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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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ENZYME - LINKED IMMUNOSORBENT ASSAY FOR ESTIMATION OF IMMUNOGLOBULIN G ANTIBODIES AGAINST PERTUSSIS IN HUMAN SERA

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Bul Bio - NCIPD Ltd. - Sofia, Bulgaria

SUMMARY

Estimation of pertussis antibody in human sera is very important for investigation of efficacy of vaccination against whooping cough. An enzyme-linked immunosorbent assay was development for measuring immunoglobolin G antibodies against pertussis in human sera. The assay was done in plastic plates coated with bacterial cells. The bacterial agglutination test is time consuming, thus challenging us to develop more rapid and simple alternative in vitro test. Simple to perform, economical and precise, the ELISA seems to be a very practical procedure for seroepidemiological purposes.

Key words: pertussis, whooping cough, ELISA

Whooping cough is a respiratory infection caused by Bordetella pertussis. Pertussis is a serious disease of infants and death is greatest in these age groups (3). The disease has been effectively controlled in developed area by vaccine prophylaxis (8).

The specific immunoprophylaxis of humans with combine bacterial vaccines leads to production of specific antibodies that have main role in pertussis prevention (9). For evolution of immunization procedures and the vaccine itself, antibody levels against pertussis are useful to show the immune status of a population. However, the agglutination - type assay to study to assess the immune response to Pertussis vaccine and Bordetella pertussis infection in human serum is not practical for seroepidemiological and diagnostic purposes, regardless of its sensitivity and reproductivity (4, 11). The agglutination test is easier to perform and have low cost: however, the assay is only semiquantitative and titers are reported in steps of serum dilutions at intervals (2, 10). Direct immunofluorescent antibody staining of nosopharyngeal secretions has been used for diagnosing whooping cough, but this test lacks specificity, and false - positive results of up to 40% have been described (3). More precise results might be obtained with an Enzyme - linked immunosorbent assay (ELISA) that would define titers over a continuos range (11).

The ELISA method is more simple, economical and precise and permits to perform rapidly the levels of antibodies in humans. Knowledge of the immune status of population against pertussis may have practical implication (7, 10). It may assist in checking the efficacy of immunization schedules adopted and the persistence of immunity.

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ABBREVIATIONS USED IN THIS PAPER: OUs - optical units of opacity; PBS - phosphate buffered saline; OPD - ortophenilenediamine; PT - pertussis toxin; FHA - fimbrial hemagglutinin.

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In this paper we present our results for the development and standardization of an ELISA for immunoglobolin G antibodies against pertussis in human sera.

MATERIALS AND METHODS

Human sera.

Sixty human sera were tested for pertussis antibodies using laboratory developed ELISA Sera separated from clotted blood were stored at - 20°C in small vials until use (3). According to person's age serum samples were divided in three groups - children younger than 10 years, adolescents from 10 to 20 years old and persons over 20 years.

Bacterial Agglutination (BA) Test (10).

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

ELISA (3).

Polystyrene plates with U - shaped 96 wells (Nunc immunoplates, Denmark) were coated with bacterial cells in different working dilutions (batch 36, National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria). To prepare the plates the bacterial cells were diluted in carbonate buffer (pH 9.6) and chloroform: ethanol (1:10) (4). 100 µl volumes were added to the wells and left 2 hours at 36°C and overnight at room temperature. The following morning the plates were emptied and drained over filter paper. After being washed three times with PBST (Phosphate Buffered Saline pH 7.2 containing 0.05% Tween 20), 100 µl volumes blocking buffer - PBS containing 1% BSA (Bovine Serum Albumin) were added to the wells and incubated at 37°C for 2 hour.

For the test 100 µl volumes of sera dilutions in PBST were added to the wells of sensitized plates incubated at 37°C for 120 minutes and washed three times in PBST. 100 µl volumes of peroxidase anti - human immunoglobolin G (National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria) diluted in PBST was added to each well, followed by incubation at 37°C for 60 minutes and washing three times of the plates. The plates were than left at room temperature with 100 µl volumes of a chromogen solution (10 mg of ortophenilenodiamine and 10 µl of 30% hydrogen peroxidase in 50 ml citrate - phosphate buffer pH 5.0). The reaction was stopped after 30 minutes with 100 µl of 1 N H_2SO_4. The plates were directly read at 405 nm in a MicroELISA Minireader Photometer (Bio - Tek) in optical densities. Standard curve with five points according to measured optical densities of used control serum samples, standardized by BA method, was prepared each time (Fig. No. 1).

RESULTS AND DISCUSSION

Sixty human sera were tested for pertussis antibodies using laboratory developed ELISA

Optimization of antigen.

Bordetella pertussis suspension (serotype 1, 2 and 3), (batch 36, National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria), dead and inactivated with 0.01% thiomersal was used in ELISA test. Diluting the stock made working dilutions suspensions in carbonate buffer (pH 9.6) and chloroform: ethanol (1:10). For optimization of the antigen con-

Table 1. Titers of antibody against whooping cough obtained by ELISA.

Age groups	% samples (number)	% full protection (number)	% basic immunity (number)	% negative (number)	% with titers as a criterion for disease (number)
up to 10	33.3	(20)	75.0	(15)	25.0
years old	(5)	0.0	(0)	0.0	(0)
10 - 20	33.3	(20)	55.0	(11)	35.0
years old	(7)	5.0	(1)	5.0	(1)
over 20	33.3	(20)	40.0	(8)	50.0
years old	(10)	10.0	(2)	0.0	(0)
Total	100.0	(60)	56.7	(34)	36.7
	(22)	5.0	(3)	1.6	(1)

tent in the reaction were compared the following bacterial suspension - 1 OUs, 5 OUs and 10 OUs and the results can be viewed on Fig. No.2 A - B. The experiments showed that the optimal concentration for detection of quantity of antigen is 10 OUs, dissolved in chloroform: ethanol (1: 10). This solvent was selected among several organic solvents investigated because it was easy to remove by evaporation and because its interaction with the surface of different commercially available ELISA plates did not interfere with the OD measurements in the ELISA reader (4). The following ELISA was made by coating of the wells with the concentration of bacterial cells 10 OUs dissolved in chloroform: ethanol (1:10).

Titration of serum samples.

Check - board titration of human sera was made after determination of optimal antigen concentration. Two serum samples, defined by using BA test - one with high level of pertussis antibody titer (1:640) and one with low level of pertussis antibody titer (1:40) were used for determination of optimal serum concentration. Human sera were prepared in following working concentration: 1:25; 1:50; 1:100; 1:150. The comparison assay between BA test and the results obtained after titration (Fig. No. 3) have shown that the working dilutions at 1:50 and 1:100 are the most useful for quantitative detection of pertussis antibodies in human sera. Because of the linear relationship between extinction values of ELISA analyze and the obtained results of BA test, the following tests were made in working dilution of human sera 1:50.

Titration of peroxidase anti - human immunoglobolin G specific conjugate.

Peroxidase anti - human immunoglobolin G specific conjugate prepare in National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria was used for detection of ELISA. To estimate the optimal working dilution of peroxidase anti - human immunoglobolin G specific conjugate the experiments were made by prepared five different dilutions of the conjugate: 1:250; 1:500; 1:750; 1:1000; 1:1250. The working dilutions were checked using three serum samples with different concentration of pertussis antibodies, defined by BA test. The titer of antibody in 1 - st sera is 1:640, in 2 - nd sera 1:160 and in 3 - td sera is 1:40. The performed results on Fig. No. 4 has shown that the most useful conjugate dilution for estimation of antibodies against pertussis in human sera is a working concentration 1:500. Having this concentration of anti - human Ig G the titers of antibody are closest to the titers observed at BA test. The parallelism relationship between the steps of antibody's titers and extinction values of ELISA analyze were observed.

Seroactivity of human sera.

Laboratory verification of whooping cough, which is almost exclusively based on isolation of Bordetella pertussis, is associated with several problems (11). Therefore, attempts to develop ELISA method that measures the antibody response against pertussis is very important. The high sensitivity of the ELISA allowed for antibody determination in small amounts of serum and evolution of laboratory verification methods (6).

In this study human sera were separated in three groups according to patient's age. The observed results were classified depend of titer obtained in following groups: group with antibodies level against pertussis up to 1:80 - strong negative; group with level of antibodies against pertussis between 1:81 to 1:160 - serums with basic immunity, group with level of antibodies against pertussis between 1:161 to 1:320 - serums with full protection and group with level of antibody against pertussis more than 1:321 - titer used as a

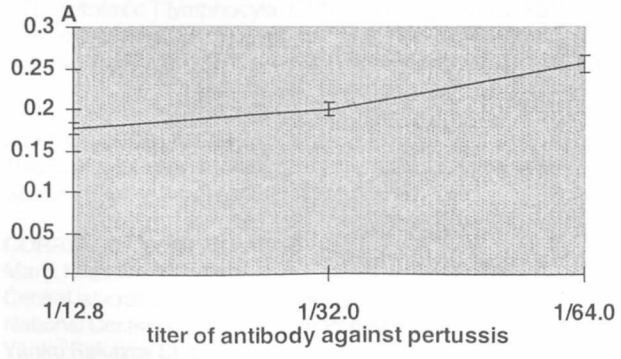
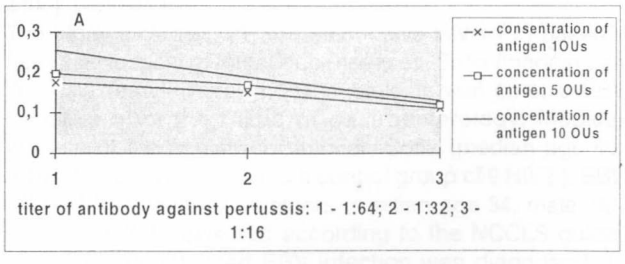


Fig. No. 1: ELISA: Standard curve.

A: Titration of different concentration of pertussis antigen dissolved in chloroform: ethanol (1:10).



B: Titration of different concentration of pertussis antigen dissolved in carbonate buffer (pH 9.6).

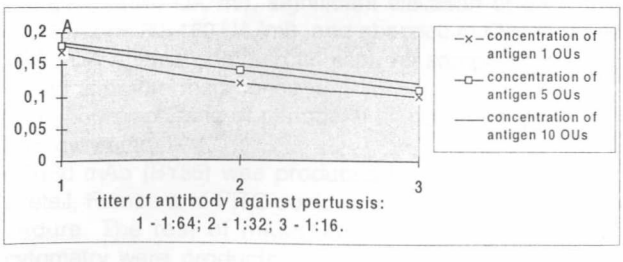


Fig. No. 2: Optimization of pertussis antigen content in ELISA.

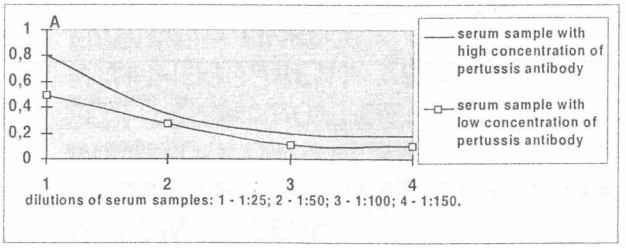


Fig. No. 3: Titration of serum samples.

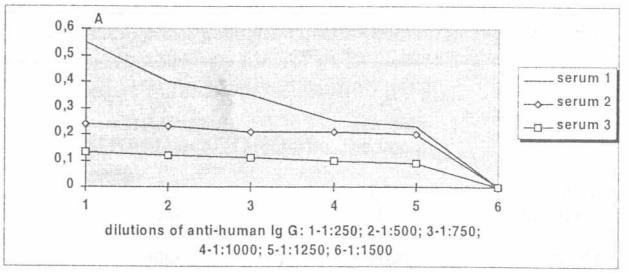


Fig. No. 4: Titration of peroxidase anti - human Ig G.

criterion for disease. The seroactivity of serum samples obtained by laboratory developed ELISA is presented on table No. 1. The ELISA test shows that 56.7% of serums in different groups of age have full protection, 36.7% have basic immunity, the level of antibodies against perussis in 5.0% is up to 1:80 and in 1.6% of human sera the titer of antibody is over titer used as a criterion for disease.

Previous pertussis vaccinations of patients with whooping cough may interfere with the antibody response to the natural disease. Glanstrom et al. (7) found that the antibody response in unvaccination patients increase Ig G titers. Most vaccination humans had a secondary type response with early increase in Ig A and after that show low level of Ig G titers (6). Vaccination results in an increase in the ELISA antibody titers to all known antigens of Bordetella pertussis (5). Children vaccinated with whole cell pertussis vaccine show increasing levels of antibody against FHA, PT and other membrane proteins (1).

The ELISA for quantitation of pertussis antibodies described in this paper should be a valuable tool for determining immunological protection against whooping cough. The test should be especially useful for population studies, since it is economical and practical for large routine purposes. When compared with the BA test, it presents several advantages, such as the possibility of precise results through the assay of only one or two serum dilutions. Because of the linear relationship between extinction values and of antibody concentrations, and the constant slope observed, serum titers could be expressed as the serum dilutions corresponding to a definite optical density value.

The ELISA was very reproducible. It should be noted that in the ELISA titers were obtained in a continuous range, in contrast to the twofold dilution step values at intervals, which are given by the BA test (10). Correction of titers for daily variations test sensitivity, as indicated by the titer variations of a reference serum also contributed to titer reproducibility.

REFERENCES:

1. Burstin, D., L. Baraff, M. Peppler. Serological response to filamentous hamagglutinin and lymphocytosis - promoting toxin of Bordetella pertussis. Inf. Immun. 41: 1150 -1156

2. Coursaget P., B. Yvonnet, E. Relyveld, J. Barres, J. Chiron. Simultaneous admonistration of DTP - Polio and Hepatitis B Vaccines in a simplified immunization program: Immune response to Diphtheria Toxoid, Tetanus Toxoid, Pertussis and hepatitis B surface antigen. Inf. Immun. 51: 784 - 787

3. Engnal, E., P. Perlmann. ELISA. The J. of Immun. 109: 129 - 135

4. Freudenberg, M. A., A. Fomsgaard, I. Mitov, C. Galanos. ELISA for antibodies to lipid A, Lipopolysaccharides and other hydrophobic antigens. Inf. 17: 322 - 328

5. Galazka, A. The immunological basis for immunization serier: Pertussis. WHO

6. Goodman, Y., A. Wort. ELISA for detection of pertussis Ig A in nasopharyngeal secretions as an indicator of recent infection. J. Clin. Microbiol. 13: 286 - 292

7. Granstorm M., A. Lindberg. Detection of antibodies in human serum against the fimbrial hamagglutinin of Bordetella pertussis by ELISA. J. Med. Microbiol. 15: 85 - 96

8. Granstorm, M., A. Lindfors, P. Askelof. Serologic diagmosis of whooping cough by ELISA assay using fimbrial hemagglutinin as antigen. The J. of Inf. Dis. 146: 741 - 745

9. Macaulay, M. The serological diagnosis of whooping cough. J. Hyg. 83: 95 - 102

10. Mertsola, J., O. Ruuskanen, T. Kuronen, M. Vijanen. Serologic diagmosis of perussis: comperison of ELISA assay and Bacterial agglutination. The J. of Inf. Dis. 147: 252 - 257

11. Preston, N. Technical problems in the laboratory diagnosis and prevention of whooping cough. Lab. Practice 19: 482 - 486

EXPRESSION OF PERFORIN AND GRANZYME B IN TOTAL CD8 T LYMPHOCYTES OF PATIENTS WITH CHRONIC HIV AND EBV INFECTIONS.

