole period of observation. For D. pteronyssinus, three periods of rises were noted (the end of May, July-August and November 1995), when the number of mites was higher than or approximated to 100 mites/g of dust and the concentration of allergens exceeded 2 μg/g of dust. The number of D. farinae did not reach 100 mites/g of dust for the whole period of observation (Figs. 2C, 2D).

The positive correlation between the number of mites (D. pteronyssinus, D.farinae) and the mite allergens exposure (Der p 1, Der p 2, Der f 1, Der f 2) was established: the coefficient of correlation (r) varied from 0.6647 to 0.9712 ($P << 0.01$).

DISCUSSION

Our data indicate the specific regional of the mite seasonal dynamics (D.farinae and D. pteronyssinus) and mite allergens exposure (Der 1 and Der 2) in Moscow' apartments, linked with the climate and mainly with the specific social and economic conditions of country life: the typical structure of dwellings (houses built of bricks and prefabricated sections) with central heating and hot water supply, standard arrangement of apartments furnished with standard furniture, bedding, toys, etc. We observed one period of the increasing of the mites populations and mite allergens (>2 μg/g of dust)- the end of August - the beginning of October - common for the all Moscow's model apartments. The analysis of literature confirms the presence of specific regional features of house dust acarofauna and mite allergens exposure in different geographical regions (10-18).

The monitored of the seasonal dynamics of pyroglyphid mites and exposure to mite allergens of groups 1 and 2 in Moscow apartments, random chosen as models, showed that each individual apartment had a number of characteristic specific features, reflected in the average number of mite, the amplitude of density and at the periods of rises and falls in the mite populations. The microclimate of the apartment was found to play an even more important role in the seasonal dynamics of house dust mites than the geographical climate of the region (17, 19, 20). Some au-

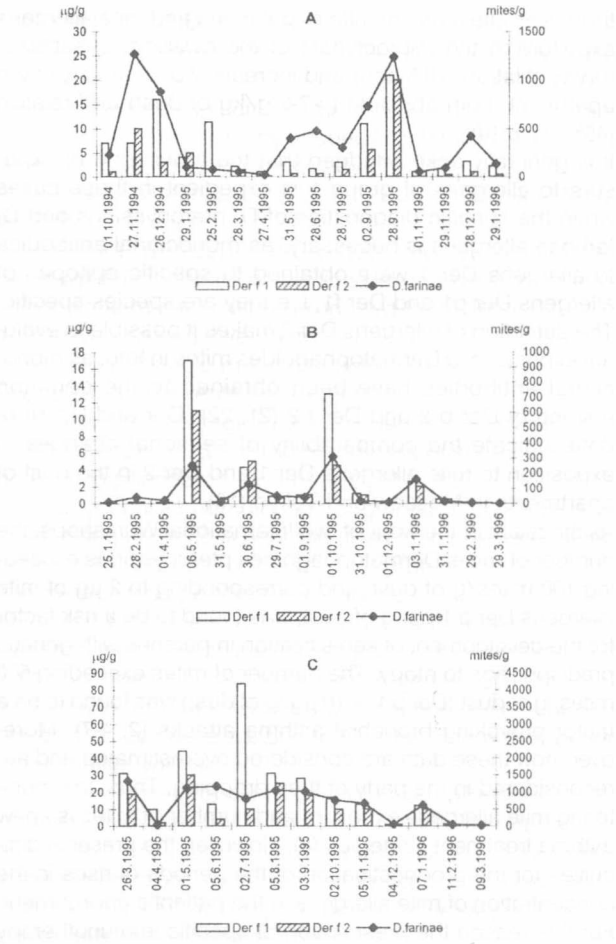

Figure 1.
Seasonal dynamic of exposure to mite allergens (Der f 1, Der f 2) and number of D.farinae in dust of: A - apt. No 1; B - apt. No2; C - No3.

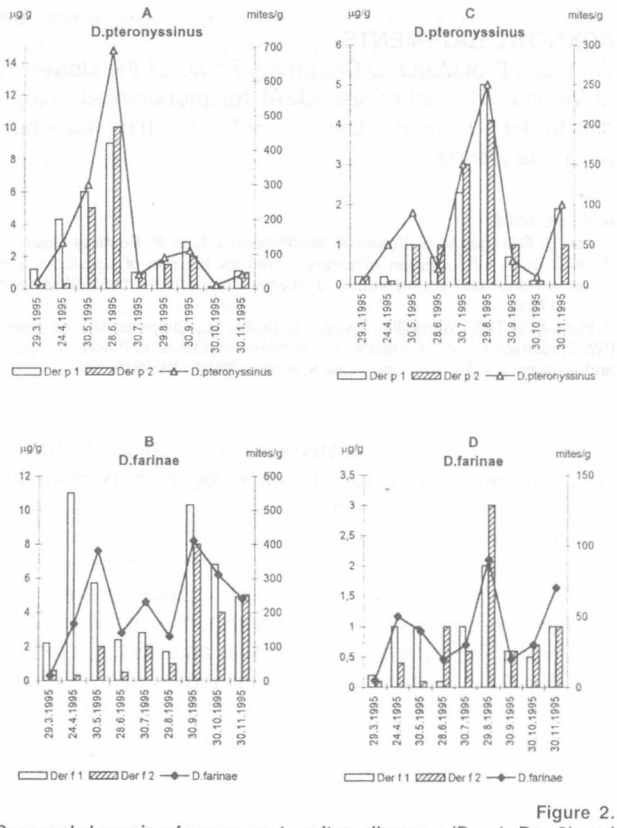

Figure 2.
Seasonal dynamic of exposure to mites allergens (Der 1, Der 2) and number of D. pteronyssinus and D.farinae: A, B - apt. No 4; C, D - apt. No 5.

thors attribute a rise of mite populations and mite allergens exposure to the microclimate of the dwelling: unsatisfactory ventilation <0.5 ach) and increased air humidity in the apartment, both absolute (>7-9 g/kg of dust) and relative (45%) (4, 6,10).