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SUMMARY

The mechanisms affecting CD8 T cell functions in the course of chronic viral infections and their phenotypic equivalents are a matter of intensive research. Together, granzyme B (GzmB) and perforin (Per) are essential mediators of cytotoxicity. Using flow cytometry, we studied GzmB/Per co-expression in total CD8 T cells of subjects with chronic untreated HIV-1 or EBV infection, in relation to CD8 T cell differentiation subsets defined by CD160/CD27/CD28 expression and the Treg subset CD4+CD25high. Further on, we looked for association between the patterns of GzmB/Per expression and markers of disease progression. GzmB+Per+ and GzmB+Per- CD8 T cells were increased in HIV and EBV patients, but only the single-positive subset was significantly increased in both patients groups as compared to controls (mean 41 % and 11 % vs. 5%). Moreover, the GzmB+Per - CD8 subset was disproportionately increased in HIV infection, differing significantly from both EBV ($p<0.01$) and control ($p<0.001$) groups. The level of GzmB+Per- CD8 T cells in HIV patients correlated with the level of antigen-primed CD28- CD8 T cells lacking CD160 expression ($R= 0.6$, $p<0.001$). While the discordant GzmB/Per expression in HIV patients did not correlate with CD4 AC or CD38ABC as a marker of viral replication, a positive correlation was established with the percentage of CD4+CD25^{high} Treg cells. In conclusion, the downregulation of CD160 on CD8 T cells in the course of chronic HIV infection may be a significant correlate of impaired cytotoxic functions, at least in part related to CD4+ Treg mediated suppression.

Key words: perforin, granzyme B, HIV infection, EBV infection, CD160

INTRODUCTION

Virus-specific CD8+ cytotoxic T lymphocytes (CTL) play a major role both during the acute and chronic phase of infections with persisting viruses as HIV-1, Epstein-Bar virus (EBV) or cytomegalovirus (CMV), [1-3]. CTL suppress viral replication by secretion of soluble factors or by direct cytotoxicity. The latter occurs primarily through perforin-mediated vectorial release of serine protease granzymes into the target cell. In humans, granzymes A and B are the most abundant, and granzyme B (GzmB) induces both caspase-independent and caspase-dependent cell death [4]. GzmB is synthesized earlier in the course of antigen-driven CTL differentiation, while perforin [Per] expression is a later event. The simultaneous Per and GzmB expression is required for eliciting cytotoxicity, as demonstrated in particular for HIV-1-specific CTL [5-7].

Chronic EBV and CMV infections are usually kept under control in immune competent subjects, while progression of HIV infection is inevitable in the vast majority of the infected, despite the presence of HIV-specific CTL. The reasons for this inefficiency have been largely investigated. It has been demonstrated that CD4 T cells are essential to maintain effective CD8 T cell memory. Thus, the loss of essential helper CD4 T cells may be the underlying cause for progressive loss of CD8 T-cell function, combined with HIV-specific viral escape mechanisms [8]. An increased level of CD4+ Treg cells was reported in HIV+ patients, as well as the suppressive effect of CD4 Tregs on CD4 and CD8 T cell-mediated virus-specific response [9;10]. A number of studies have evoked the concept of "arrested maturation" of HIV-specific CD8 T cells resulting in the accumulation of CD8 T cells with intermediate phenotype in parallel with a low level of Per expression [11]. However, the importance of this phenomenon is not clear yet, as "intermediate" CTL were demonstrated to exert cytolytic functions in both HIV and EBV infection [12;13].

To further elucidate the issue of CD8 T cell functional incapacity in HIV infection we studied the co-expression of GzmB and Per in total CD8 T cells of subjects with untreated HIV-1 chronic infection, in comparison to immunocompetent HIV-1(-) subjects with chronic EBV hepatitis, and healthy HIV(-) controls. We have previously demonstrated that the expression of the co-stimulatory cytotoxic CD8 T cell surface antigen, CD160, correlates well with HIV-specific IFNγ expression, and the response to HAART [14]. Further on the expression of CD160 differentiated between HIV+ patients with detectable and suppressed HIV-, EBV- and CMV-specific IFNγ responses [15]. Now we tested the hypothesis that the inefficient CD8 T cell function in HIV and EBV infection is associated with discordant maturation of cytotoxic mediators, and may be related to the downmodulation of CD160 expression and the suppressive effects of CD4+ Treg cells.

MATERIALS AND METHODS

Study population

The study included: 21 treatment-naive HIV-1 (+) patients from the Hospital of Infectious Diseases, Sofia (median age 35; male/female ratio 11/9); 11 patients with chronic EBV hepatitis from the Clinic of gastroenterology, Medical Insitiute of the Ministry of Interior, Sofia (median age 40; male /female ratio 7/4) and a control group of 9 HIV (-), EBV VCA IgM (-) healthy volunteers (median age 34, male /female ratio 2/7), selected according to the NCCLS guidelines [16]. Reactivated EBV infection was diagnosed as elevation of serum aminotransferases of more than 6 months duration, combined with elevation of EBV CA IgG (range 160(640 UA/ml), significant elevation of EA-R and EA-D (range 80÷160 UA/ml), and absence of EBV VCA IgM (IFA FDA Atlanta commercial kits). All samples were collected after informed consent.

Immunophenotyping of peripheral blood lymphocytes and flow cytometry

CD160 mAb (BY55) was produced by INSERM, Unit 659, Creteil, France and FITC-conjugated after a standard procedure. The rest of mAbs, kits and reagents for flow cytometry were products of BD Biosciences (San Jose, CA). Absolute CD4+ and CD8+ T cell counts were deter-

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ABBREVIATIONS USED IN THIS PAPER:

CTL - cytotoxic T lymphocyte, CMV - cytomegalovirus, EBV - Epstein Bar virus, Gzm B - granzyme B, HAART - highly active antiretroviral therapy, HIV - human immunodeficiency virus, Per - perforin

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Table 1. Characteristics of the patients and control groups

characteristic	Controls	EBV	HIV
		Mean (min-max) values	
CD4AC (cells/μl)	846(590-1299)	965 (464-1645)	392 (102-1003)
CD8AC (cells/μl)	441 (294-718)	538(374-815)	1498 (320-5492)
CD4/CD8 ratio	1.9 (1.4-2.9)	1.8 (1.1-3.1)	0.4 (0.03-1.1)
CD4+CD25^{high} T (%)	3.9 (2.6-5.3)	4.9 (2.6-9.1)	7.3 (2.5-18)
CD38ABC	1352 (676-2393)	2581 (1039-7486)	5759 (1247-20511)
Estimated infection duration (mo)	-	10.8 (6 - 36)	27 (12-48)

mined in whole heparinized blood by multicolor immunofluorescent staining and lysis/no wash procedure using TruCOUNT tubes (BD). CD8+ T cell subsets were defined using the CD160/CD27/CD28/CD8 combination of mAbs and routine lysis/wash technique. The regulatory CD4 T cell subset was defined with the CD4/CD25/CD8 combination of mAbs. Immune activation was assessed according to the quantitative expression of CD38 (CD38 antibody binding capacity, ABC) on CD8 T cells using the QUANTIBRITE PE fluorescence quantitation kit and, QUANTIBRITE software according to manufacturer's instructions. At least 5000 cells for surface staining, or 25000 cells for intracellular staining analysis were collected using a FACSCanto flow cytometer and DIVA software (BD).

Intracellular staining for cytolytic molecules.

At least 2x10^5 peripheral blood mononuclear cells (PBMCs)/ sample were stained. After refrigeration, samples were permeabilized with FACS Permeabilizing solution (BD Biosciences, San Jose, CA) for 10 min at 37°C, washed with 0.5% BSA in PBS, and labelled either with GzmB-FITC/Per - PE or relevant intracellular isotype control mAbs combined with CD8-PerCP. Staining was performed at RT for 30 min, followed by washing and fixing with 300 μl 0.5% formaldehyde.

Statistical analysis

Data was analyzed using GraphPad PRISM software v.4. Correlations between tested variables were assessed according to Spearman's correlation coefficient, and one-way ANOVA was used to compare differences between the three groups, with $P < 0.05$ considered as significant.

RESULTS

1. A discordant increase of granzyme B single-positive CD8 T cells is observed in patients with chronic HIV and EBV infection.

We evaluated the expression of GzmB and Per in CD8^{high} - gated PBMC from 21 HIV infected subjects, in comparison to 11 individuals with reactivated EBV infection and 9 controls. GzmB content in CD8 T cells varied greatly in the three groups: HIV patients range 11 - 77%, EBV patients range 2 - 66% and controls range 2 - 30%. Perforin expression also varied in patients groups: HIV range 4 -36 %, EBV range 1 - 23%, and less in controls, 0 -8 %. The co-expression of the two effector molecules defined three major CD8 T-cell subsets: double-negative (GzmB-Per-), single-positive (GzmB+Per-), and double-positive (GzmB+ Per+). Per was always co-expressed with GzmB, so that GzmB-Per+ cells were not identified, (Fig.1). The mean level of double-positive (GzmB+Per+) CTL was elevated in HIV and EBV patients groups as compared to controls (means 11% and 7% vs. 3%), but differed significantly only between HIV+ patients and controls ($p < 0.001$), (Fig.2A). In contrast, the percentage of GzmB+/Per- CD8 T-cells was significantly increased in both HIV and EBV patients groups as compared to healthy controls (mean 41% vs. 5%, $p<0.0001$ and 19% vs. 5%, $p<0.05$ respectively). However, the discordant elevation of single-positive (GzmB+Per-) CD8 T-cells was more important in HIV patients as they differed significantly from the EBV group ($p<0.01$), (Fig.2B). The discordant expression of effector molecules was best described by the ratio of double-positive (GzmB+Per+) to total GzmB+ CD8 T-cells: a mean of 20% (range 5 - 42) in HIV patients, as compared to a mean of 31% (range 12 - 48) in EBV patients and a mean of 55% (range 17 - 100) in controls (Fig.2C).

2. Granzyme B and perforin expression do not correlate with CD27/CD28 defined CD8 T-cell subsets.

The significant differences in GzmB expression established between HIV and EBV groups might reflect the differentiation state of the respective T cell pools, as GzmB expression is confined to the antigen-primed CD8 T subset [17]. According to the expression of the costimulatory receptors CD28 and CD27, antigen-experienced CD8 T-cells can be subdivided into early (CD28+/CD27+), intermediate (CD28+/CD27+), and terminally differentiated effectors (CD28-/CD27-), [12]. An increased expression of CD160 was detected in each of the CD27/CD28 defined subsets in the course of HIV infection [15].

As expected, early (CD28+CD27+) CD8 T cells, were significantly decreased in HIV and EBV patients as compared to controls ($p<0.0001$), (Fig.3A). This was accompanied by an increase of both intermediate (CD27+CD28-) and terminal (CD27-CD28-) subsets, (Fig. 3B and 3C). Importantly, a significant difference existed between HIV and EBV patients regarding only the intermediate subset: a mean of 31 % vs. 15 % respectively ($p<0.001$), (Fig. 3B).

Based on this result, we envisaged a possible correlation between the increased expression of GzmB+/Per- CD8 T cells and the intermediate CD8 T cell subset in HIV patients. However, the percentage of double positive (GzmB+/Per+) or single-positive (GzmB+Per-) CD8 T cells did not correlate with the percentage of intermediate or late CD8 effectors, as defined by CD27/CD28 expression in any of the studied groups. In fact, the percentage of single-positive CD8 T cells in HIV patients was invariably higher than that of CD27+CD28- CD8 T indicating that in HIV infection expression of Per no longer accompanies the disappearance of CD27 as established during the normal differentiation.

3. The GzmB+/Per- subset in HIV patients correlates with CD28- T cells lacking CD160 expression.

In healthy donors the expression of CD160 molecule on CD8 T cells correlates with granzyme B and perforin expression and is confined to a subset with high cytotoxic activity [18]. Based on this, we looked for a possible association between the expression of cytotoxic effector molecules and CD160 in patients with chronic viral infections. When analyzing CD28/CD27 CD8 T cell subsets subdivided according to CD160 expression, we established an intriguing result in the HIV(+) group: the percentage of functionally inactive GzmB+/Per- CD8 T cells correlated strongly and positively with the CD28-CD160- subset ($R=0.6$, $p<0.01$), (Fig.4). This relation was not observed in patients with EBV hepatitis (data not shown). Thus, CD160 expression may specifically indicate the functional "fitness" of CTL in chronic HIV infection.

4. Relationship of GzmB/Per expression in total CD8 T cells

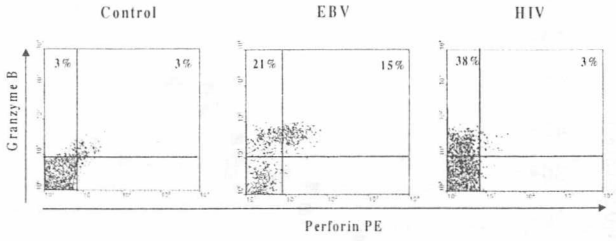

Fig.1. GzmB/Per co-expression in CD8 T cells of HIV- controls, patients with chronic EBV infection and patients with chronic HIV infection. Flow-cytometry analysis of PBMC stained with Gzm-FITC/Per-PE/CD8-PerCP. Representative examples of the three groups are shown; percentage of GzmB+Per+ and GzmB+Per- CD8 T cells is noted.

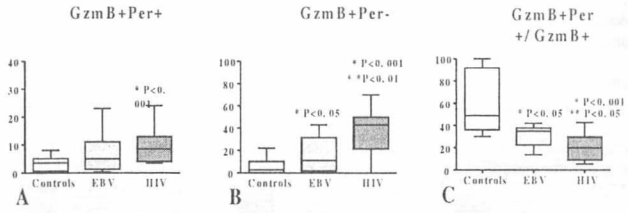

Fig.2.Discordant expression of GzmB and Per in total CD8 T cells is most pronounced in HIV + patients. Box-and-whiskers plots represent the distribution of GzmB+Per+ CD8 T cells (A), GzmB+Per- CD8 T cells (B) and GzmB+Per+/GzmB+ CD8 T cells ratio (C), in HIV- controls, patients with chronic EBV and patients with chronic HIV infection. Between-group statistically significant differences are noted.
* Significant differences between patients groups and healthy controls.
** Significant differences between patients with HIV and EBV infection.

of HIV patients to CD4 AC, immune activation and percentage of Treg cells.

Since the individual values of Per and especially of GzmB varied greatly within the patients groups, we searched for a possible association with parameters related to disease severity and progression. No correlation was established between GzmB/Per expression and: CD4 and CD8 AC and percentage, CD4/CD8 ratio or CD38ABC of CD8 T cells as a surrogate marker of viral replicative activity and immune activation. This was in spite of the variable individual values, especially of CD38ABC in both groups (Table 1).

CD4+ Treg cells are involved in the control of excessive immune activation and cytotoxic activity in chronic viral infections. Therefore we verified whether Tregs expression was related to the discordant GzmB/Per expression in the studied patients groups. We quantified the CD4+CD25high subset as the expression of high-density CD25 was shown to be a marker for immunosuppressive Treg activity in human CD4+CD25+ T cells [10]. The frequency of CD4+CD25high T cells was modestly, but significantly elevated in HIV-infected individuals, in comparison to EBV patients and healthy controls ($P<0.05$), (Table 1). Further on, a significant positive correlation existed between the percentage of CD4+CD25high T-cells and the percentage of total GzmB+ CD8 T-cells in HIV+ patients ($p<0.0001$, Spearman $R=0.6$). No such correlation was observed in the other patients group (EBV+). Thus, CD4+ Treg cells might at least in part be responsible for the perturbed functional differentiation of CD8 T cells in HIV infection.

DISCUSSION

Cytotoxic T lymphocyte responses are of crucial importance in both the acute and chronic phases of persistent viral infection. However, despite the presence of HIV-specific CD8 T cells most HIV+ patients fail to contain viral replication in the chronic phase without specific therapy. Moreover, the total CD8 T cell effectors activity is perturbed in these patients, as evidenced by suppressed antigen-specific responses to other viruses [12,19,20]. Accumulating data about reactivated EBV infection in apparently immunocompetent individuals also indicate a probable defect at the level of CTL signaling/effector mechanisms [21]. The most contradictory concept regarding virus-specific cellular immune responses is that of the correlation between phenotypically defined subsets prevailing in the different infection and the efficiency of virus-specific effector mechanisms. This issue is not only of practical importance, but is related to the models of antigen-driven T cell differentiation and the mechanisms of its perturbation in pathologic conditions.