It is generally acknowledged that the monitoring of exposure to allergens of group 1 is expedient in those cases when the specific diagnostics of D. pteronyssinus and D. fari-nae allergens is necessary, as monoclonal antibodies to allergens Der 1 were obtained to specific epitopes of allergens Der p1 and Der f1, i. e they are species-specific. The detection of allergens Der 2 makes it possible to evaluate exposition to Dermatophagoides mites in toto, as monoclonal antibodies have been obtained to the common epitope of Der p 2 and Der f 2 (21, 22). Our and literature data indicate the comparability of seasonal changes in exposition to mite allergens Der 1 and Der 2 in the dust of apartments in Moscow and Tokyo (18).

As the result of the work of two International Workshops, the number of mites Dermatophagoides pteronyssinus exceeding 100 mites/g of dust, and corresponding to 2 μg of mite allergens Der p 1 per g of dust, was found to be a risk factor for the development of sen-sitization in persons with genetic predisposition to atopy. The number of mites exceeding 500 mites/g of dust (Der p 1 = 10 μg/g of dust) was found to be a factor provoking bronchial asthma attacks (2, 4-7). Moreover, now these data are considered overestimated and are reconsidered in the party of their dropping. Thus, the monitoring mite allergens exposure and number of mites is anew asthma treatment strategies (8). However, the presents difficulties for the prognostication of the periods of rises in the concentration of mite allergens in the patient's environment. For this reason the prescription of specific immunotherapy to an individual patient must be preceded by the determination of exposition to mite allergens. All these facts indicate that it is necessary to monitor the number of mites and exposition to mite allergens in different regions of the world.

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STUDY OF THE FC FUNCTION OF THE IMMUNOGLOBULIN IN IMMUNOVENIN-INTACT

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SUMMARY

Immunovenin-intact (immunoglobulin for intravenous use) contains immunoglobulin in its native form and is presented as ready to use liquid. The recommendation of the European Pharmacopoeia to prove the nativity of the IgG molecules (their Fc function) by the assessment of the level of rubella antibodies was followed. The measurements of these antibodies during the shelf life of the preparation showed that their levels were preserved and this gave assurance of the efficacy and stability of Immunovenin-intact. The method of rubella radial haemolysis was applied to prove that the Fc function of the immunoglobulin in this preparation was not impaired.

Key words: *Fc function, immunoglobulin, Immunovenin-intact, rubella antibodies, radial haemolysis*

Immunovenin-intact is a plasma derivative that belongs to the group of life-saving preparations. The product meets the requirements of the European Pharmacopoeia, 2002. The preparation is well characterized for protein concentration, pH, purity, sterility, pyrogenicity, viral safety, etc. The following key markers on the nativity of the immunoglobulin are regularly recorded: 1) anticomplementary activity; 2) molecular size distribution; 3) prekallikrein activator activity. The fourth key marker is the Fc function of the immunoglobulin in the product. The regular assessment of the Fc function of the immunoglobulin in Immunovenin-intact would give better assurance of its clinical efficacy. This is important because the indications for immunoglobulin therapy are numerous and their scope is increasing. Now the indications may be grouped into three main categories: adjuvant therapy in severe life-threatening infections; replacement therapy in primary and secondary antibody deficiency states; immunomodulation in immunoinflamatory conditions and autoimmune diseases.

The European Pharmacopoeia describes a method for the testing of Fc function, which requires a rubella antigen of very high activity (above 256 haemaglutination units). That reagent was not available at the time of the study.

The study was directed to the application of the method of single radial haemolysis (as approved by the British Pharmacopoeia) for the testing of the Fc function of immunoglobulin in Immunovenin-intact and to the comparison of the results with these obtained by two other methods for determination of rubella antibodies (haemagglutination inhibition test and indirect ELISA-IgG antibodies).

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ABBREVIATIONS USED IN THIS PAPER:

SRH - single radial haemolysis
HIT - haemagglutination inhibition test

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MATERIALS AND METHODS

1. 29 lots of Immunovenin-intact (5% IgG) with expiry dates from November 2001 to March 2004, produced by BB-NCIPD Ltd were tested. The samples were heat treated for 20 minutes at 56 °C to remove any unspecific inhibitors. They were diluted 1:2 with phenobarbitone buffer.
2. International standard of EDQM - Human Immunoglobulin BRP
3. Rubella antigen with biological activity 1:128 haemaglutination units determined by the reaction of inhibition of haemaglutination (produced by NCIPD)
4. Positive and negative contol sera
5. Reaction of single radial haemolysis (1, 2, 3, 4).

The following reagents were used:

- red blood cells of pigeon (8% suspension in phenobarbitone buffer, pH = 7,2) or red blood cells of sheep (5% suspension in phenobarbitone buffer, pH = 7,2)
- freshly prepared complement of guinea pig (1:73 titre)
- petri dishes with 1,5% agarose (Merck) in phenobarbitone buffer, pH = 7,2 with 0,05% sodium azide.

The method was performed as follows:

a) the red blood cells were sensitized with rubella antigen by mixing them 1:1 with the antigen and leaving them at +4 °C for 1 hour; then the cells were centrifuged at 1500 rpm/min for 5-10 min and washed twice with phenobarbitone buffer; the washed cell were suspended in phenobarbitone buffer to their initial volume

b) the agar plates (test and control) were prepared:

- 0,6 ml sensitized red blood cell suspension was mixed with 3,5 ml 1,5% agarose (warmed to 43 °C), and poured into a sterile petri dish; it was allowed to set and labeled "test"
- the control plate was prepared in the same way but the red blood cells were not sensitized
- wells were punched out; 10 µl samples of the studied Immunovenin-intact and the controls were filled in them; the samples were doubled
- the plates were left in a moist chamber till the next day; then the agarose was covered with the fresh complement (diluted 1:2 with phenobarbitone buffer) and the plates were incubated at 37 °C for 4-5 hours. The haemolysis was visualized and the diameters of the zones of red cell lysis were measured in mm
- zones with diameter less or equal to 5 mm were read negative.