In addition to IFNγ-response, perforin and granzyme B are indispensable mediators of CD8 T cell cytotoxic activity [22]. In our study we assessed GzmB and Per co-expression in total CD8 T cells of untreated patients with chronic HIVand EBV infection and looked for its correlation with T cell effector and regulatory subsets.

According to our results, GzmB and Per expression was enhanced in both HIV and EBV patients. However, while perforin levels were comparable in the two patients groups, the increase of GzmB was much more important in HIV patients, resulting in a discordant prevalence of single-positive (GzmB+Per-) CD8 T cells lacking cytotoxic ability. Previous studies on total CD8 T cells of HIV+ patients have reported either diminished [12], comparable [22], or substantially increased [23] perforin expression in comparison to HIV(-) donors. Having in mind that the intensity of Per expression seems to correlate tightly with replicative senescence [24], such disparity in results might result from difference in the duration and activity of viral replication, the amount of additional antigenic stimuli, etc. Much more significant, according to us, is the established discordant enhancement of GzmB and Per expression, indicating that in the course of antigen-driven CD8 T-cell differentiation the expression of Per, hence - the effective cytotoxicity, is selectively inhibited. This result is in line with the data of Haridas for HIV+ children and adolescents. However, the latter had already been subjected to HAART and the authors discussed the possibility that the discordance was a consequence of therapy. We demonstrate that in untreated HIV+ patients with similar and comparatively short disease duration the regulation of GzmB/Per expression is primarily and profoundly perturbed.

The expression of GzmB/Per is normally regulated in strict concordance with CTL differentiation: GzmB upregulation follows the antigen-driven proliferation, in parallel with CD28 downregulation, while Per expression is a later event, accompanied by the disappearance of CD27 [25]. Based on this rationale, "intermediate" CD27+CD28- CD8 T have low Per expression and are inefficient effectors, while CD27-CD28-Perhigh CD8 T that accumulate in the course of antigen-driven differentiation are potent cytotoxic cells. A phenomenon considered typical of HIV-driven CTL differentiation is the accumulation of "intermediate" or pre-terminally differentiated CTL, both at the level of HIV-specific and total T cells. This concept of "arrested maturation" seems to be supported by the discordant overexpression of GzmB [22]. However, a direct correlation between the CD27+CD28- subset and the single-positive GzmB+Per-CD8 T was not demonstrated. Moreover, contradictory data about the cytolytic potential of CD27+CD28- cells, both in HIV and EBV chronic infection do exist [12,13]. Attempts for precise correlation of GzmB/Per expression to other markers including CD45RA, CCR7 or CD62L have also failed [23]. In our hands, GzmB and Per expression did not correlate with the CD8 T cell differentiation CD27/CD28 subsets. In contrast, GzmB/Per expression in HIV patients was re-

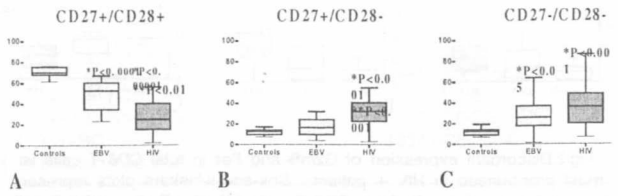

Fig.3. Significant decrease of early CD8+ T subpopulations in HIV and EBV patients is compensated by an increase of both intermediate and terminally differentiated CD8 T cell subsets. Box-and-whiskers plots represent the distribution of early CD27+CD28+ (A), intermediate CD27+CD28- (B) and late CD27-CD28- CD8high T cells in HIV- controls, patients with chronic EBV infection and patients with chronic HIV infection. Between-group statistically significant differences are noted.
* Significant differences between patients groups and healthy controls.
** Significant differences between patients with HIV and EBVinfection.

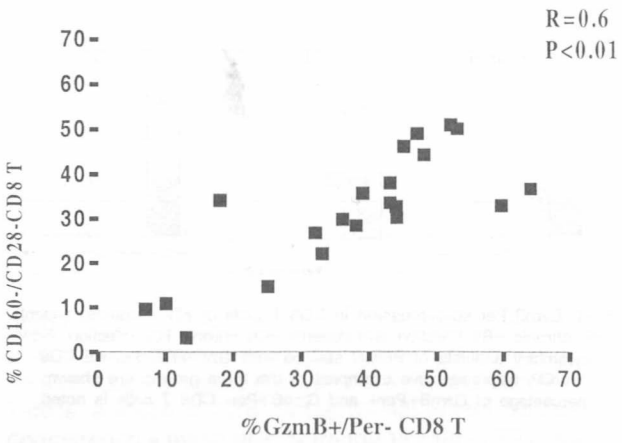

Fig.4. Correlation between GzmB+Per- CD8 T cells and CD160-CD28- CD8 T cells in HIV-infection.

lated to the expression of the functional CD160 molecule. According to our previous results, expression of CD160 in chronic HIV-1 infection is significantly increased on both CD28+ and CD28- subsets and is predictive of a good response to HAART [14]. This co-stimulatory receptor increases the effector potential of CD8 T cells by enhancing HIV-specific IFNγ response. We also observed that HIV patients with generally suppressed CD8 T cell effector functions (including HIV, EBV and CMV-specific responses) were deficient in CD160 expression [15]. In the present study we extend these results by demonstrating that HIV+ patients specifically lacking perforin co-expression are those lacking CD160 expression on their antigen-primed (CD28-) T cells. Whether a direct causal relationship exists between these two facts or they result from a perturbed common regulatory mechanism remains to be elucidated. One such mechanism could be the overexpression of the CD160 specific ligand, HLA-C, or the increased IL-2 levels in the settings of extensive by-stander activation, both leading to CD160 downmodulation in vitro. The important message is that in HIV+ patients with prevalence of CD160-CD28- T cells both IFNγ-mediated and GzmB/Per T cellular responses are perturbed at the level of the total T cell pool.

GzmB/Per co-expression varied widely in both HIV and EBV patients groups. Unlike Haridas et al. [22] we established no significant correlations between the level of GzmB+Per- CD8 T cells and CD4 and CD8 percentage, AC and ratio, or CD38 ABC as a surrogate marker of viral replication. However, the mean disease duration in Haridas group was much higher (10.6 years), all patients were infected perinatally, with extremely high viral loads and supposed early damage of CTL functions, and finally - these patients were treated for different periods, which might add to the observed differences with our results. It should be underlined however, that in a different study, after our HIV+ patients were subjected for 6 months to HAART, we observed a significant difference in the CD4 AC gains, that was in negative correlation with the baseline percentage of GzmB+Per- CTL (manuscript in preparation). Thus, the loss of concordant GzmB/Per expression would be certainly associated with the severity of infection and the possibilities for immune recovery in the long run.

The role of CD4 T cell depletion as a possible mechanism of perturbed CD8 T-cell differentiation and function in HIV infection seems logical, but its relative importance is uncertain. While the need of T cell help for maintaining the memory pool was demonstrated in mice [26, 27], it was also demonstrated that impaired Per expression and antiviral cytotoxicity were not specific to HIV but to chronic antigenic exposure in general [7]. Our data support the second view, as far as the observed discordant GzmB/Per expression did not correlate directly with CD4AC in HIV patients. Further on, a similar tendency of discordant GzmB/Per expression, although less significant, was observed in EBV patients.

It has been lately proposed that T-cell effector functions in HIV infection are at least in part suppressed by CD4+ Treg cells. Experimental data have shown that the absence of antigen-specific CD4 T-cell proliferation in chronically infected HIV patients may not be due to depletion but to anergy, mediated by the suppressive activity of Treg cells [28,29]. Further on, a significant increase of CD4+CD25+ cells with characteristics of Tregs was observed in HIV+ patients, containing HIV-specific cells that secreted TGFβ and IL10 in response to p24 [9]. In line with these data, we reported a significantly increased CD4+CD25^{high} T-cell expression in HIV+ patients. An interesting observation was the correlation of this subset with the level of GzmB+ cells, disproportianately increased in HIV patients. The fact that in our patients group the discordant GzmB/Per expression did not correlate with CD38ABC but rather with the level of CD4+ Tregs suggests that at least in the early stage of chronic infection Tregs might counterregulate the excessive immune activation, however suppressing in parallel CD8 T cell effector functions. On this background we may speculate that perforin and granzyme B expression in CD8 effector T cells are differentially regulated by Tregs resulting in suppressed Per expression and impaired cytotoxic T-cell response. At the molecular level such a differential regulation is supported by the well-established inhibitory effect of TGFβ on Stat5/IL-2 signaling, which in turn is involved in perforin expression [30,31].

Although we did not observe a similar correlation in the EBV patients group, data exist about Treg-mediated suppression of perforin expression in chronic HCV infection [32]. In fact the observed differences between HIV and EBV patients may be related to the duration of antigenic stimulation and the amount of antigenic burden rather than to the specific infection. It was proposed that the quality of CD8 T cell memory response is much less affected in recently reactivated latent viral infections (as is the case of EBV hepatitis) in comparison to long-term active chronic infections (as chronic HIV), [20]. Never-the-less in the long run similar changes may be expected in chronic EBV infection.

In conclusion, we report a discordant expression of GzmB and Per in chronic HIV infection, that is independent of the differentiation state of antigen-primed CD8 T cells but is

associated with lack of CD160 co-stimulation and increase of the CD4+CD25high subset. This observation deserves further investigations, in order to prove its prognostic significance and relevance in the settings of other chronic viral infections.

REFERENCES

1. Appay V, Nixon DF, Donahoe SM, et al. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med 2000;192: 63-75
2. Benito JM, Lopez M, Soriano V. The role of CD8+ T-cell response in HIV infection. AIDS Rev 2004;6: 79-88
3. Catalina MD, Sullivan JL, Brody RM, et al. Phenotypic and functional heterogeneity of EBV epitope-specific CD8+ T cells. J Immunol 2002;168: 4184-4191
4. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 2003;3: 361-370
5. Andersson J, Behbahani H, Lieberman J, et al. Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection. AIDS 1999;13: 1295-1303
6. Andersson J, Kinloch S, Sonnerborg A, et al. Low levels of perforin expression in CD8+ T lymphocyte granules in lymphoid tissue during acute human immunodeficiency virus type 1 infection. J Infect Dis 2002;185: 1355-1358
7. Zhang D, Shankar P, Xu Z, et al. Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. Blood 2003;101: 226-235
8. Lieberman J, Shankar P, Manjunath N, et al. Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. Blood 2001;98: 1667-1677
9. Weiss L, Donkova-Petrini V, Caccavelli L, et al. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. Blood 2004;104: 3249-3256
10. Kinter AL, Hennessey M, Bell A, et al. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. J Exp Med 2004;200: 331-343
11. Champagne P, Ogg GS, King AS, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature 2001;410: 106-111
12. Appay V, Dunbar PR, Callan M, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 2002;8: 379-385
13. Hislop AD, Gudgeon NH, Callan MF, et al. EBV-specific CD8+ T cell memory: relationships between epitope specificity, cell phenotype, and immediate effector function. J Immunol 2001;167: 2019-2029
14. Nikolova MH, Muhtarova MN, Taskov HB, et al. The CD160+ CD8high cytotoxic T cell subset correlates with response to HAART in HIV-1+ patients. Cell Immunol 2005;237: 96-105
15. Muhtarova MN, Nikolova M, Vezenkov L, et al. The co-stimulatory Molecule CD160 differentiates between HIV1+ patients with detectable and suppressed cytotoxic activity. Probl Inf Parasit Dis 2004;32: 20-22
16. NCCLS.2000. How to define and determine reference intervals in the clinical laboratory. Approved Guideline -second ed.NCCLS document C28-A2[ISBN 1-56238-406-6],NCCLS. PN 19087-1898 USA 2000;
17. Trimble LA, Shankar P, Patterson M, et al. Human immunodeficiency virus-specific circulating CD8 T lymphocytes have down-modulated CD3zeta and CD28, key signaling molecules for T-cell activation. J Virol 2000;74: 7320-7330
18. Anumanthan A, Bensussan A, Boumsell L, et al. Cloning of BY55, a novel Ig superfamily member expressed on NK cells, CTL, and intestinal intraepithelial lymphocytes. J Immunol 1998;161: 2780-2790
19. Wherry EJ, Teichgraber V, Becker TC, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol 2003;4: 225-234
20. Wherry EJ, Ahmed R. Memory CD8 T-cell differentiation during viral infection. J Virol 2004;78: 5535-5545
21. Katano H, Ali MA, Patera AC, et al. Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation. Blood 2004;103: 1244-1252
22. Haridas V, McCloskey TW, Pahwa R, et al. Discordant expression of perforin and granzyme A in total and HIV-specific CD8 T lymphocytes of HIV infected children and adolescents. AIDS 2003;17: 2313-2322
23. Chen G, Shankar P, Lange C, et al. CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. Blood 2001;98: 156-164
24. Papagno L, Spina CA, Marchant A, et al. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. PLoS Biol 2004;2: E20
25. Shankar P, Russo M, Harnisch B, et al. Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. Blood 2000;96: 3094-3101
26. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science 2003;300: 339-342
27. Grakoui A, Shoukry NH, Woollard DJ, et al. HCV persistence and immune evasion in the absence of memory T cell help. Science 2003;302: 659-662
28. Seder RA, Grabstein KH, Berzofsky JA, et al. Cytokine interactions in human immunodeficiency virus-infected individuals: roles of interleukin (IL)-2, IL-12, and IL-15. J Exp Med 1995;182: 1067-1077
29. Clerici M, Lucey DR, Berzofsky JA, et al. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. Science 1993;262: 1721-1724
30. Bright JJ, Kerr LD, Sriram S. TGF-beta inhibits IL-2-induced tyrosine phosphorylation and activation of Jak-1 and Stat 5 in T lymphocytes. J Immunol 1997;159: 175-183
31. Yu CR, Ortaldo JR, Curiel RE, et al. Role of a STAT binding site in the regulation of the human perforin promoter. J Immunol 1999;162: 2785-2790
32. Rushbrook SM, Ward SM, Unitt E, et al. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. J Virol 2005;79: 7852-7859

CLINICAL CASE OF TRICHINELLOSIS IN A SPLEENECTOMIZED PATIENT

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SUMMARY

A prolonged course of moderately severe form of trichinellosis was observed in a patient, with previous spleen and kidney ectomy. After etiological treatment with Zentel and prolonged pathogenic therapy, the patient went trough a half-year dispensary observation, until recovery. Peculiarities observed in clinical and laboratory constellations were described.

Key words: Helminthic diseases, Trichinellosis, Immune deficiency

Trichinellosis is a parasitic zooanthroponotic disease with environmental and synantropic focus of distribution, which is periodically registered in different regions of Bulgaria in the form of epidemic outbreaks and sporadic cases /2/. Various clinical forms of trichinellosis, which differ in severity, are distinguished - abortive, benign, moderately severe, severe and complicated form, that can lead to fatal outcome. Reconvalescence is a prolonged process, consistent of myalgia and muscle weakness, which can persist for months and years /1,3/. Regarding this, some pathogenic aspects of trichinellosis are still unclear. Immunodeficient conditions are a factor that complicates the clinical course of some parasitic diseases (including trichinellosis), or leads to reactivation of latent invasions /4/.

The aim of present work was to represent a case with prolonged course of trichinellosis in immunocompromised patient with concomitant chronic glomerular nephritis and past traumatical nephrectomy and spleenectomy.

CLINICAL CASE

The patient with initials B.H.R. is a 30 years old female, who had previous spleenectomy and nephrectomy at the age of 7, as a result of traumatic accident. At the age of 12 she was diagnosed with glomerular nephritis, and in 2001 arterial hypertension was found. In 2004, on clinical and laboratory examinations, anemia, micotic eczema and protein in urine were found. Currently she was put on dispensary observation in the Nephrology & Transplantation Clinic at the "Aleksandrovska Hospital". Her present hospital attendance was registered on 27.12.2004, in relation to a severe disease with one month' duration (high temperature 39-40 °C, diarrhea, troat and neck pain, lids' edema, eyes' irritation, and pain in eyes and muscles). She felt general fatigue, heartbeating, limbs weakness, and laboratory examinations revealed elevated leucocytes count (WBC count - 22 000 x 10^9 /l), elevated eosinophils (55 %), elevated Creatinine (148 mol/l). Her initial therapy included antibiotic and antipiretics, with no essential effect, which led to her further hospital attendance. During that period, for several days her husband also had temperature, general fatigue and muscle pain. The patient used to tasting and eating raw minced meat (including pork), while cooking her home-made meals.

On her hospital attendance, the patient was afebrile, with no present edemas, but complained of general fatigue and pains in extremitas, neck and shoulder region. She tired quickly after walking and a digital tremor in hands was present. Respiratory and cardio-vascular systems were free of pathological findings, arterial blood pressure was 130/90 mmHg, liver was not enlarged, and no edemas were present.

Laboratory examinations: ESR - 23 mm/h, WBC - 30 to 13,5 x10^9/ l, Eosinophils - 67 % to 25 %, AST (SGOT) - 30 U/l, ALT (SGPT) - 71 U/l, CPK - 62, Serum Creatinine - 172 mol/l, Urine examination: proteinuria 1,68 g/24 hours, sterile uroculture.

Instrumental examinations:

Abdominal ultrasonography: Right kidney with thickened parenchime structure and unclear margins between parenchyma and pyelon.

Thorax X-ray: deformed bronchial tree lines

Electrocardiogram: Left ventricular hypertrophy.

Electromyography: In some muscular regions of the arm and lower extremities miogenic changes were found. In legs, especially in thigh musculature, anterior-radix pathology was present.

Consultations: Cardiologist: no data for myocarditis; Haematologist: no data for haematological disease, Oftalmologist: Hypertonical retinal angiosclerosis; Parasitologist: according to previous anamnestic history and from clinical data: moderately severe form of Trichinellosis. Confirmation for this conclusions were the following data: outset of the disease had been a diarrheal syndrome, which was followed by fever and oedema, myalgia, fatigue, tachicardia and laboratory confirmed leucocytosis, high blood eosinophilia, elevated liver enzymes, elevated ESR, present electromiography data of muscular damage, although serological testing for trichinellosis in ELISA was negative.

CONCLUSIONS

The patient leading symptoms were from trichinellosis as intercurrent disease, and they prevaled over manifestations from chronical glomerular nephritis in her sole right kidney (protein was found in urine and elevated serum creatinine was present).

Trichinellosis was treated with albendazole /Zentel/, tablets of 200 mg in dose of 15 mg/kg for 14 days. Pathogenic treatment was conducted with Clemastin, Calcium-vit.C, Piroxicam and bed-regime. Patient's condition improved - muscle pains disappeared, appetite was restored, WBC count dropped to 13,5 x10^9 /l, and eosinophils dropped to 24 %. Dexydrocortisone treatment for kidney disease was post-pointed for a later period after regression of the severe phase of trichinellosis, because of its immunosuppressive action. The patient proceeded to conduct pathogenic therapy at home, and followed free of physical pressure regime.

She attended to the hospital again on 24.02.2005 /stayed to 16th of March/, with complaints on frequent and painfull urination with stinging, and ambulatory examinations revealed bacteria in her urine. She was still having muscle weakness, but without pain, with slight tremor in her hand fingers. Clinically transitient tachicardia was present (up

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ABBREVIATIONS USED IN THIS PAPER:

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to 110 beats / minute). Laboratory data from her 2 nd hospital attendance: ESR 14-12 mm/h; WBC 15,7 - 13,7 x 109 / l; Eosinophils 35% - 18 %, ASAT 17 U/l, ALAT 19 U/l, Creatinine 143-125 mol/l; Protein in urine up to 0,8 g/l; Uroculture - sterile. Pathogenic treatment for kidney disorder was conducted with Urbason - initial dose of 60 mg and subsequent reducing of daily dose.

In the following months during ambulatory observation, there was a tendency for eosinophils drop - they reached normal values of 4 % on 05.06.2005.

In July 2005, after sun exhibition at a seaside resort, she felt fatigue - as "muscle fever", and laboratory examinations discovered elevated WBC - 14,1 x 109 /l and elevated Eosinophils - 36 %.

She attended to the hospital for the 3 rd time on 23.08.2005, because of increasing proteinuria, edemas and bacteriuria, which was treated with Augmentin. Laboratory data showed: ESR - 12 mm/h, WBC - 14,0 - 17 x 109/l; eosinophils - 35 % - 1%, Creatinine 144 - 159 mol/l, CPK - 36 U/l, ASAT 17 U/l, ALAT -14 U/l. Her serology testing for trichinellosis was again negative. She was consulted with haematologist on 26.08.2005, who concluded that there was no data for systemic haematological disease. Treatment for kidney disorder with Methyl-prednisolon was initiated, and eosinophil count dropped to 1%. Protein in urine and urinary infection subsided. She left the hospital with recommendations to attend regularly to control examinations and laboratory testing, during her dispensary observation period.

DISSCUSSION

It is known that trichinellosis can suppress the specific immune and nonspecific inflammatory reactions, which can lead to secondary infections (pneumonia, urinary infection and others). In immunocompromised patients we had observed a more dramatic course of the disease - a severe course of trichinellosis in a diabetic patient, and a lethal outcome of trichinellosis in a patient with leucemia /5/.

The represented clinical case of concomitant spleenectomy and chronic glomerular nephritis, was immunomodulated towards humoral and cell immunity. This can explain the peculiarities in the clinical course of trichinellosis in the described case with concomitant disease:

1. Hypereosinophilia - which reached 67 %, during the acute stage of moderately severe form of trichinellosis.
2. Secondary bacterial infection - cystitis.
3. Prolonged period of elevated counts of WBC and eosinophils.
4. Prolonged period of clinical reconvalescence /over 6 months/.
5. Negative results from ELISA testing for trichinellosis.
6. Worsening of glomerular nephritis.
7. Worsening of myositic syndrome and eosinophils elevation, after continuous sun exposure.
8. Possible invasion with Trichinella pseudospiralis (seronegative, prolonged myosytis, restricted consumption of pork due to religious reasons, though muscle biopsy was not performed).

Some famous relationships between immune deficiency and parasitic invasion reveal that:

* Accomplishment of full biological cycle of the parasite is facilitated, especially in those protozoal and helminth agents, which can perform their whole cycle in humans, including T. spiralis.
* Host's parasites population is intensively increasing. Growing intensity of invasion leads to complicated clinical symptoms.
* Parasite life in the host is prolonged, and so the parasitic disease is prolonged.
* Reactivation of latent invasion in relation to immune deficiency is possible.

These peculiarities have to be considered in the diagnostication of a parasitic invasion in immunocompromised patients and adequate therapeutic approach has to be followed.

Dispensary observation with periodical clinical and laboratory control examinations in these patients, have to be performed until their final sanation.

REFERENCES:

1. D. Vutchev: Late clinical consequences in trichinellosis. Hygiene and Health Protection, 2000, 2, 44-48 p. /in Bulgarian/
2. R. Kurdova et al.: Indigenous and imported parasitic diseases in Bulgaria and measures for their surveillance and control /2002/. Informational Journal of NCIPD, 2003, 5, 5-29 p.
3. Pawlowski, Z. Clinical aspects in man in Trichinella and Trichinellosis, ed. W.Campell. Plenum press - New York. 1986, 367-401.
4. Clinical Approach to Infection in the Compromised Host. Eds.R.Rubin, L. Young. Kluwer Acad. Plenum Publishers. New york, 2002, 265-334.
5. Jacobson. E., H. Jacobson. Trichinosis in an immunosupressed human host. - Am.J.Clin. Pathol., 68, 791-794.

ANTICESTODAL ACTIVITY OF NEW BENZIMIDAZOLE DERIVATIVES

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SUMMARY

Two chemicals containing a condensed thiazole nucleus in their structure, are investigated in the present study. The compounds are tested in vitro on isolated scolices of Echinococcus granulosus in concentrations from 5 to 320 mg/ml and in vivo on laboratory animals - white mice infected with Hymenolepis nana in dosage 20 mg/ml. One of the benzimidazoles, shown prominent activity is subject to additional investigations.

Key words: echinococcosis, chemotherapy, benzimidazoles

Research of new chemicals for therapy of cestodoses is more limited than investigation of compounds with antinematodal activity. In the past, medical plants as extractum filicis maris aethereum, pomegranate, pumpkin seeds were applied as anticestodal drugs. Even after the synthesis and brought into use of effective antihelminthics such as niclosamide and prasiquantele the problem for radical therapy of cestodoses still exists. For example hymenolepidosis - a parasitosis caused by largely distributed taenia Hymenolepis nana, is difficult for treatment and the larval stage of the cestode Echinococcus granulosus is treated mainly surgically with frequently occurring relapses. /1,2/ Mebendazole and albendazole are the most often prescribed benzimidazoles with antihelminthic activity which explains the assessment of newly synthesised benzimidazoles for the treatment of cestodoses. /3,4,5/

The aim of the present study was to investigate newly synthesized benzimidasole derivatives for anticestodal activity. Their efficacy was experimentally explored on scoleces of Echinococcus granulosus (in vitro activity) and on laboratory mice naturally infected with Hymenolepis nana (in vivo activity).

MATERIAL

Two compounds designated Bz I and Bz II were selected among structural analogues of well known chemicals with proven antiparasitic efficacy on intestinal and tissue nematodes. Both contained in their structure a condensed thiazole nucleus and were diluted in DMSO in different concentrations. (Fig.1)

Scoleces from hydatid cyst of a patient with hepatic echinococcosis were used to evaluate the in vitro activity of the compounds. The cyst was obtained by operation and contained vital scoleces and daughter cysts.

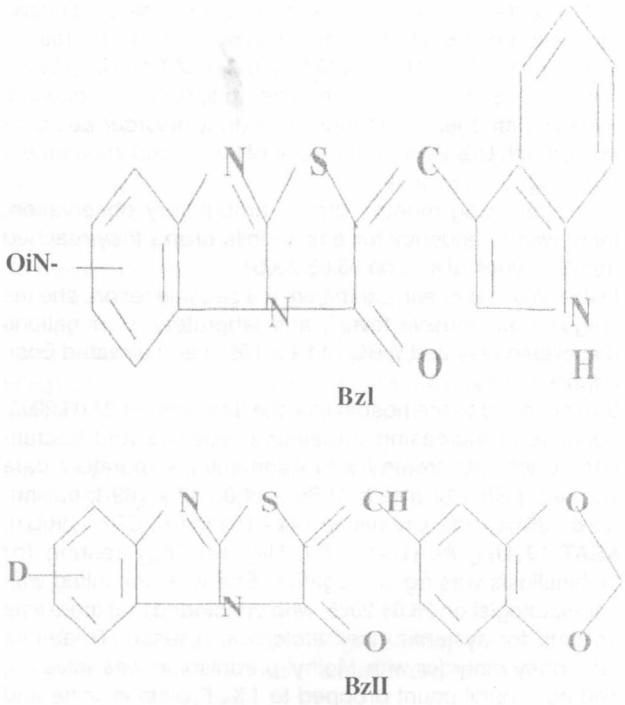

Figure 1. Structure of benzimidazole derivatives Bz I and Bz II

The in vivo activity was estimated on white mice (about 20g in weight), naturally infected with Hymenolepis nana. They were divided in tree groups: 10 mice treated with the benzimidazole derivatives; 3 mice handled only with DMSO; 3 mice left without treatment.

METHODS

Assessment of in vitro activity

For estimation of in vitro activity, Bz I and Bz II were diluted in DMSO in different concentrations - 5, 10, 20, 40, 80, 160, 320 mg/L. Vital scoleces of Echinococcus granulosus were isolated by centrifugation of the cystic fluid in PBS three times at 1500 g for 10 min. Scoleces in the sediment were incubated with the different concentrations of Bz I and Bz II at 37°C overnight in wet camera. After the incubation, vitality of the scoleces was assessed according to the method of Nakanishi. Degenerative changes were estimated in 100 scoleces.

Assessment of in vivo activity

The mice infected with H.nana were divided in three groups: group I -mice treated with Bz I or Bz II in dosage 20 mg/kg body weight, once a day for 3 days; group II - mice treated only with the solvent DMSO - 0,25 ml once a day for 3 days; group III - mice left without treatment. Microscopic examination of the feces was carried out after five days in three consecutive days. Partial autopsy was accomplished on the small intestines - the site of localization of H.nana (the adult form). The lumen was examined stereomicroscopicaly for the cestode.

RASULTS AND DISCUSSION

When Bz I and Bz II were assessed for in vitro activity on scoleces of E.granulosus, the first compound (Bz I) revealed degenerative changes in 25% of the scoleces when given in concentrations 40-320 mg/1. In the lower concentrations the derivative showed no effectiveness. The second chemical (Bz II) showed more prominent activity - 75% devitalization of the scoleces in concentration 5mg/ml up to 100% effectiveness in concentration 320 mg/ml.

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ABBREVIATIONS USED IN THIS PAPER:

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The evaluation of in vivo activity of benzimidazole derivatives showed lack of effectiveness on the adult form of H.nana. Eggs of the cestode were detected in all mice from the three control groups. Two mice from each control group were dissected and examined for adult forms in the small intestines. H.nana was observed in all of them.

As the result of conducted experiment, in vitro activity on isolated scoleces of E.granulosus was observed for both compounds but it was more prominent for Bz II reaching 100% in high concentrations. These results gave reasons for further examinations of their activity on laboratory animals - in vivo experiment. The second experiment revealed that tested benzimidazoles had no effectiveness against the adult stage of H.nana.

Research of more perspective benzimidazole (Bz II) are anticipated in higher doses on larger number of laboratory animals.

REFERENCES

1. Boeva, W., G.Genov, S.Nikiforov. Echinococcosis. Medicina and phiscultura, 1983, 127-128./in Bulgarian/
2. Krotov, A. Basic experimental therapy of helminthoses. Medicina, Moscva. 1973, 272, 215-216./in Russian/
3. Campbell, E.D. Chemotherapy of parasitic diseases. R.Rew.Pleum.Press. New York, 1986, 655.
4. Davis, A., H.Dixon, Z.Pawlowski. Multicenter clinical trials of benzimidazolecarbamates in human cystic echinococcosis (phase 2). Bull.WHO 1989,67,503-508.
5. Horton, R. Chemotherapy of Echinococcus infection in man with albendazole. Trans.Royal Soc.Trop.Med.Hyg., 1989, 85, 97-102.

STOOL ANTIGEN TEST FOR DIAGNOSIS OF HELICOBACTER PYLORI INFECTION

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SUMMARY

Helicobacter pylori is now generally accepted as the main etiological agent in chronic active gastritis and peptic ulce and risk factor for the development of gastric cancer. The two major categories of diagnostic methods for diagnosis are invasive tests, or those that require endoscopy, and noninvasive, or nonendoscopic tests. The aim of this study was to systematically review the experience on H. pylori stool immuno assay antigen tests as a relatively new noninvasive diagnostic test. The review was intended to evaluate the usefulness of different stool antigen tests in the pretreatment diagnosis of H. pylori infection and after eradication therapy; in adult and in pediatric patients; and factors which affect the accuracy of the stool antigen tests.

Key words: Helicobacter pylori, stool antigen test, diagnosis

Helicobacter pylori is a small, curved, highly motile, Gram negative bacillus that colonises only the mucus layer of the human stomach. Since its discovery in 1984, it has been recognized as the principal cause of peptic ulcer disease and as the main risk factor for the development of gastric cancer. H pylori is one of the commonest bacterial pathogens in humans, however, most infected people (> 70%) are asymptomatic (1)

Main diagnostic methods for detection of H. pylori infection Invasive and non-invasive methods may be used to diagnose H. pylori infection. Upper esophagogastroduodenal endoscopy is considered the reference method of diagnosis. During endoscopy, biopsy specimens of the stomach and duodenum are obtained and after that the diagnosis of H. pylori can be made by the biopsy urease test (a colorimetric test based on the ability of H. pylori to produce urease; it provides reading of results 20 min to 1-3 hours after inoculation), histological identification of organisms and culture of biopsy specimens for H. pylori, which requires an experienced laboratory and is necessary when antimicrobial susceptibility testing is desired.