6. Haemagglutination inhibition test (HIT)- the generally accepted method for the detection of rubella antibodies was applied
7. Indirect ELISA test kits for rubella IgG antibodies of ETI-Rubek plus (dua Sorin, Italy), ImmunoComb II Rubella IgG (Orgenics, Israel) or Platelia Rubella IgG (Bio Rad) were used

RESULTS AND DISCUSSION

The method of single radial haemolysis was performed as described above. It was based on the lysis of sensitized red blood cells with rubella antigen with the complex antigen-antibody formed in the presence of the studied product and with the addition of complement. The sensitized red blood cells were incorporated in the agarose gel and the area of the lysis was related to the amount of rubella antibodies (immunoglobulin with preserved Fc function). The method was applied to demonstrate that it could be used for the regular evaluation of the Fc function of immunoglobulin in Immunovenin-intact instead of the method published in the European Pharmacopoeia, 2002 (the method in the Pharmacopoeia required an antigen of very high activity that was not available at the time of the study). The use of pigeon or sheep red blood cells did not have signifi-

cant impact on the results. It seemed important to use fresh complement.

The expiry term of Immunovenin-intact is two years. The samples of the lots were divided in groups according to their state of expiry. The results of their testing by radial haemolysis (RH) were compared to the results by reaction of inhibition of haemaglutination (RIH) and presented in the following Table:

No of lots	Expiry term	SRH - diameter of haemolysed zone	HIT - titre
2	expired	8-10 mm	1:256/512
7	end of expiry term	10-14 mm	1:512/1024
4	1 1/2 year after release	7-10 mm	1:16/1024
6	1 year after release	10-14 mm	1:256/1024
6	1/2 year after release	10-13 mm	1:256/1024
4	at release	10-13 mm	1:512/1024
Immunoglobulin standard BRP		10-12 mm	1:128
(+) control serum	7-8 mm	1:16/32	
(-) control serum		-	

In all samples of Immunovenin-intact, tested by ELISA, the antirubella IgG content was above the highest standard of each kit.

The diameters of the haemolysed zones of 26 samples were equal or above to the results obtained with the immunoglobulin standard. The diameters were 10-14mm. Three samples (one - expired lot, two - 6 months before expiry) showed haemolysis at 7-8 mm. The agglutination inhibition titre of the one expired lot was 1:256 that was comparable to the results with the lots during their term of use. The smaller haemolysis diameters of the other two lots could be related to the lower HIT titres (1:16 and 1:128).

On the basis of these lower results, it may be concluded in this case that the Fc function in the product was impaired. All other samples showed HIT titre above the titre of the immunoglobulin standard and their results with the single radial haemolysis test were in concurrence. Beside the practically equal results in radial haemolysis for 26 of the 29 samples, the titres in RIH for 27 samples were 1:256 and above (the immunoglobulin standard gave 1:128 titre) that reflect the high level of rubella antibodies in the studied product and give another proof of its stability in the liquid form. The high rubella antibody content was confirmed by the ELISA test also.

The method of single radial haemolysis could be applied in the screening of Immunovenin-intact lots to evaluate the Fc function of the immunoglobulin in the product and this was an additional assurance of its efficacy and stability in the liquid form.

CONCLUSION

The state of intactness of the immunoglobulin in Immunovenin-intact was studied by the evaluation of its Fc function with the method of single radial haemolysis for the detection of rubella antibodies. The results obtained by this method were compared with these obtained by the haemaglutination inhibition test and ELISA. They were in good correlation.

The measurements of these antibodies during the shelf life of the preparation showed that their levels were preserved and this gave assurance of the efficacy and stability of Immunovenin-intact. The method of rubella radial haemolysis gave proof that the Fc function of the immunoglobulin in this product was not impaired.

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COMPARATIVE CHARACTERIZATION OF ANTIGENS ISOLATED FROM *TOXOCARA CANIS*

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SUMMARY

Toxocarosis is parasitic disease in humans, caused by the larval form of dog nematode Toxocara canis. For diagnosis of the disease different serological tests are used which demonstrate the presence of specific antibodies in serum specimens of suspected patients. In the development of the immunological methods two kinds of antigens are used mainly - somatic from adult forms and excretory-secretory (metabolic) from parasite larvae cultivated in vitro. The aim of the presence work is to make comparative characterization of these two antigens using SDS-electrophoresis and to study their specificity in enzyme -linked immunosorbent assay (ELISA). The results showed that by electrophoretic separation in SDS-PAGE the two kinds of antigens differ in number of isolated fractions and their molecule mass in kDa. The testing in ELISA with sera from patients, reacted negative for toxocarosis in another tests and from patients with other parasitic diseases, showed that E/S antigen isolated from T.canis larvae is more useful and possess enough specificity as a diagnostic reagent.

Key words: *Toxocara canis, somatic antigen, E/S antigen, SDS-PAGE, ELISA*

Toxocarosis is a parasitic disease in humans, caused by the larval form of dog nematode Toxocara canis. The clinical signs of the disease induced by larval migration in organs and tissues are united in complex larva migrans. The specific laboratory diagnosis of this parasitosis is difficult, because T.canis larvae don't complete their life cycle in humans. For the practice the accurately diagnosis is connected with Toxocara IgG antibodies detection in sera samples of suspective for toxocarosis patients. The search of species antigens and their applying in contemporary immunological tests as ELISA and Western blotting is an object of many investigations. Up to now the serological diagnosis of toxocarosis is connected with the application of two antigens mainly - total extract from adult worms and excretory-secretory (E/S) products obtained from *in vitro* cultivated larvae. The aim of this study is to compare the both mentioned kinds of antigens isolated from Toxocara canis by SDS-PAGE and determine their specificity in ELISA.

MATERIALS AND METHODS

I. ANTIGENS

1. Total extract from adult T.canis:

Mature T.canis were harvested from naturally infected puppies (till 6 months) after anthelmintic treatment. Parasites were killed by freezing and then partially grinded in a Poter tissue grinder in saline. This crude material was centrifuged for 30 min. at 12000 rpm. The supernate was collected and kept at 4°C. The pellet was homogenized again by sonication for 10 min. and centrifuged under the previous conditions. The second supernate was collected, mixed with the supernate obtained after the first centrifugation and used as a total antigen from adult T.canis.