Non- invasive tests are serological tests that measure specific H. pylori IgG antibodies can determine if a person has been infected. Circulating IgG antibodies to H. pylori can be detected by enzyme linked immunosorbent assay (ELISA) antibody test. These tests are generally simple, reproducible, inexpensive, and can be done on stored samples. They have been used widely in epidemiological studies, including retrospective studies to determine the prevalence or incidence of infection. Current serological tests are unable to distinguish active from past infection. Another diagnostic method is the breath test. In this test, the patient is given either 13C-or 14C-labeled urea to drink. H. pylori metabolizes the urea rapidly, and the labeled carbon is absorbed and measured as CO_2 in the patient's expired breath to determine whether H. pylori is present. Faecal antigen tests (a simple sandwich ELISA) can be used to detect the presence of H pylori antigens shed in the faeces. These new tests have sensitivities and specificities similar to those of the 13C- urea breath test. Non-invasive tests are useful for primary diagnosis, when a treatment indication already exists, or to monitor treatment success or failure. They are also useful in patients who cannot tolerate endoscopy, children, and in epidemiological population studies. In 2000, a consensus report stated that two non-invasive tests, urea- breath test and stool antigen test, could be used both safely and cost effectively to screen for H pylori positive patients (below the age of 45) without alarm symptoms (2). Table 1 presents a comparison between different diagnosting tests (sensitivity and specificity), according to R. P. H. Logan and M. M. Walker, BMJ, 2001 (1)

Well-established noninvasive tests are serology and the 13C- urea breath test. Serology, however, is not suitable for an early follow-up examination due to the slow reduction of the anti-H. pylori antibody titer after successful treatment and urea breath test needs expensive instrumentation and a specialized technician for it. Since infected individuals excrete H. pylori in stool specimens (3, 4, 5, 6), a sufficiently accurate test using faeces would be an important alternative to 13°C-urea breath test. Stool antigen test is determined as rapid, highly sensitive (90%) and specific before therapy, convenient to the patient and acceptable for monitoring eradication, cost is lower than that of 13°C-urea breath test. At present, new data are available with respect to the performance of this new test.

This review was intended to evaluate the usefulness of different stool antigen tests in the pretreatment diagnosis of H.pylori infection, after eradication therapy in adult and in pediatric patients.

Stool antigen test. Stool antigen ELISA H. pylori test represents an accurate and relatively novel non-invasive concept for diagnosis of infection and can be used for daily routine in clinical practice. Some of the stool antigen tests are polyclonal antibody based stool enzyme immunoassay. For example the Premier Platinum Hp SA, has been evaluated extensively. In 2001, a meta analysis of 4769 untreated patients in 43 separate studies using the Premier Platinum Hp SA kit showed a mean sensitivity of 92.1% and 91.9% for specificity (7).

FemtoLab Cnx is another stool antigen ELISA test based on monoclonal antibodies to detect H.pylori antigen in faeces that has been tested principally in initial when compared with urea breath test and serology (8). Dia.Pro Hp Ag is also an ELISA kit that uses monoclonal antibodies, which are affinity purified (9). These three stool antigen enzyme immunoassay kits (Premier Platinum Hp SA, FemtoLab Cnx, and Hp Ag) have been compared with biopsy based methods for the detection of H pylori in previously undiagnosed patients in the study of J. Andrews et al, 2003 (9). One hundred and eleven adults with dyspepsia referred for endoscopy provided a stool sample for testing and had biopsies taken. Patients were considered H pylori positive if two out of three invasive tests were positive or if culture alone was positive. The sensitivities and specificities of the Premier Platinum Hp SA, FemtoLab Cnx, and Hp Ag stool antigen kits when compared with biopsy based diagnosis were, 63.6%, 88.0%, and 56.0% and 92.6%, 97.6%, and 97.6%, respectively. The most promising kit appeared to be FemtoLab Cnx, which had a sensitivity of 88.0% and a specificity of 97.6% when tested on the 72 patients who had not received medications in the preceding four weeks .Both the Premier Platinum Hp SA and HpAg had lower sensitivities of 63.6% and 56.0%, respectively, and similar specificities of 92.6% and 97.6%, respectively, in the 72 patients not on medication predictive values. FemtoLab Cnx has a high negative

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ABBREVIATIONS USED IN THIS PAPER: ELISA - enzyme linked immunosorbent assay

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Table 1. Comparative sensitivity and specificity of tests for H pylori infection, according to R. P. H. Logan and M. M. Walker, BMJ, 2001

Test	Sensitivity	Specificity
Invasive		
Histology	88-95%	90-95%
Culture	80-90%	95-100%
Urease test	90-95%	90-95%
Non-invasive		
^{13}C-UBT	90-95%	90-95%
^{14}C-UBT	86-95%	86-95%
Serology		
ELISA	80-95%	80-95%
Stool Ag	90-95%	90-95%

UBT - urea breath test

predictive value, suggesting that it might be useful in screening out uninfected patients. In the similar study with three different stool antigen tests Veijola L, et al, 2005 demonstrated that the performance of all three stool antigen tests in the post-treatment setting was slightly inferior to that of the urea breath test and serology, with monoclonal antibody-based tests showing better results (10).

ImmunoCard-STAT HpSA is a new rapid simple noninvasive immunoassay, based on a lateral flow chromatography technique, for the qualitative detection of Helicobacter pylori antigens in human stool. The test is based on a proprietary monoclonal antibody; it is one-step procedure and gives the result in only 5 minutes, with accuracy comparable to the Premier Platinum HpSA. The single test format provides new flexibility to the Helicobacter pylori stool antigen testing. It is the convenient choice when testing a limited number of specimens and a simpler procedure is desired (11, 12, 13).

Initial (pretreatment) diagnosis of H. pylori infection. The stool antigen test has been widely studied in the initial (pretreatment) diagnosis of H. pylori infection). The largest European study reported to date included 501 patients undergoing endoscopy and a 13C urea breath test (14). According to the gold standard, patients are considered positive if they have only the culture or two of the three diagnostic tests positive. In this study patients were considered positive if they had a positive result on rapid urease testing and histologic examination or a positive culture for H. pylori. The stool test had a high sensitivity and specificity compared with the urea breath test. Studies from the United States have had similar results (15). In his systematic review up to May 2003 Gisbert JP et al, 2004, presents data about the diagnostic accuracy of the Helicobacter pylori stool antigen test on the basis of monoclonal ELISA tests (16). Mean sensitivity, specificity, positive predictive value and negative predictive value were 91%, 93%, 92% and 87%, respectively. Analysis of the eight studies (1399 patients) in which pretreatment evaluation of the monoclonal stool antigen test was performed showed better ($p < 0.001$) results (96%, 97%, 96% and 97%, respectively), with a clearer distinction between positive and negative results.

Post-treatment diagnosis of H. pylori infection In the post-therapy setting, a multicenter European trial evaluated the sensitivity and specificity of the stool test and the urea breath test (compared with multiple endoscopic tests as the gold standard), 4 weeks after eradication therapy in 235 patients (17). The gold standard for comparisons in that study was histologic examination plus culture. The sensitivity of the stool test was 95.6% with confidence interval 89.6% to 100%), and the specificity was 94.7 with confidence interval 91.5% to 97.9%. A smaller (142 patients) single-center study reported poorer results, with a sensitivity of 70%, confidence interval 50% to 86%) and a specificity of 82%, confidence interval 75% to 89%, 6 weeks after completion of eradication therapy (18). In that study, a single test (urea breath test) was used as the gold standard for eradication. The choice of gold-standard test greatly affects the results of noninvasive testing for H. pylori (19). In the review of the literature about post-treatment studies Gisbert JP et al, 2004 presents thirty-nine studies (3147 patients) evaluated the stool antigen test for the confirmation of H. pylori eradication 4-8 weeks after therapy, with accuracies of 86%, 92%, 76% and 93% for mean sensitivity, specificity, positive predictive value and negative predictive value, respectively (16). Results were similar when a gold standard based on at least two methods was used. Relatively low accuracy was reported in some post treatment studies with the polyclonal stool antigen test. However, excellent results ($p < 0.001$) were achieved in all the six studies evaluating the monoclonal stool antigen test 4-8 weeks post treatment. Results evaluating the stool antigen test < 4 weeks post treatment are contradictory.

4. Evaluation of a stool antigen test in children

Helicobacter pylori infection is usually acquired in early childhood. Noninvasive methods for detection of H. pylori are required to study its incidence, transmission, and clearance of infection in childhood. They should be easy to perform, inexpensive, and have a high diagnostic accuracy, especially in infants and toddlers. Both serology and the 13C-urea breath test do not fulfill all these requirement. Children and teenagers has been tested in some developed countries with stool antigen test, showing a sensitivity and specificity above 90%, however, its accuracy in developing countries and in children under 6 years is not well established. Some authors present data about a monoclonal stool antigen EIA as excellent in diagnosing H. pylori infection in symptomatic children with accuracy independent of the laboratory, production lot used, or the child's age. But because of small number of children <6 years of age infected with H. pylori, further validation of the test is recommended for young infected children (20).

In symptomatic children, the H. pylori stool antigen test (HpSA) test seems suitable to monitor the success of anti-H. pylori therapy (21). Sabbi T et al, 2005 concluded that among noninvasive and easily applicable tests, particularly in small children, H. pylori stool antigen test is simple, suitable, and has high accuracy for the screening of H pylori-positive patients. The H. pylori faecal antigen revealed a sensitivity of 97%, a specificity of 98%, and a positive predictive value of 97% (22). Using a novel rapid monoclonal enzyme immunoassay stool antigen for Helicobacter pylori detection (Immunocard STAT!HpSA the highest performance was observed in children older that 10 years, with a sensitivity level of 100%, contrasting with a lower level, 75%, in those younger than 5 years. The overall sensitivity, specificity, and positive and negative predictive values were 86.2%, 92.9%, 78.1%, and 95.8%, respectively, with an accuracy of 91.4%. A good negative predictive value was observed in all age groups, particularly in older children achieving 100% (23)

Some authors concluded that only when increasing numbers of patients were tested and separated into subgroups by age it became apparent that the accuracy of most tests is lower in young children if the same cut-off values are used as established for older children or adults. Therefore, statements such as "a test has been validated with good results in children" must be interpreted with caution, unless different age groups are considered with sufficient numbers of infected and non-infected children in each age group (24).

According to Canadian Helicobacter Study Group Consensus Conference, 2005 (25) the 13°C-urea breath test is

currently the best noninvasive diagnostic test for H. pylori infection in children; there is currently insufficient evidence to recommend stool antigen tests as acceptable diagnostic tools for H. pylori infection; serological antibody tests are not recommended as diagnostic tools for H. pylori infection in children. According to Megraud F. et al, 2005. The urea breath test appears to be an excellent test for diagnosis of H. pylori infection for children and adolescents. Comparing the current non-invasive tests for Helicobacter pylori infection in children and adolescents, the 13C-urea breath test had the best sensitivity in all age groups, followed by serology, stool test, and antibody detection in urine. A trend for better sensitivity with an increase in age was observed except for the stool test (26). Raguza D. et al concluded that the test is efficient in adolescents and children; however there is a need for further studies with a greater number of patients for evaluation of its accuracy in infants (27). Factors which affect the accuracy of the stool antigen tests Some factors exists which can affect the results obtained with faecal antigen tests. The age of the patient is one of them ant it was discussed in the previous section about the children.

An inadequate storage of the specimens may explain the poor results of the stool test. Storage of stools at -20°C instead of -70°C may have contributed to the lower sensitivities of the kits in some studies when compared with other published results. However, most of the other studies stored faeces at -20°C before testing, and this is the recommended temperature in the kit inserts (9, 26).

Proton-pump inhibitors seem to affect the accuracy of the stool antigen test (16)

Although most studies showed that stool antigen test is an accurate method to confirm H. pylori eradication more 4 weeks after treatment, these favorable results were not confirmed in some studies (7). Further investigation is necessary to explain these discrepancies, as well as to clarify the precise time for confirmation of eradication after therapy, the appropriate cut-off point for the stool antigen test, and which factors influence it. Proton pump inhibitors seem to affect the accuracy of stool antigen test, but the negative effect disappears 1-2 weeks after stopping treatment. Finally, the stool antigen test seems to be a highly cost-effective method for the diagnosis of H. pylori infection (7, 16).

The stool antigen test is technically feasible in patients with upper gastro-intestinal bleeding although the true diagnostic accuracy in this group of patients remains to be more fully assessed. Neither the polyclonal enzyme-linked immunosorbent assay stool antigen test nor the rapid immunochromatographic stool antigen test can be recommended to diagnose H. pylori infection in patients with upper gastrointestinal bleeding. However, the monoclonal enzyme-linked immunosorbent assay stool antigen test is highly sensitive for detecting the infection in patients with this complication, although more studies are necessary to evaluate the specificity of the method (28).

As the interest in Helicobacter pylori has not declined in these years, it is clear that improved methods for the diagnosis and follow up treatment of the infection have been developed in the last decades. Stool antigen test is noninvasive test, which also is accurate, simple and cost effective. Therefore, the stool antigen test is potential diagnostic tool to be employed in many different clinical settings from epidemiological studies to paediatric investigation, from pre-endoscopic screening strategies to post-monitoring.