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2. Excretory/secretory (E/S) antigen from T.canis larvae:

Excretory/Secretory (E/S) products were obtained from T.canis larvae according to the method described by Fairbairn (2). In vitro hatched live larvae were cultured in Eagle·s minimal essential medium (MEM) as described by Savigny (6). The culture was carried out at 37° C and the medium was changed weekly. Collected fluid containing E/S products was centrifuged, dialysed against water and concentrated by ultrafiltration (Amicon PM10). This concentrated fluid was used as E/S antigen for SDS-PAGE and ELISA.

Protein content was determined by absorbance at 280 nm.

3. Sera samples. Followed groups of sera were used in the study:

A. From patients, negative for toxocarosis in another tests (Ridascreen Toxocara IgG) - 6 sera samples

B. From patients with other parasitic diseases - 4 sera samples

II. METHODS

1. Polyacrylamide gel electrophoresis (SDS-PAGE):

SDS - electrophoresis was carried out on MiniProtean II (BioRad) apparatus according to the manufacturer·s instructions under reduced conditions. Apparent molecular weights of tested Toxocara antigens were estimated by mobility relative to standard marker proteins (Sigma) between 14 000 –70 000 Da.

2. Enzyme linked immunosorbent assay (ELISA):

Indirect ELISA was performed according to van Knapen (7). Polystyrene strips were coated with solutions prepared from both kinds of antigens and contained 2 мg protein per ml carbonate buffer (pH 9.6). The initial sera dilution was 1:200 and the dilution of the antihuman goat IgG conjugated with horse radish peroxidate (HRP) was 1:4000 in PBS BSA Tween 20. The optical density was measured by ELISA Reader Uniscan (Labsystems) at 492 nm. Extinction value greater with 0.150 then extinction value of negative control serum was accepted as a cut off.

RESULTS AND DISCUSSION

In this study a comparative characterization by SDS-PAGE and ELISA of two kinds of antigens from parasite nematode Toxocara canis was accomplished.

SDS-PAGE analysis revealed the composition of tested antigens. Figure 1 and Table 1 show that the total antigen from adult worms is made up of more then 10 major components ranging in molecular weight from 108 000 to 15 000 kDa. Bands between 37 000 and 20 000 kDa are the brightest and cut clearly. Our results support the observations of other authors (1,3) detected in total toxocara extract using the same method till 30 fractions ranging from 330 000 to 18 000 Da. The differences between number of isolated fraction in the antigen, haracterized by us and reported by other authors probably due to a some variety of the application of polyacrylamide electrophoresis as well as to standard marker proteins have been used.

When E/S antigen by SDS-PAGE was separated a considerably reduction of the number of isolated fractions was observed. Figure 1 and Table 1 show 6 components in this kind of antigen with molecular weight 63 000, 52 000, 40 000, 35 000, 29 000, 20 000 kDa. According to the previous studies (4) E/S antigen contains fractions with molecular weight 26 000-32 000, 55 000-70 000, 120 000 and 400 000 kDa. Antigens with molecular weight 32 000 kDa and 70 000 kDa are secreted in the most quantity and that with molecular weight 400 000 kDa can be detected with Shiff reagent for polysaccharides. Unfortunately till now we could not isolate in our

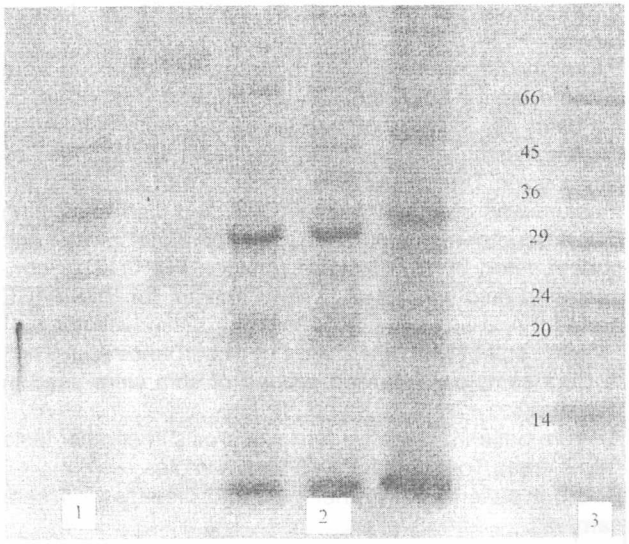

Figure 1.
SDS-PAGE of *Toxocara canis* antigens

L E G E N D : **1.** E/s antigen from in vitro cultivated larvae; **2.** Somatic antigen from adult forms; **3.** Molecular weight standards (sigma)

antigen high molecular weight fractions, but as for some new investigations (5) specific for toxocarosis are fractions with lower molecular weight ranging between 24 000 and 35 000 kDa.

The second aim of the study is to test in ELISA the specificity of the two isolated *toxocara* antigens. The results obtained are performed on Table 2. There were tested ten sera samples divided into three groups:

- 6 sera samples from patients serologically negative for toxocarosis
- 1 serum sample from patient with proved ascaridosis
- 3 sera samples from patients with trichinellosis (clinical and serological proved).

Sera samples negative for toxocarosis tested with total antigen showed extinction values equal or higher than accepted cut off value. Sera samples from patients with ascaridosis and trichinellosis showed positive reaction.

When sera samples were tested in ELISA with E/S antigen the extinction values showed negative results for more of them. False-positive reaction was observed only in one patient with trichinellosis. Our observations confirm those from other authors, who have also founded cross - reactions between E/S antigen from T.canis larvae and sera from patients with trichinellosis (8). Perhaps these cross-reactions occurred because of some common epitopes between E/S antigens from Trichinella spiralis ant Toxocara canis larvae. Further investigations by ELISA in large number of patients will give more reliable results about the cross-reactivity observed.

The results obtained in ELISA reveal that total antigen from adult T.canis is not useful as a diagnostic reagent for toxocarosis because the cross - reactions with sera samples from patients with another nematodoses. Our observations indicate that E/S antigens possess enough specificity for application in serological tests for diagnosis of human toxocarosis.

Table 1. SDS-PAGE analysis of toxocara canis antigens

№ Antigen of fractions	Number in kDa	Molecular weight
1. Somatic from adult	10	108, 100, 94, 88, 62, 37, 25, 23, 20,15
2. E/S from in vitro cultivated larvae	6	63, 52, 40, 35, 29, 20

Table 2. Results in ELISA testing of the two toxocara antigens

Antigen	Patients sera samples	Number of sera samples	ELISA optical density at 492 нм	Result
1. Somatic from adult forms	negative for toxocarosis	6	1.253	+
			0.512	-
			0.604	±
			1.179	+
			1.314	+
			1.513	+
	ascaridosis	1	1.369	+
	trichinellosis	3	1.121	+
			0.550	±
			1.097	+
(+) c. - 1.91; (-) c. - 0.39				
2. E/S from in vitro cultivated larvae	negative for toxocarosis	6	0.333	-
			0.313	-
			0.219	-
			0.244	-
			0.204	-
			0.187	-
	ascaridosis	1	0.305	-
	trichinellosis	3	0.534	+
			0.270	-
			0.315	-
(+) к. - 1.65; (-) к. - 0.215				

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EXSTRADIGESTIVE DISEASES ASSOCIATED WITH *H. PYLORI* INFECTION

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SUMMARY

***H. pylori* gastric infection is a chronic process lasting for decades. The persistent infection induces a chronic inflammatory and immune response able to induce lesions both locally and remote to the primary site of infection. *H. pylori* infection may be a risk factor for the development of coronary heart disease. In a recent cross-sectional study Mendall et al, confirmed that CHD is associated with C reactive protein and with high serum levels of IL-6 and TNF-a demonstrated predictors of myocardial infarction and stroke. These cytokines are associated with high levels of fibrinogen, sialic acid, triglycerides and reduced HDL-cholesterol. Following *H. pylori* infection, a link, between increased levels of cytokines and an increased level of C reactive protein, leading to CHD, can be hypothesized. A significant amelioration of both - Raynaud phenomenon and migraine symptoms has been described after *H. pylori* eradication. The prevalence of CagA(+)*H. pylori* strain was higher in infected subjects with autoimmune thyreoiditis than in infected controls. An association with chronic idiopathic urticaria has been proposed with the pathogenetic mechanism related to an increase in gastric vascular permeability during *H. pylori* infection, resulting in a greater exposure of the host to alimentary allergens.**

Key words: *H. pylori infection, extradigestive diseases, coronary heart diseases*

H. pylori is the main aetiological factor for gastritis and peptic ulcer disease. Its eradication is associated to healing of these diseases and significant reduction of ulcer recurrence and rebleeding. The characteristics of the infecting strain may influence the clinical outcome of the infection. The postulated role of *H. pylori* in the pathogenesis of extradigestive manifestations is based on the facts that : 1) local inflammation has systemic effects; 2) *H. pylori* gastric infection is a chronic process lasting for decades and 3) persistent infection induces a chronic inflammatory and immune response able to induce lesions both locally and remote to the primary site of infection.

Coronary heart disease

A number of studies have been published recently on the association between *H. pylori* infection and CHD. *H. pylori* infection, which is accompanied by a persistent inflammatory response, may be a risk factor for the development of coronary heart disease. If such is the case, it could at least partially explain the link observed between childhood poverty and adult CHD(2); on the other hand, older age, low socioeconomic status and being part of specific ethnic groups are all associated with both *H. pylori* infection and CHD and thus would be expected to confound the association. Furthermore, some studies have not supported this hypothesis(3, 4). However, if *H. pylori* itself is responsible for the association, then this would be of great potential importance as the infection is treatable with a single course of antibiotics, and reinfection rarely occurs. In a recent literature review on chronic infections and CHD, Danesh et al.(5) pointed out the lack of homogeneity in *H. pylori* studies: few studies with a powerful design, heterogeneity in the definition of CHD, biased control groups, large variation in the adjustment of potential confounding factors, different sampled population.

An interesting aspect of this work was the method used to score the way counfounders were controlled in these studies. Using Danesh et al's method and updating with recent publications, the aim of our (18) meta-analysis on the association between *H. pylori* infection and CHD was to evaluate the degree of heterogeneity between studies and how the adjustment for confounding factors influenced the pooled odds ratio. The pooled odds ratio for the studies adjusted for control population score was 1.99 (95% CI [1.67-2.36]) when the control population was not appropriate and 1.25 (95% CI [1.06-1.47]) when the control population was randomly chosen in the general population. The studies with no appropriate control population were very heterogeneous ($p<0.001$) while studies with an appropriate population control were less heterogenous with a p value borderline for significance (0.053).

The stomach is a large organ and billions of *H. pylori* are present at any moment. The inflammation induced is intense as is the humoral response. It is, therefore, plausible that this host response has consequences outside of the target organ. Different but not necessarily alternative mechanisms are hypothized to be involved. Tumor Necrosis Factor alfa (TNFa) which is elevated in blood following *H. pylori* infection(12) is a good candidate. Neri et al. also found an increased level of soluble TNFa receptor I in sera of *H. pylori* patients suffering from CHD(11). Patel et al. demonstrated that *H. pylori* infection was accompanied by acute phase reactants such as fibrinogen, white cell count and sialic acid, which are predictors of CHD(13, 14). However, such findings were not confirmed by other works(8,15). In a recent cross-sectional study Mendall et al.(16), confirmed that CHD is associated with C reactive protein and with high serum levels of IL-6 and TNF-a demonstrated predictors of myocardial infarction and stroke(17). These cytokines are associated with high levels of fibrinogen, sialic acid, triglycerides and reduced HDL-cholesterol. Following *H. pylori* infection, a link, between increased levels of cytokines and an increased level of C reactive protein, leading to CHD, can be hypothesized.

Raynaud phenomenon and prymary headache

H. pylori infection has been linked with functional vascular disorders (19,20). A direct or indirect release of vasoactive substances has been proposed as the pathogenic mechanism. The persistent immunological and inflamatory response to *H. pylori* infection may influence peripheral vessels through an immune- mediated release of vasoactive substances such as cytokines (interleukin, interferon-r, TNF-6), prostaglandins, oxyradicals, C- reactive protein or fibrinogen. Most of these factors are persistently enhanced during *H. pylori* infection.