REFERENCES

1. Robert P H Logan, Marjorie M Walker. ABC of the upper gastrointestinal tract Epidemiology and diagnosis of Helicobacter pylori infection. BMJ 2001; 323:920-922.
2. Malfertheiner P, Megraud F, O'Morain C, et al. Current concepts in the management of Helicobacter pylori infection-the Maastrict 2-2000 consensus report. Aliment Pharmacol Ther 2000; 2:167-80.
3. Kelly, S., M. Pitcher, S. Farmery, and G. Gibson. 1994. Isolation of Helicobacter pylori from feces of patients with dyspepsia in the United Kingdom. Gastroenterology 107:1671-1674.
4. Mapstone, N. P., D. A. F. Lynch, F. A. Lewis, A. T. R. Axon, D. S. Tompkins, M. F. Dixon, and P. Quirke. 1993. PCR identification of Helicobacter pylori in faeces from gastritis patients. Lancet 341:447.
5. Namavar, F., R. Roosendaal, E. J. Kuipers, P. de Groot, M. W. van der Bijl, A. S. Pena, and J. de Graaff. 1995. Presence of Helicobacter pylori in the oral cavity, oesophagus, stomach and faeces of patients with gastritis. Eur. J. Clin. Microbiol. Infect. Dis. 14:234-237.
6 Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver. 1992. Isolation of Helicobacter pylori from human faeces. Lancet 340:1194-1195.
7. Gisbert JP, Pajares JM. Diagnosis of Helicobacter pylori infection by stool antigen determination: a systematic review. Am J Gastroenterol 2001; 96:2829-38.
8. Makristathis A, Barousch W, Pasching, et al. Two enzyme immunoassays and PCR for the detection of Helicobacter pylori in stool specimens from paediatric patients before and after eradication therapy. J Clin Microbiol 2000;38:3710-14.
9. J Andrews, B Marsden, D Brown, V S Wong, E Wood, M Kelsey. Comparison of three stool antigen tests for Helicobacter pylori detection. J Clin Pathol 2003; 56:769-771
10. Veijola L, Oksanen A, Lofgren T, Sipponen P, Karvonen AL, Rautelin H.Comparison of three stool antigen tests in confirming Helicobacter pylori eradication in adults. Scand J Gastroenterol. 2005; 40:395-401
11. Trevisani L, S. Sartori, M. R. Rossi, M. Ruina, V. Matarese, S. Gullini & V. Abbasciano. Evaluation of a new rapid immunoassay for the detection of Helicobacter pylori in faeces: a prospective pilot study Alimentary Pharmacology & Therapeutics 2005; 21: 485.
12. Wu IC, Ke HL, Lo YC, Yang YC, Chuang CH, Yu FJ, Lee YC, Jan CM, Wang WM, Wu DC. Evaluation of a newly developed office-based stool test for detecting Helicobacter pylori: an extensive pilot study. Hepatogastroenterology 2003; 50:1761-5.
13. Li YH, Guo H, Zhang PB, Zhao XY, Da SP. Clinical value of Helicobacter pylori stool antigen test, ImmunoCard STAT HpSA, for detecting H pylori infection World J Gastroenterol. 2004; 10:913-4.
14. Vaira D, Malfertheiner P, Megraud F, Axon AT, Deltenre M, Hirschl AM, et al. Diagnosis of Helicobacter pylori infection with a new non-invasive antigen-based assay. HpSA European study group. Lancet. 1999; 354:30-3.
15. Vakil N, Affi A, Robinson J, Sundaram M, Phadnis S. Prospective blinded trial of a fecal antigen test for the detection of Helicobacter pylori infection. Am J Gastroenterol. 2000; 95:1699-701.
16. Gisbert JP, Pajares JM. Stool antigen test for the diagnosis of Helicobacter pylori infection: Helicobacter. 2004; 9:347-68.
17. Vaira D, Malfertheiner P, Megraud F, Axon AT, Deltenre M, Gasbarrini G, et al. Noninvasive antigen-based assay for assessing Helicobacter pylori eradication: a European multicenter study. The European Helicobacter pylori HpSA Study Group. Am J Gastroenterol. 2000; 95:925-9.
18. Forne M, Domynguez J, Fernandez-Banares F, Lite J, Esteve M, Galy N, et al. Accuracy of an enzyme immunoassay for the detection of Helicobacter pylori in stool specimens in the diagnosis of infection and post treatment check-up. Am J Gastroenterol. 2000; 95:2200-5.
19. Vaira D, Vakil N. Blood, urine, stool, breath, money, and Helicobacter pylori. Gut. 2001; 48:287-9.
20. Koletzko S, Konstantopoulos N, Bosman D, Feydt-Schmidt A, van der Ende A, Kalach N, Raymond J, Russmann H. Evaluation of a novel monoclonal enzyme immunoassay for detection of Helicobacter pylori antigen in stool from children. Gut. 2003; 52:804-6.
21. Konstantopoulos N, Russmann H, Tasch C, Sauerwald T, Demmelmair H, Autenrieth I, Koletzko Ss. Evaluation of the Helicobacter pylori stool antigen test (HpSA) for detection of Helicobacter pylori infection in children. Am J Gastroenterol. 2001; 96:677-83.
22. Sabbi T, De Angelis P, Colistro F, DalliьOglio L, di Abriola GF, Castro M. Efficacy of noninvasive tests in the diagnosis of Helicobacter pylori infection in pediatric patients. Arch Pediatr Adolesc Med 2005; 159:238-41.
23. Kalach N, Nguyen VB, Bergeret M, Boutros N, Dupont C, Raymond J. Usefulness and influence of age of a novel rapid monoclonal enzyme immunoassay stool antigen for the diagnosis of Helicobacter pylori infection in children. Diagn Microbiol Infect Dis. 2005; 52:157-160.
24. Koletzko S. Feydt-Schmidt A. Infants differ from teenagers: use of noninvasive tests for detection of Helicobacter pylori infection in children. Eur J Gastroenterol Hepatol. 2001; 13: 1047-52.
25. Jones NL, Sherman P, Fallone CA, Flook N, Smaill F, van Zanten SV, Hunt R, Thomson A. Canadian Helicobacter Study Group Consensus Conference: Update on the approach to Helicobacter pylori infection in children and adolescents - An evidence-based evaluation.Can J Gastroenterol. 2005; 19:399-408.
26. Megraud F; European Paediatric Task Force on Helicobacter pylori. Comparison of non-invasive tests to detect Helicobacter pylori infection in children and adolescents: results of a multicenter European study. J Pediatr. 2005; 146:198-203.
27. Raguza D, Granato CF, Kawakami E. Evaluation of the stool antigen test for Helicobacter pylori in children and adolescents. Dig Dis Sci. 2005; 50:453-7.
28. Gisbert JP, Trapero M, Calvet X, Mendoza J, Quesada M, Guell M, Pajares JM. Evaluation of three different tests for the detection of stool antigens to diagnose Helicobacter pylori infection in patients with upper gastrointestinal bleeding. Aliment Pharmacol Ther Ther. 2004; 19:923-9.

FOUR CASES OF IMPORTED BRUCELLOSIS IN BULGARIA

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SUMMARY

Brucellosis is a typical zoonosis infecting domestic animals and frequently humans occupationally linked to them. Isolated cases of imported infection have been observed in Bulgaria though this disease was considered to be eliminated since 1974. The social and economic changes and migration of the population in the last decade make brucellosis current issue in our country. The aim of our study is to make a clinical and epidemiological surveillance of four cases of imported brucellosis from Greece and to display the risk of employment in enzootic regions. The patients are with manifested brucellosis appearing as systemic infection with multiorgan location. The diagnosis was proved by complex epidemiological and routine clinical, laboratory and serological methods. In conclusion familiarity with manifestations of brucellosis, the optimal laboratory techniques for its diagnosis and preliminary health education for the people going to work in risk endemic regions could help physicians protect the public against this reemerging, under-recognized zoonosis.

Key words: brucellosis, systemic infection, imported infection

INTRODUCTION

Brucellosis is zoonosis of domestic animals (goats, sheep, cows and pigs) and is most commonly involving humans occupationally linked to them (6, 7, 9, 10). It is an important public health problem throughout the world. It is one of the most widely seen infections and nearly half a million cases are declared annually (8). Endemic infections occur especially in the Mediterranean, Middle East, Latin America and Asia (8). Yet Hippocrates described cases with lasting fevers among population of Aegean Sea islands (Thasos). Nowadays the prevalence of the disease in the islands and continental part of Greece is high among animals and humans (11). In Bulgaria the first case of human brucellosis was found in 1903 in the city of Lom (1, 2). Till 1974 twenty cases have been declared and after that the infection was considered eliminated. That is why the essential risk of brucella transmission is its import from Greece by the Bulgarians working in the Greek cattle farms. The risk occupations in the endemic regions are stockmen, abattoir workers, veterinarians, etc. Transmission of brucellosis to humans occurs through the consumption of infected, unpasteurized animal-milk products, through direct contact with infected animal parts (such as the placenta by inoculation through ruptures of skin and mucous membranes), and through the inhalation of infected aerosolized particles (4, 5). Human brucellosis is traditionally described as a disease of protean manifestations. However, fever is invariable and can be spiking and accompanied by rigors, if bacteremia is present, or may be relapsing, mild, or protracted (11). It is accompanied by malodorous perspiration, especially at night, anorexia, asthenia, headache, arthralgia, and weight loss. Treatment of human brucellosis is long and tough. In 1986, the World Health Organization issued guidelines for the treatment of human brucellosis. The guidelines discuss two regimens, both using doxycycline for a period of six weeks, in combination with either rifampin for six weeks or streptomycin for two to three weeks (12, 13).

The aim of our study is to make a clinical and epidemiological surveillance of four cases with imported brucellosis from Greece and to reveal the risk of working in enzootic foci.

MATERIAL AND METHODS

A clinical and epidemiological surveillance of four patients with imported brucellosis was made at the Clinic of Infectious Diseases in Plovdiv, Bulgaria for the period from March, 2005 to April, 2006. For the diagnosis "brucellosis" complex epidemiologic and routine clinical and laboratory methods were used. The microbiological confirmation of diagnosis was done in the National laboratory of extremely dangerous infections in NCIPD - Sofia, by using the following tests: Rose Bengal, Widal, Coombs IgM and IgG, and serum agglutination test.

RESULTS

First case:

G.V.B. file number 1035 is a 44 years old man who has been working in a dairy farm near Csanti (Greece) till 15.03.2005. He declared a high mortality among young animals. Everyday he was in contact with them and also with placentas and dead animals. He announced that repeatedly consumed unpasteurized milk. The clinical manifestation of the disease started 15 - 20 days after his return in Bulgaria. The anamnesis revealed the following symptoms: permanent high temperature up to 39.5°C, nightly sweating, headache, expressed arthralgic and adynamic syndrome, weight loss, and visual impairment, which is described in literature (9). Objective examination displayed a man in moderate damaged common status, intoxicated and dehydrated, hepatomegaly - 2 cm under costal arch proved sonographycally, lymph nodes were not significantly enlarged, normal pulmonary and neurological status. Cardiac sonography revealed dilation of the left chamber of degenerative type. A round formation was found above the right macula. Laboratory values were normal. Serological test of Coombs IgM showed titer 1:160.

Second case:

I.G.B., file number 1034 is a son of G.V.B. He was 25 years old, working in the same place and in the same conditions like the father. His complains were based on subfebrile temperature and weakness. Enlarged lymph nodes were the only finding. Serological examination (Coombs IgM) revealed titer 1:320.

Third case:

M.G.T., file number 0502 was a 47 years old woman who had been working in a cattle farm in Greece till 01.01.2006. Her complaints started from 15.01.2006, 15 days after her return home. She had pain in her waist, myalgia, arthralgia predominantly in lower limbs, subfebrile body temperature (37.3° - 37.5°), sweating, abdominal pain, and visual disturbances. The diagnosis brucellosis was proved serologically in the clinic of internal medicine and after that the patient entered the clinic of infectious diseases with marked intoxication, no temperature and slight hepatomegaly. The laboratory examinations revealed anemic syndrome: (HB 114g/l, Ht 0.35, RBC 4.43.1012 / l). Positive Coombs test showed titer 1:2560.

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ABBREVIATIONS USED IN THIS PAPER:

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Table 1. Clinical and laboratory manifestation of brucellosis in 4 patients

Symptoms	No of patients
Temperature to38°C/above 38°C	2/2
Sweating	3
Myalgia	3
Arthralgia	3
Headache	2
Waist pain	1
Abdominal pain	1
Weakness	4
Weight loss	1
Visual impairment	2
Lymphadenopathy	3
Anemia	2
Hepatomegaly	2
Splenomegaly	1
Myocarditis	1

Forth case:

I.P.P., file number 814 was 51 years old man who worked from 01.01.2005 to 01.02.2006 in a sheep farm near Cavala, Greece. He told about a high mortality among the pregnant sheep in the period from December 2005 to January 2006. The milking was done manually with no gloves. He didn't consume unpasteurized milk or milk products. The initial complaints appeared 35 days after his return from Greece and include fever up to 39°C accompanied by rigors, sweating, lost of appetite, nausea, marked weakness. Clinical examination revealed manifested intoxication, moderate lymphadenopathy, hepatomegaly (3 cm under costal arch) and splenomegaly (1cm). Myocarditis was found by objective and sonographic criteria. Serologic test of Coombs IgM showed titer 1:800. Laboratory values revealed anemic syndrome (Hb 79-73g/l, platelet count 180-167.109, fibrinogen 6.0g.l and ESR to 70 mm.

All patients were treated etiologically by the guidelines of W.H.O with doxacycline and rifampin for 6 weeks. The positive effect of the treatment was obvious on the third week after its commencement.

DISCUSSION AND CONCLUSION

This study presents four typical cases with manifested brucellosis imported from abroad. The course of the disease is like systemic infection with multiorgan location, which does not differ from those described in the available literature (14, 15). The most commonly described symptoms among all cases are adynamia, temperature, sweating, myalgia, and arthralgia. The treatment is protracted and leads to disability of long duration. There is a resurgence of interest in brucellosis is Bulgaria during the last two years according to this study and the described by Baev and al. twenty cases of imported infection from Greece in Sliven in 2005 (3). The changes in socioeconomic and political circumstances leading to labor emigration of our citizens in enzootic places of brucellosis like Greece makes this diseases an imported infection in Bulgaria. Therefore it is of great importance for the physicians in our country to be able to discriminate quickly between true brucellosis and other diseases appearing in the differential diagnosis of this infection. The diagnosis is based on epidemiological criteria confirming occupational risk, the most common clinically manifested symptoms of brucellosis and microbiological examinations. The early diagnosis and the subsequent appropriate treatment prevent infection from relapsing and developing into chronic forms. Health education of labor immigrants in enzootic regions and prophylactics of brucellosis are necessary.

REFERENCES:

1. Ангелов С., В. Ганов, И. Куюмджиев, Бруцелозата у животните и човека, София, 1951,21-19.
2. М. Тихолова, А. Гоцева, М. Баев, М. Ченков. Клинични проучвания върху първите случаи с бруцелоза при епидемичен взрив в България. Научни трудове на втората национална конференция по инфекциозни болести. Кърлежопреносими инфекциозни болести. Монография, 2006, стр. 188-192.
3. М. Баев, М. Ченкова, М. Косева, Ц. Андонова, Я. Чукова, Р. Иванова. Епидемиологичен, клиничен и клинико-лабораторен анализ на 20 случая на бруцелоза, наблюдавана в инфекциозно отделение - Сливен през периода март-юни 2005. Научни трудове на втората национална конференция по инфекциозни болести. Кърлежопреносими инфекциозни болести. Монография, 2006, стр. 181-187.
4. Chand P, Rajpurohit BS, Malhotra AK, Poonia JS. Comparison of milk-ELISA and serum-ELISA for the diagnosis of Brucella melitensis infection in sheep. Vet Microbiol. 2005 Jul 1;108(3-4):305-11.
5. Troy SB, Rickman LS, Davis CE. Brucellosis in San Diego: epidemiology and species-related differences in acute clinical presentations. Medicine (Baltimore). 2005 May;84(3):174-87.
6. Gall D, Nielsen K. Serological diagnosis of bovine brucellosis: a review of test performance and cost comparison. Rev Sci Tech. 2004 Dec; 23(3):989-1002.
7. Godfroid J, Cloeckaert A, Liautard JP, Kohler S, Fretin D, Walravens K, Garin-Bastuji B, Letesson JJ. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res. 2005 May-Jun;36(3):313-26.
8. Ardic N, Ozyurt M, Sezer O, Erdemoglu A, Haznedaroglu T. Comparison of Coombs' and immunocapture-agglutination tests in the diagnosis of brucellosis. Chin Med J (Engl). 2005 Feb 5;118(3):252-4.
9. Hatipoglu CA, Yetkin A, Ertem GT, Tulek N. Unusual clinical presentations of brucellosis. Scand J Infect Dis. 2004;36(9):694-7.
10. Elbeltagy KE. An epidemiological profile of brucellosis in Tabuk Province, Saudi Ar abia. East Mediterr Health J. 2001 Jul-Sep;7(4-5):791-8.
11. Pappas G, Akritidis N, Bosilkovski M, Tsianos E. Brucellosis. N Engl J Med. 2005 Jun 2;352(22):2325-36.
12. Solera J, Martinez-Alfaro E, Saez LMeta-analysis of the efficacy of rifampicin and doxycycline in the treatment of human brucellosis. Med Clin (Barc) 1994;102:731-8.
13. Colmenero JD, Fernandez-Gallardo LC, Agundez JA, Sedeno J, Benitez J, Valverde E. Possible implications of doxycycline-rifampin interaction for treatment of brucellosis. Antimicrob Agents Chemother 1994;38:2798-802.
14. Young EJ. An overview of human brucellosis. Clin Infect Dis, 1995;21:283-9.
15. Colmenero JD, Reguera JM, Martos F, et al. Complications associated with Brucella melitensis infection: a study of 530 cases. Medicine 1996;75:195-211.