Prymary headache has recently been implicated as related to *H. pylori* infection. In some investigation clinical attacks complitely has been disapeared in the succefully treated patients or the intensity, duration and frequency of attachs has been reduced. The actuale question should be whether headache is relieved following cure of *H. pylori* infection and thus a low prevalence of *H. pylori* does not

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ABBREVIATIONS USED IN THIS PAPER:
CHD - Coronary Heart Diseases

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exclude a role for *H. pylori*. The treatment trials are incomplete in that there are other infections besides *H. pylori* that can cause headache (sinus infection).
A significant amelioration of both - Raynaud phenomenon and migraine symptoms has been described after *H. pylori* eradication (21).

Autoimmune diseases and *H. pylori*

Autoimmune thyreoiditis, Schonlein-Henoch purpura etc.
The prevalence of CagA(+)*H. pylori* strain was higher in infected subjects with autoimmune thyreoiditis than in infected controls (22). The pathogenic mechanisms relating *H. pylori* to any of these immunological diseases are unknown. An alternative hypothesis is the production of autoantibodies induced by the bacteria through cross-reactions mechanism. *H. pylori* have similar epitopes to those found on gastric epithelium, salivary gland ducts and endometrium.

Skin diseases

An association with chronic idiopathic urticaria has been proposed with the pathogenetic mechanism related to an increase in gastric vascular permeability during *H. pylori* infectionq resulting in a greater exposure of the host to alimentary allergens. The possibility that patients with urticaria develop specific IgE against *H. pylori* is an attractive explanation that requires confirmation. Although a few trials of chronic urticaria have reported improvements in several patients following *H. pylori* eradication (23, 24) these studies were small, non-randomized and lacked any comparison groups. *H. pylori* infection was reported in 84% of 31 patients with rosacea. Disappearance of the clinical manifestations after *H. pylori* eradication has also been described, but rosacea was already known to be an antibiotic- responsive skin condition and the link is therefore tenuous (25). A possible association between *H. pylori* infection and idiopathic sideropenic anemia has been sugested. The resolution of anemia has been observed in some cases after cure of the infection. Several case reports have suggested reversal of iron deficiency anaemia refractory to iron replacement after *H. pylori* eradication (26,27) as has one trial of antibiotics in 30 *H. pylori* positive patients with persistent iron deficiency refractory to iron replacement therapy (with significant improvements in heamatological parameters such as serum ferritin values). Another interesting clinical manifestation related to *H. pylori* infection is growth retardation. A possible pathogenic mechanism to explain the relationship relates to the increased level of circulated cytokines, such as TNFa. associated with *H. pylori* infection.
Future studies should follow three directions. First epidemiological studies should be large enough for moderate-sized effects to be assessed or registered reliably. The effect of residual confounders shoud be kept to a minimum. Second pathogenic mechanism shoud be investigated by comparison of plasma concentration of inflamatory markers in patients positive and negative in serum for microorganism. Third, because antibiotics are not specific for *H. pylori* infection appropriate controls include those with the same condition and without *H. pylori* infection placebo controls and longer follow-up after therapy.

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TRANSMISSION OF HELICOBACTER PYLORI: EVIDENCE FOR FECAL-ORAL ROUTE

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SUMMARY

The evidence for the fecal-oral route of transmission of *H. pylori* is still controversial. *H. pylori* DNA has been detected in feces of infected subjects by some researchers but not others. It has been demonstrated that *H. pylori* can live for several days in milk and tap water in its infectious bacillary form and in river water for several months in a coccoid form. Transmission has been linked to consumption of vegetables grown in regions where human feces is used as fertilizer. Although the principal reservoir for *H.pylori* infection appears to be people, *H.pylori* has been isolated from nonhumans primates and domestic cats. The most recent reservoir suggested for *H.pylori* transmission is the housefly. However, evidence is lacking that *H.pylori* can be isolated from flies that have been in contact with *H.pylori* infected feces and that *H.pylori* can be transmitted from contaminated flies to food in a quantity sufficient to infect humans. In fact, nosocomial transmission of *H.pylori* is the only proven mode of transmission. Understanding the route(s) by which *H.pylori* infection spreads is fundamental for development of strategies for the infection's control.

Key words: *H.pylori, transmission, fecal-oral route*

Although is now clear that the reservoir of *Helicobacter pylori* is limited to the stomach of humans, the routes of transmission still remain unproven. As for other pathogens of the digestive tract, it is logical to consider a fecal-oral transmission as a probable "path". However, the evidence is still controversial. Several studies performed DNA fingerprinting to determine the specific strains of *H. pylori* harbored by family members' (8). They found the same strain of *H. pylori* to be present in a small percentage of spouses and siblings. These studies, taken as a whole, lend support to the concept of **interfamilial clustering** of *H. pylori* infection. They suggest that person-to-person transmission occurred in these families possibly because of close interpersonal contact, that family members shared a genetic predisposition to *H. pylori* infection, that family members were exposed to a common source of infection, or that spouses' childhood socioeconomic class was similar.

H. pylori DNA has been **detected in feces** of infected subjects by some researchers (9,10) but not others (13). Recently Gramley et al found detectable *H. pylori* DNA in the feces of 73% of infected subjects. Isolation of *H. pylori* by fecal culture has been performed by a number of investigators from around the world (9,10,14). Recently, Parsonnet et al were able to culture *H. pylori* from catharic- induced diarrheal stools in 7 of 14 *H. pylori* infected subjects but not from normal stools (11). Studies by Hazell et al. in China (15) and Webb et al in United Kingdom noted serum antibodies to *H. pylori* and Hepatitis A, a sensitive marker of fecal- oral exposure, but did not find strong evidence supporting community- wide fecal- oral spread of *H. pylori* via food or water.

It has been demonstrated that *H. pylori* can live for several days in **milk and tap water** in its infectious bacillary form (16,17) and in river water for several months in a coccoid form. However, the idea of coccoid forms is very controversial. While experimental tests have shown that, under physical or chemical stress, *H. pylori* is able to convert to a viable but nonculturable coccoid form (18), researchers have failed to convert a coccoid to a bacillary form in culture (19). In addition, it has not yet been determined whether *H. pylori* can revert from its coccoid to its infectious form in humans. Support for waterborne transmission comes from epidemiological studies conducted in Colombia, rural China, and Lima, Peru, that found that water source may be related to risk of *H. pylori* infection (21). The finding of *H. pylori* positive drinking and sewage water samples by PCR assays in Peru and Japan (15,20) provides additional evidence that waterborne transmission may be important, especially in areas of the world that have high rates of *H. pylori* infection and less than adequate water quality.

Transmission has been linked to consumption of **vegetables** grown in regions where human feces are used as fertilizer.

It has been suggested that *H.pylori* infection may, in some instances, be a zoonosis. Although the principal reservoir for *H.pylori* infection appears to be people, *H.pylori* has been isolated from **non-human's primates and domestic cats** (23,24,26). Under controlled laboratory conditions, human *H.pylori* has been shown to infect monkeys. However, even if *H.pylori* occurs naturally in monkeys, they are unlikely to represent a major route of transmission to humans, since close contact between nonhuman primates and humans is limited in most of the world. Handt et al was the first to report isolation of *H.pylori* from domestic cats (24). Their laboratory was able to experimentally infect naive cats with *H.pylori*, to culture *H.pylori* from feline salivary and gastric sections, and to find *H.pylori* DNA in feline feces and dental plaque. A study in Germany by Rothenbacher et al. (25) found that adults who owned a cat as a child had a significantly higher prevalence of *H.pylori* infection.

Gnotobiotic pigs have been successfully infected with *H.pylori*. However, there is currently no convincing evidence that swine are a reservoir for *H.pylori*, even though the monogastric pig stomach is anatomically and physiologically similar to human and nonhuman primate stomachs (26).

Exposure to sheep was implicated in two recent epidemiological studies. In a study of children from the Colombian Andes, Goodman et al. (21) reported that children who "played with sheep" had a higher risk of *H.pylori* infection (OR=4.5). A study by Dore et al. (2) revealed that the prevalence of *H.pylori* was significantly higher among Sardinians shepherds occupationally exposed to sheep than among their nonexposed family members or among Sardinian blood donors. While tending sheep shepherds live usually in small enclosures by themselves or sometimes with their families, far from villages. The enclosures lack elementary sanitation and are potentially at high risk for diseases transmitted by the fecal- oral route.

Nevertheless, we can not exclude the possibility that *H. pylori* could survive in sheep. This hypothesis is currently under investigation.

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H.pylori has been cultured from stool and has been identified in feces by PCR. *H.pylori* can survive in milk (31) for several days, suggesting that milk contaminated by feces containing feces could be potentially infectious to humans. Alternatively, animals might transmit the infection to humans through the environment contaminated by feces containing *H.pylori* or the coccoid forms. If animals, on occasion, play a role in transmission, the cycle of infection might include phases in the environment, animals /sheep or dogs/, and human beings.

The most recent reservoir suggested for *H.pylori* transmission is the **housefly.** In controlled experimental studies, Grubel et al. found that houseflies infected with *H.pylori* in the laboratory could harbor viable *H.pylori* bacteria in their intestines as well as on their body hairs (4,27). However, evidence is lacking that *H.pylori* can be isolated from flies that have been in contact with *H.pylori* infected feces and that *H.pylori* can be transmitted from contaminated flies to food in a quantity sufficient to infect humans. In a recent experimental study by Osato et al. researchers were unable to recover *H.pylori* from houseflies that were fed human feces infected with *H.pylori* (3). The fact that they were unable to recover *H.pylori* from houseflies that were exposed to stool containing approximately 9x107 CFU per g of stool indicates that even higher levels of viable organisms must be present in nature to ensure positive recovery from flies. Previous data have shown that such high levels of viable *H.pylori* may not be present in the extragastric environment (3,4). Therefore, it seems less likely that the domestic housefly serves as a vector for *H.pylori* transmission.

Endoscopy is a common medical procedure used to diagnose and manage gastrointestinal disease. Because of the complex structure of the endoscope and difficulty in disinfecting it, the possibility of iatrogenic infection in-patients following endoscopy is a potential risk factor not only for *H.pylori* but also for other infectious diseases such as hepatitis C, hepatitis B, tuberculosis and possibly HIV (28,29). **In fact, nosocomial transmission of *H.pylori* is the only proven mode of transmission**. According to Tytgat et al, the rate of iatrogenic infection may approximate four per 1000 endoscopies (0.4%) when the prevalence of *H.pylori* is around 60% (30). In 1990 the working party report to the World Congresses of Gastroenterology recommended that the endoscope be soaked in 2% activated glutaraldehyde for at least 5-10min. Furthermore, because biopsy forceps typically penetrate the gastric mucosa and are difficult to clean sterilization of the forceps or, preferably use of disposable forceps is essential (30).

Understanding the route(s) by which *H.pylori* infection spreads is fundamental for development of strategies for the infection's control.

Knowledge of the Epidemiology and mode of transmission of *H.pylori* may be useful in identifying high- risk populations, especially in areas that have high rates of gastric lymphoma, gastric cancer and gastric ulcer. Different studies show contradictory results for a fecal- oral route as well as for other types of transmission. The difficulty is linked to the lack of adequate markers for each route of transmission and to the problems in accurately diagnosing *H.pylori* infection, especially in children, where symptoms are commonly nonexistent. Studies have to be designed in families from high *H.pylori* infection prevalence areas.

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CALGEVAX

COMPOSITION

It is composed of lyophilized living bacteria of Calmette-Guerin (BCG). Each ampoule contains 37.5 mg living BCG (1.0 - 3.0 x 10^8 live units). As stabilizer 8% sodium glutamate is added. The preparation contains no conservants.

INDICATIONS

CALGEVAX is prescribed as an additional treatment for all types of malignant tumors requiring adjuvant therapy, especially for vesicle tumors, malignant melanoma, etc.

CALGEVAX is especially active in case of superficial tumors of the bladder. It is applied intravesically in the following cases:

- for prophylaxis of recidivations after transurethral resection of superficial tumors;
- for treatment of carcinoma in situ of the bladder.