PRELIMINARY CHARACTERIZATION OF THE RESISTANCE MECHANISMS IN CARBAPENEM-RESISTANT CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA

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SUMMARY

A total of 59 clinical isolates of Pseudomonas aeruginosa resistant to all beta-lactams, including to carbapenems, were collected during 2001-2005 from 5 university hospitals in Sofia to assess the current levels of antimicrobial susceptibilities and to preliminary evaluate the resistance mechanisms. Antimicrobial susceptibilities were detected by a disk diffusion method and Etest (AB Biodisk). Polymerase chain reaction amplification and sequencing of $bla_{IMP-like}$, $bla_{VIM-like}$, and bla_{VEB-1} were performed. The antibiotic resistance rates were: to ceftazidime - 100 %, cefepime - 100%, imipenem - 100%, meropenem - 100%, amikacin- 86.4%, gentamicin - 86.4%, ciprofloxacin - 81.4%. IMP- and VIM-type metallo-beta-lactamases (MBLs) from molecular class B were not detected. Structural genes for VEB-1 extended-spectrum beta-lactamases (ESBLs) were found in 34 (57.6 %) of the isolates. In conclusion, the resistance to all beta-lactams, including toward carbapenems, in the studied strains of P. aeruginosa was not related to enzymatic hydrolysis by MBLs. It was resulted from the combination of different mechanisms such as: OprD deficiency, ESBLs production, derepression of AmpC cephalosporinase and overexpression of active efflux systems. VEB-1 appears to have a significant presence among carbapenem-resistant P. aeruginosa isolates from Sofia.

Key words: Pseudomonas aeruginosa, carbapenem resistance, resistance mechanisms, metallo-beta-lactamases, extended-spectrum beta-lactamases.

INTRODUCTION

Pseudomonas aeruginosa is a clinically important pathogen with intrinsic resistance to various antimicrobial agents. This intrinsic resistance to a number of structurally unrelated antimicrobials results from the synergy between multidrug resistance (MDR) efflux systems and a low degree of outer membrane permeability (7, 27). Moreover, P. aeruginosa possesses an inducible chromosomally encoded AmpC cephalosporinase belonging to Ambler class C (4). This enzyme, expressed at low levels, confers resistance to aminopenicillins and to first- and second-generation cephalosporins.

The carbapenems, imipenem and meropenem, are anomalous beta-lactams in their antipseudomonal behaviour, as well as in their beta-lactamase stability (18). The resistance to these antibiotics is mostly mediated by OprD loss, which primarily confers a resistance to imipenem but also confers a low grade resistance to meropenem (18, 26). MDR efflux systems which mediate the resistance to quinolones, chloramphenicol, and many other antimicrobial agents, also contribute to the carbapenem resistance (14). The strains which overexpress the MexA-MexB-OprM system or express the MexE-MexF-OprN system exhibit the carbapenem resistance by pumping the drug out or repressing the transcription of oprD, respectively (17, 20, 25). Carbapenem resistance mediated by acquired metallo-beta-lactamases (MBLs) from molecular class B (4) is increasingly reported among Pseudomonas spp. and Acinetobacter spp. in the recent years (19, 24). Four families of acquired MBLs are described: IMP, VIM, SPM and GIM (36). IMP- and VIM-types are geographically scattered; SPM enzymes have, to date, only been reported from Brasil (34), GIM-type carbapenemases- only from Germany (5). The emergence of MBLs-producing isolates of P. aeruginosa is becoming a severe therapeutic problem (32). The purpose of the present study was to preliminary characterize the resistance mechanisms to antipseudomonal beta-lactams among carbapenem-resistant clinical isolates of P. aeruginosa using phenotypic methods and polymerase chain reaction (PCR).

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ABBREVIATIONS USED IN THIS PAPER: MBLs- metallo-beta-lactamases, ESBLs- extended-spectrum beta-lactamases, MDR- multidrug resistance, MIC- minimal inhibitory concentration, DDM- disk diffusion method, DDS test- double disk synergy test, MH agar- Mueller-Hinton agar, NCCLS- the National Committee for Clinical Laboratory Standards, PCR- polymerase chain reaction.

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MATERIALS AND METHODS

Bacterial isolates

A collection of 59 non-duplicate, non-susceptible to all antipseudomonal beta-lactams, including carbapenems (according to the disk diffusion method of the National Committee for Clinical Laboratory Standards (NCCLS)), isolates of P. aeruginosa were used for this study. The strains were isolated from 5 university hospitals in Sofia during 2001-2005 and were as follows: from urine (30.5 %), bronchopulmonary sample (32.2 %), wound (5.1 %), blood culture (10.2 %), throat swab (6.8), nasal swab (8.5 %), rectal swab (5.1 %) and ocular swab (1.7 %). The bacterial identification was performed by BBL Enteric/Nonfermenter ID System (Becton Dickinson, USA). The isolates were stored at -70°C in 20 % skimmed milk until used in this study.

Antimicrobial susceptibility testing

The susceptibilities to 15 antimicrobial agents of the P. aeruginosa isolates were first determined by the disk diffusion method (DDM) on Mueller-Hinton (MH) agar plates with antibiotic-containing disks provided by Becton Dickinson (USA), Mast Diagnostics (USA) and Bul Bio (Bulgaria). The minimal inhibitory concentrations (MICs) of selected antibiotics were determined using Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. The obtained results from DDM and Etest were interpreted according to the NCCLS-2004 recommendations (23). Control strains included P. aeruginosa ATCC 27853.

Screening for extended-spectrum beta-lactamases (ESBLs)

The presence of ESBLs was investigated by the double disk synergy (DDS) test, as described by Jarlier V. et al. (11). Ceftazidime (30 μg), cefepime (30 μg), cefpirome (30 μg) and aztreonam (30 μg) disks were placed next to amoxicillin/claculanic acid (20/10 μg)-containing disk at a distance of 20 mm (center to center) on MH agar plate inoculated with the test organism. After overnight incubation at 37°C, an enhancement of the inhibition zone around at least one of these disks toward the clavulanate-containing disk indicated the presence of ESBLs. All studied strains were additionally tested by a DDM with imipenem - (10 μg) and ceftazidime-containing (30 μg) disks for presence of synergism (37).

Table 1. Oligonucleotides used as primers for amplification and sequencing.

Primer	Target pairs	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
IMP-F IMP-R	IMP-type MBLs	GAA GGY GTT TAT GTT CAT AC (Y=C or T) GTA MGT TTC AAG AGT GAT GC (M=A or C)	587	56
VIM-F VIM-R	VIM-type MBLs	ATG GTG TTT GGT CGC ATA TC TGG GCC ATT CAG CCA GAT C	510	60
VEB-F VEB-R	VEB-1 ESBL	CGA CTT CCA TTT CCC GAT GC GGA CTC TGC AAC AAA TAC GC	643	58

F- forward, R- reverse; MBLs- metallo-beta-lactamases.

Screening for metallo-β - lactamases (MBLs)

The presence of MBLs belonging to molecular class B (4) was studied by the modified Hodge test of Lee K. et al. (15). The indicator organism, Escherichia coli ATCC 25922 at a turbidity of 0.5 McFarland standard, was used to swab inoculate the surface of a MH agar plate, and the test strain was heavily streaked from the center to the plate periphery. After the plate was allowed to stand for 15 min at room temperature, an imipenem-containg disk (10 μg) was placed at the center, and the plate was incubated overnight. The presence of a distorted inhibition zone was interpreted as a positive result for carbapenem hydrolysis screening.

Polymerase chain reaction (PCR) amplification and sequencing

The total DNA from P. aeruginosa isolates was extracted by boiling. Detection of bla_{VEB-1}, $bla_{IMP-like}$ and $bla_{VIM-like}$ genes in the investigated strains was performed by PCR with specific primers listed in table 1 (16, 24). PCR was carried out with 2 μl of the template DNA, 0.25 μM of each primer (Alpha DNA, Canada), 0.2 mM deoxyribonucleoside triphosphates, 1x Reaction Buffer, 2 mM $MgCl_2$ and 1.5 U of Taq DNA polymerase (Prime Taq™ DNA Polymerase, GENET BIO, Korea) in a total volume of 25 μl. The DNA was amplified in a Techgen PCR thermocycler (Techne, England) using the following protocol: an initial denaturation (94°C, 5 min) followed by 30 cycles of denaturation (94°C, 45 s), annealing (56°C- 60°C, 30 s- 45 s) and extension (72 °C, 45 s- 1 min), and a single final extension of 7 min at 72 °C. The PCR products were separated in a 1% agarose gel for 45 min at 150 V, stained with ethidium bromide (0.5 °g/ml), and detected by UV transillumination (λ=312 nm). The amplified genes were identified on the basis of fragment size (643 bp for bla_{VEB-1}, 587 bp for $bla_{IMP-like}$ and 510 bp for $bla_{VIM-like}$). Selected VEB-1 PCR products were purified with an ExoSAP-IT reagent (Amersham Biosciences Corp., USA). Sequencing reactions were performed using the same blaVEB-1-specific primers and BigDyeTerminator v.3.1. kit (Applera Corp., CA, USA) in an automated sequencer (ABI 310 Sequence Genetic Analyzer; Applied Biosystems, Foster City, USA). The nucleotide and deduced amino acid sequences were analyzed with software available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The GenBank accession number of the published sequence is DQ333895.

Statistics: Alternative analysis.

RESULTS

Antimicrobial susceptibility of the studied carbapenem-resistant P. aeruginosa isolates

According to the DDM/ NCCLS-2004 (23), the studied strains of P. aeruginosa (n=59) were determined as resistant to all wide used antipseudomonal beta-lactams: carbenicillin, azlocillin, piperacillin, ceftazidime, cefoperazone, cefepime, cefpirome, aztreonam, imipenem and meropenem. Additionally, the antimicrobial activity of the following antimicrobial agents- piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin and ciprofloxacin, has been tested by minimal inhibitory concentrations (MICs) determinations using Etest (AB Biodisk, Solna, Sweden). The MICs-rates against the investigated clinical strains of P. aeruginosa and P. aeruginosa ATCC 27853 are presented in table 2. As it is shown in the table, the rates of antimicrobial resistance toward the selected beta-lactams, aminoglycosides and fluoroquinolones were very high (varied from 81.4 % to 100 %), except to piperacillin/tazobactam (50.8 %).

The MICs of meropenem were a fourfold lower than these of imipenem in 7 (11.9 %) of the studied isolates of P. aeruginosa. 88.1 % of the isolates (52/59) were MDR (resistant to at least three of the drugs: ceftazidime, imipenem, gentamicin and ciprofloxacin) (12).

Screening for ESBLs and MBLs

ESBLs were detected in 28 isolates of P. aeruginosa (47.5 %) by the DDS test (11). Synergy between imipenem and ceftazidime was observed in 27 of the strains (45.8 %). MBLs were detected in none of the studied strains according to the modified Hodge test (15).

PCR detection of molecular class A ESBLs and class B MBLs

Because of the expressed in vitro synergism between ceftazidime and imipenem, it was carried out the molecular-genetic study for a presence of VEB-1 clavulanic acid inhibited ESBLs from molecular class A (37). Of the 59 isolates, 34 (57.6 %) were determined as VEB-1 producers. The sequence for the blaVEB-1 genes amplified from 5 different isolates was identical for all isolates and 100 % identical to the known VEB-1 sequence (GenBank DQ333895). The absence of MBLs (according to the modified Hodge test) was confirmed by PCR with specific group primers detected IMP- and VIM-types carbapenemases.

DISCUSSION

The studied strains of P. aeruginosa were resistant to all wide used antipseudomonal beta-lactams, including toward the carbapenems (imipenem and meropenem). In Bulgaria the average imipenem resistance rate was approximately 18 % for 2004, according to data of the National Program BulSTAR (1). It was lower than the resistance rate to imipenem in Turkey and Brazil, but higher than that in USA at the same time (8, 9, 13). Over 88 % of the isolates in the present study were determined as MDR strains (12).

The DDS test for detection of ESBLs was positive in 47.5 % of the studied strains, but 57.6 % of them were determined as producers of VEB-1 ESBLs by PCR. These differences

Table 2. MICs[a] of selected antibiotics against 59 carbapenem-resistant Pseudomonas aeruginosa isolates (Etest, AB Biodisk).

Antimicrobial agent	MIC range (mg/l)	MIC50[b] (mg/l)	MIC90[b] (mg/l)	Number (%) of nonsusceptible (I+R) strains*	MIC against P. aeruginosa ATCC 27853
Piperacillin	8 - >256	256	>256	55 (93.2)	1.5
Piperacillin+tazobactam[c]	8 - >256	64	>256	30 (50.8)	2
Ceftazidime	16 - >256	>256	>256	59 (100)	1.0
Cefepime	64 - >256	>256	>256	59 (100)	2
Imipenem	8 - >32	>32	>32	59 (100)	1.0
Meropenem	6 - >32	>32	>32	59 (100)	0.5
Amikacin	1 - >256	128	>256	51 (86.4)	1.0
Gentamicin	0.750 - >256	64	256	51 (86.4)	0.5
Ciprofloxacin	0.032 - >32	>32	>32	48 (81.4)	0.5

[a] MICs- minimal inhibitory concentrations.
[b] MIC50 and MIC90- MICs at which 50 % and 90 % of isolates are inhibited, respectively.
[c] tazobactam at a fixed concentrations of 4 mg/l.
* Interpretation according to the NCCLS-2004; I- intermediate susceptible, R- resistant.

show that the DDS test (11) may not be useful for detection of ESBLs in P. aeruginosa. The false negative results due to naturally occurring chromosome-encoded AmpC cephalosporinase that may be overexpressed; to the simultaneous presence of extended-spectrum oxacillinases or to combined mechanisms of resistance, such as impermeability and efflux (37). The synergy between ceftazidime and imipenem may be obscured in some cases by the induction effect of imipenem on the expression of the chromosomal cephalosporinase, resulting in a concomitant line of antagonism between ceftazidime- and imipenem-containing disks (37).

In this study, the resistance to extended-spectrum cephalosporins, such as ceftazidime, among 57.6 % of the strains was due mainly to hydrolysis by VEB-1 ESBLs. In the rest of strains, other mechanisms were implicated. The carbapenem resistance observed in 100 % of P. aeruginosa isolates was not related to enzymatic hydrolysis by MBLs from molecular class B.

It was established a high frequency of spread of ESBLs among the studied ceftazidime- and carbapenem-resistant isolates of P. aeruginosa, which contrasts with data from analogous investigations in Europe (6). The present study demonstrated a widespread dissemination of bla_{VEB-1} in clinical isolates of P. aeruginosa in the monitored university hospitals in Sofia. Recently, Strateva T. et al. studied the prevalence of VEB-1-type beta-lactamases among 132 ceftazidime-resistant strains of P. aeruginosa isolated from 2001 to 2005 from the same university hospitals in Sofia (33). The frequency was 56.8 % and it was significantly higher ($p<0.01$) than the determined rate in an analogous study carried out by Bachvarova A. et al. (36.8 %) among ceftazidime-resistant isolates of P. aeruginosa isolated during 1998-2003 from distinct regions of Bulgaria (2). For the last three years in Bulgaria there is an increasing trend in VEB-1-producing P. aeruginosa isolation. VEB-1 and VEB-1-like enzymes are widespread in Asia (Thailand, Kuwait, India, China) (10, 29, 37) and has been detected only in France from the European countries (22).

The VEB-1-producing strains of P. aeruginosa showed a resistance to all beta-lactams except to piperacillin/tazobactam, typically of producers of clavulanic acid- and tazobactam-inhibited ESBLs from molecular class A (4). The cross-class resistance to aminoglycosides and ciprofloxacin was high among the VEB-1 producers. This poses the substantial risk of treatment failures as in ESBL producers from Enterobacteriaceae (31). As described previously, VEB-1 was the first class A enzyme found to be encoded by an integron-located gene cassette (28). Integrons are in fact expression vectors for different antibiotic resistance genes that are included as gene cassettes and are neighbored. In the bla_{VEB-1}-containing integrons of P. aeruginosa, the veb-1 cassette is often associated with aminoglycoside resistance gene cassettes (10).