CALGEVAX is used for treatment of malignant melanoma as well as for non-specific immunostimulating adjuvant therapy in other malignancies (tumors of the lungs, mammary glands, acute leukemia, lymphosarcoma, osteosarcoma, etc.)

ADMINISTRATION AND DOSAGE

Intravesical administration: CALGEVAX is applied most early 14 days after a biopsy, transurethral resection or traumatic catheterization.

It is carried out following a strictly aseptic technique and under specialist's control. Three or four ampoules of CALGEVAX are needed for each intravesical application. The standard application scheme consists of one intravesical infusion per week during six weeks (induction therapy). The maintaining therapy depends on the individual case.

The following schema may be applied: monthly infusion in the course of at least 6-12 months or weekly, during 3 consecutive weeks, at 3, 6, 12, 18, 30 and 36 months after the first infusion.

Percutaneous application: On the spot of scarification 0.5 ml of the preparation is applied. One ampoule of CALGEVAX is dissolved in 0.5 ml saline. For one scarification is used one ampoule.

PACKAGE

A box with five or ten ampoules CALGEVAX.

On each ampoule and box are marked: the name of the producer, the preparation name, the BCG contents in mg, the number of the lot and expiry term.

SHELF LIFE

Not more than 24 months from the date of control test and not more than 30 months from the date of production.

STORAGE

In a dry and dark place at +2°C - +8°C.

PRODUCER
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UROSTIM

COMPOSITION

Each tablet UROSTIM contains 25 mg for children and 50 mg for adults of freeze-dried with excipient lysate and killed bacterial bodies of the following microbial species: Escherichia coli; Proteus mirabilis; Klebsiella pneumoniae; Enterococcus faecalis.

INDICATIONS

UROSTIM is intended for peroral immunotherapy and immunoprophylaxis of acute recurrent or chronic non-specific infections of the urinary tract in children and adults, regardless of the agent type. It is recommended for the treatment of cystites, pyelonephrites, uretrites, asymptomatic bacteriuria, etc. Can be used with antibiotic therapy and especially for treatment of patients with suppressed immune reactivity obtained in result of a long antibiotic therapy.

CONTRAINDICATIONS

The drug has an excellent tolerance and no contraindications.

ADMINISTRATION AND DOSAGE

For the purposes of immunotherapy UROSTIM is administrated as follows:
Adults: 50 mg daily. Basic course of treatment: one tablet of 50 mg in the morning before breakfast, every day for 10 days.
Maintenance treatmen: for long-term therapeutic effect 1 tablet of 50 mg UROSTIM is recommended in the morning before breakfast in three consecutive months.
Children: 25 mg daily for children 3 to 14 years of age. One tablet of 25 mg daily according to the above dosage scheme.
In case in which antibiotic therapy is needed UROSTIM should be given in addition to the antibotic treatment.

DOSAGE FORM AND PACKAGES

tablets of 25 mg for children and 50 mg for adults, 30 tablets in one package.

EXPIRY DATE

2 years

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Face A Healthy New Day -

UROSTIM

DENTAVAX

COMPOSITION

Each tablet DENTAVAX contains 36.0 mg freeze-dryed lysate and killed bacterial bodies of the following microbial species: Lactobacillus acidophilus, Streptococcus pyogenes, Staphylococcus aureus, Klebsiella pneumoniae, Candida albicans.

INDICATIONS

DENTAVAX has a prophylactic and therapeutic action in case of inflammatory and infectious diseases of the oral cavity and parodonte:

- gingivitis erythematosa and ulcerosa;
- superficial or profound parodontosis;
- stomatitis, glossitis, aphtae, etc.;
- before and after tonsillectomies and after dental extractions;
- Ulcerations and secondary infections caused by artificial dentures.

ADMINISTRATION AND DOSAGE

Tablets for oral administration, which are left to dissolve in the mouth by sucking. In case of acute inflammation - 4-6 tablets daily during 15-30 days. In case of chronic inflammation - 3-4 tablets daily during 15-30 days.

Depending on the clinical data the treatment can be carried out 2-3 times per year.

ATTENTION

Eating and drinking are not allowed for one hour after the preparation has been taken in order not to disturb its local effect.

EXPIRY DATE

2 years

DOSAGE FORM AND PACKAGES

40 tablets of 36 mg in blisters in a box or 40 tablets of 36 mg in a plastic sealed bottle.

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Dentavax

A Smile That Speaks of Health -

bulbio@bulbio.com

RESPIVAX!

Choose Health! Choose -

RESPIVAX

COMPOSITION

Each tablet RESPIVAX contains 25 mg for children and 50 mg for adults of freeze-dried lysate and killed bacterial bodies of the following microbial species: Streptococcus pneumaniae, Haemophilius influenzae, Staphylococcus aureus, Klebsiella pneumoniae.

INDICATIONS

RESPIVAX is intended for oral immunotherapy and immunoprevention of non-specific respiratory diseases and has a very good therapeutic effect in children and adults with recurrent and chronic infections of the respiratory pathways, throat and nose included.

RESPIVAX is practically suitable for patients with hypersensitivity to antibiotics and for infections caused by antibiotics-resistant bacteria. It can be used in combination with any other treatment and allows repeated dose administration without risk of dependence.

CONTRAINDICATIONS

The drug has an excellent tolerance and no contraindications.

ADMINISTRATION AND DOSAGE

For the purposes of immunotherapy RESPIVAX is administrated as follows:
Adults: 50 mg daily. Basic course of treatment: one tablet of 50 mg in the morning before breakfast, every day for 10 days.
Maintenance treatmen: for long-term therapeutic effect 1 tablet of 50 mg RESPIVAX is recommended in the morning before breakfast for 10 days in three consecutive months.
Children: 25 mg daily for children 3 to 14 years of age. One tablet of 25 mg daily according to the above dosage scheme.
In case in which antibiotic therapy is needed RESPIVAX should be given in addition to the antibotic treatment.

DOSAGE FORM AND PACKAGES

tablets of 25 mg for children and 50 mg for adults, 30 tablets in one package.

EXPIRY DATE

2 years

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RESPIVAX