Carbapenem- hydrolyzing IMP- and VIM-type metalloenzymes belonging to Ambler class B (4) were not detected in the present study. The investigated carbapenem-resistant strains of P. aeruginosa from Sofia did not harbored blaVIM-like, which contrasts with the widespread detection of these genes worldwide, especially in the neighbour countries, such as Greece and Turkey (3, 21, 30, 35).

The resistance against imipenem is usually mediated by OprD loss, and the imipenem does not select for the multidrug efflux pumps (26). The organisms which lost OprD showed an MIC of meropenem a fourfold lower than that of imipenem. However, the MIC values for our clinical strains of P. aeruginosa showed that 52 of 59 studied strains (88.1 %) were also resistant to meropenem (MICs$\geq$16 mg/l) and the most of the isolates (48 of 59) were resistant to ciprofloxacin. Thus, we suspected that other resistance mechanisms such as efflux systems might play a role for the carbapenem resistance in these isolates (14).

In conclusion, the resistance to ceftazidime was due mainly to hydrolysis by clavulanic acid-inhibited VEB-1 ESBLs among the investigated strains. This work underlines that VEB-1-type ESBLs may be identified in different parts of the world and appear to have a significant presence among ceftazidime-resistant P. aeruginosa isolates in Bulgarian hospitals. The current high prevalence of VEB-1 in P. aeruginosa may be a hidden reservoir for the transfer of bla_{VEB-1} genes to other gram-negative aerobes in our country. The carbapenem resistance was resulted from a combination of non-enzymatic mechanisms, such as OprD deficiency and active efflux. The obtained results justify the necessity for additional research by Western blot analysis for exact detection and characterization of the non-enzymatic resistance mechanisms toward carbapenems (the presence of OprD and expressions of MexAB-OprM and MexEF-OprN efflux).

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REFERENCES

1. Велинов, Ц., М. Петров, А. Бъчварова, Т. Кантарджиев. Етиологична структура и резистентност на най-честите причинители на инфекции в България- BulSTAR 2004. Инфектология. 2006; XLIII, специално издание: 8-11.
2. Bachvarova, A., T. Velinov, M. Petrov et al. Widespread detection of VEB-1-type extended-spectrum beta-lactamases among nosocomial Pseudomonas aeruginosa isolates in Bulgaria: a nationwide multicentre study. Clin. Microbiol. Infect. 2005; 11 (suppl. 2): 233.
3. Bahar, G., A. Mazzariol, R. Koncan et al. Detection of VIM-5 metallo-beta-lactamase in a Pseudomonas aeruginosa clinicval isolate from Turkey. J. Antimicrob. Chemother. 2004; 54: 282-283.
4. Bush, K., G. A. Jacoby, and A. A. Medeiros. A functional classification scheme for beta-lactamases and its correlation with molecular structure.

Antimicrob. Agents Chemother. 1995; 39: 1211-1233.
5. Castanheira, M., M. A. Toleman, R. N. Jones et al. Molecular characterization of a beta-lactamase gene, blaGIM-1, enciding a new subclass of metallo-beta-lactamase. Antimicrob. Agents Chemother. 2004; 48: 4654-4661.
6. De Champs, C., L. Poirel, R. Bonnet et al. Prospective survey of beta-lactamases produced by ceftazidime-resistant Pseudomonas aeruginosa isolated in a French hospital in 2000. Antimicrob. Agents Chemother. 2002; 46: 3031-3034.
7. Dubois, V., C. Arpin, M. Melon et al. Nosocomial outbreak due to multiresistant strain of Pseudomonas aeruginosa P12: efficacy of cefepime-amikacin therapy and analysis of beta-lactam resistance. J. Clin. Microbiol. 2001; 39: 2072-2078.
8. Flamm, R., K. Weaver, C. Thornsberry et al. Factors associated with relative rates of antibiotic resistance in Pseudomonas aeruginosa isolates tested in clinical laboratories in the United States from 1999 to 2002. Antimicrob. Agents Chemother. 2004; 48: 2431-2436.
9. Gencer, S., O. Ak, N. Benzonana et al. Susceptibility patterns and cross resistance of antibiotics against Pseudomonas aeruginosa in a teaching hospital of Turkey. Ann. Clin. Microbiol. Antimicrob. 2002; 1: 2.
10. Girlich, D., T. Naas, A. Leelaporn et al. Nosocomial spread of the integron-located veb-1-like cassette encoding an extended-spectrum beta-lactamase in Pseudomonas aeruginosa in Thailand. Clin. Infect. Dis. 2002; 34: 603-611.
11. Jarlier, V., M.-H. Nicolas, G. Fournier, A. Philippon. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 1988; 10: 867-878.
12. Karlowsky, J.A., D. C. Draghi, M. E. Jones et al. Surveillance for antimicrobial susceptibility among clinical isolates of Pseudomonas aeruginosa and Acinetobacter baumannii from hospitalized patients in the United States, 1998 to 2001. Antimicrob. Agents Chemother. 2003; 47: 1681-1688.
13. Kiffer, C., A. Hsiung, C. Oplustil et al. Antimicrobial susceptibility of Gram-negative bacteria in Brazilian hospitals: the MYSTIC Program Brazil 2003. Braz. J. Infect. Dis. 2005; 9: 216-224.
14. Kohler, T., M. Michea-Hamzehpour, S. F. Epp, and J.-C. Pechere. Carbapenem activities against Pseudomonas aeruginosa: respective contributions of OprD and efflux systems. Antimicrob. Agents Chemother. 1999; 43: 424-427.
15. Lee, K., Y. Chong, H. B. Shin et al. Modified Hodge and EDTA-disk synergy tests to screen metallo-beta-lactamase- producing strains of Pseudomonas and Acinetobacter species. Clin. Microbiol. Infect. 2001; 7: 88-91.
16. Lee, S., Y.-J. Park, M. Kim et al. Prevalence of Ambler class A and D beta-lactamases among clinical isolates of Pseudomonas aeruginosa in Korea. J. Antimictob. Chemother. 2005; 56: 122-127.
17. Li, X.-Z., H. Nikaido, and K. Poole. Role of MexA-MexB-OprM in antibiotic efflux in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 1995; 39: 1948-1953.
18. Livermore, D. M. Of Pseudomonas, porins, pumps and carbapenems. J. Antimicrob. Chemother. 2001; 47: 247-250.
19. Livermore, D. M., and N. Woodford. Carbapenemases: a problem in waiting? Curr. Opin. Microbiol. 2000; 3: 489-495.
20. Maseda, H., H. Yoneyama, and T. Nakae. Assignment of the substrate-selective subunits of the MexEF-OprN multidrug efflux pump of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 2000; 44: 658-664.
21. Mavroidi, A., A. Tsakris, E. Tzelepi et al. Carbapenem-hydrolyzing VIM-2 metallo-beta-lactamase in Pseudomonas aeruginosa from Greece. J. Antimicrob. Chemother. 2000; 46: 1041-1042.
22. Naas, T., L. Poirel, A. Karim, P. Nordmann. Molecular characterization of In50, a class 1 integron encoding the gene for the extended-spectrum beta-lactamase VEB-1 in Pseudomonas aeruginosa. FEMS Microbiol. Lett. 1999; 176: 411-419.
23. National Committee for Clinical Laboratory Standards. 2004. Performance standards for antimicrobial susceptibility testing: 14th informational supplement. NCCLS document M100-S14. National Committee for Clinical Laboratory Standards, Wayne PA.
24. Nordmann, P., and L. Poirel. Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. 2002; 8: 321-331.
25. Ochs, M., M. McCusker, M. Bains, and R. Hancock. Negative regulation of the Pseudomonas aeruginosa outer membrane porin OprD selective for imipenem and basic amino acids. Antimicrob. Agents Chemother. 1999; 43: 1085-1090.
26. Pai, H., J.-V. Kim, J. Kim et al. Carbapenem resistance mechanisms in Pseudomonas aeruginosa clinical isolates. Antimicrob. Agents Chemother., 2001; 45: 480-484.
27. Pechere, J.-C., and T. Kohler. Patterns and modes of beta-lactam resistance in Pseudomonas aeruginosa. Clin. Microbiol. Infect. 1999; 5 (suppl. 1): S15-S18.
28. Poirel L., T. Naas, M. Guibert et al. Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum beta-lactamase encoded by an Escherichia coli integron gene. Antimicrob. Agents Chemother. 1999; 43: 573-581.
29. Poirel, L., V. Rotimi, E. Mokaddas et al. VEB-1-like extended-spectrum beta-lactamases in Pseudomonas aeruginosa, Kuwait. Emerg. Infect. Dis 2001; 7: 468-470.
30. Poumaras, S., A. Tsakris, M. Maniati et al. Novel variant (blaVIM-4) of the metallo-beta-lactamase gene blaVIM-1 in a clinical strain of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 2002; 46: 4026-4028.
31. Procop G. W., M. J. Tuohy, D. A. Wilson et al. Cross-class resistance to non-beta-lactam antimicrobials in extended-spectrum beta-lactamase-producing Klebsiella pneumoniae. Am. J. Clin. Pathol. 2003; 120: 265-267.
32. Sanchez, A., S. Salso, E. Culebras, J. J. Picazo. Carbapenem resistance determined by metalloenzymes in clinical isolates of Pseudomonas aeruginosa. Rev. Esp. Quimioter. 2004; 17: 336-340.
33. Strateva, T., V. Ouzounova-Raykova, B. Markova et al. Widespread detection of VEB-1-type extended spectrum beta-lactamases among nosocomial ceftazidime-resistant Pseudomonas aeruginosa isolates in Sofia, Bulgaria. J. Chemother. 2007; 19 (2): in press.
34. Toleman, M., A. Simm, T. Murphy et al. Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY amtimicrobial surveillance programme. J. Antimicrob. Chemother. 2002; 50: 673-679.
35. Tsakris, A., S. Pournaras, N. Woodford et al. Outbreak of infections caused by Pseudomonas aeruginosa producing VIM-1 carbapenemase in Greece. J. Clin. Microbiol. 2000; 38: 1290-1292.
36. Walsh, T. R., M. A. Toleman, L. Poirel, P. Nordmann. Metallo-beta-lactamases: the quiet before storm? Clin. Micribiol. Rev. 2005; 18: 306-325.
37. Weldhagen, G., L. Poirel, P. Nordmann. Ambler class A extended-spectrum beta-lactamases in Pseudomonas aeruginosa: novel developments and clinical impacts. Antimicrob. Agents Chemother. 2003; 47: 2385-2392.

OCCURRENCE OF CTX-M-3 EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING SALMONELLA BRANDENBURG IN BULGARIA

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SUMMARY

The aim of the present work was to study the genetic profile and resistance to antibiotics of a rare serotype of Salmonella causing a cluster of cases among infants and children in Bulgaria. Materials and methods: nine strains of Salmonella Brandenburg were isolated from infants and children with acute enterocolitis in the beginning of 2005. Culture, identification, serotyping and antimicrobial susceptibility testing to fourteen antibiotics have been performed. PCR with primers detecting bla-CTX-M genes and PFGE after restriction with XbaI were applied. Results: A single PFGE type of strains confirmed the occurrence of a cluster of cases caused by Salmonella Brandenburg among infants and children. The strain was found to be multidrug- resistant. It produced a CTX-M- 3 extended- spectrum beta-lactamase. Conclusion: Our findings proved the etiological role of a unique genetic variant of Salmonella Brandenburg in a cluster of cases among infants and children. This is the first report for Bulgaria of Salmonella Brandenburg producing a CTX-M-3 extended-spectrum beta-lactamase.

Key words: ESBL, CTX-M-3, Salmonella Brandenburg

Salmonellosis is one of the priority communicable diseases officially determined by the Bulgarian Ministry of Health. Health care providers are legally required to record and report cases of illness discovered in their regions. The network of microbiological laboratories functioning in the country performs the primary diagnosis and forwards outbreak- and sporadic strains to the National Reference Laboratory of Enteric Pathogens for confirmation, serotyping and antimicrobial susceptibility testing. Salmonella Brandenburg is a rare serotype causing human salmonellosis in Bulgaria. An increase in the number of cases due to Salmonella Brandenburg has been detected since the beginning of 2005. The aim of this work was to study the genetic profile and the resistance to antimicrobial agents of Salmonella Brandenburg causing a cluster of cases among infants and children in Bulgaria.

MATERIALS AND METHODS

From February to April 2005 nine strains of Salmonella Brandenburg (№№: 852, 853,854, 855, 856, 857, 858, 859, 891) have been sent to the National Reference Laboratory of Enteric Pathogens. Seven out of nine strains were recovered from the feces of sick children with acute enterocolitis. The remaining two strains originated from blood cultures of a 6-months-old and a 7-months-old babies. Children got sick at their homes with symptoms of vomiting, abdominal pain, diarrhea and fever. The age of ill patients varied between four months to fourteen years. Both sexes were affected. These cases of salmonelloses emerged in one village and three towns situated in the central part of Bulgaria. Classical methods for microbiological diagnosis have been performed: culture, identification, slide agglutination (Bio-Rad), Sven Gard techniques for phase inversion. Serotyping results were established according to the Kauffmann-White sheme (1).

Screening for resistance to fourteen antimicrobial agents: Cefotaxime, Cefoxitin, Carbenicillin, Ceftazidime, Cefuroxime, Cephalothin, Ampicillin, Amoxicillin/ Clavulanic acid, Gentamicin, Tetracycline, Chloramphenicol, Ciprofloxacin, Nalidixic acid, Trimethoprim/ Sulfamethoxazole (Biomerieux) has been done using standard Bauer-Kirby disk- diffusion method and screening for extended-spectrum beta-lactamases (ESBLs) production with the double-disk synergy method (2).

The second generation cephalosporines and the aminoglycosides served only for epidemiological purposes.

For PCR detection of bla- CTX-M genes the following primers have been applied: ALA2/P2D 5' ATGGTTAAAAAATCACTGCG 3'/ 5' CAGCGCTTTTGCCGTCTAAG 3' (3).

Pulsed Field Gel Electrophoresis (CHEF-DR II; BIO-RAD) after restriction with XbaI (Sigma) enzyme has been performed in order to study the genetic relationships between strains. Conditions of the reaction were according to the PulseNet protocol (4).

RESULTS AND DISCUSSION

A single PFGE profile identical for all tested strains of Salmonella Brandenburg was identified. This finding proved the occurrence of a cluster of cases caused by one agent. The PFGE profile of the studied Salmonella Brandenburg was characterized by thirteen bands with molecular weight ranging from 485 to 48,5 kb.

Figure 1 demonstrates the results from the PFGE typing (Figure 1).

The examined strain of Salmonella Brandenburg was multidrug-resistant to seven antimicrobial agents: cefotaxime, carbenicillin, cefuroxime, cephalothin, ampicillin, gentamicin and trimethoprim/ sulfamethoxazole.

The double-disk synergy method demonstrated the presence of an extended-spectrum beta- lactamase with profile predictive for CTX-M type of enzymes. PCR amplification with primers ALA2 and P2D revealed bla- CTX- M-3 genes in Salmonella Brandenburg (Figure.2).

Resistance of salmonellae to antimicrobial agents is increasing worldwide and Bulgaria does not represent any exception. Extended-spectrum beta-lactamases occur frequently among members of the family Enterobacteriaceae causing nosocomial outbreaks. Data obtained from a national longitudinal surveillance programme (BulSTAR) have demonstrated that eight percent of Escherichia.coli and twenty three percent of Klebsiella spp. strains in the country produced ESBLs (5). However, these enzymes remained rare in Salmonella until the second half of the 90-ties. Today the list of Salmonella serotypes expressing extended-spectrum beta- lactamases is constantly expanding (6). Recently conducted studies in Bulgaria have discovered ESBLs among the leading serotypes causing human salmonellosis: Salmonella Enteritidis, Salmonella Typhimurium, Salmonella Corvallis and Salmonella Isangi (7). Salmonellae are important food-borne human pathogens. They can cause large community outbreaks that in

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ABBREVIATIONS USED IN THIS PAPER: ESBL - extended spectrum Beta-Lactamase

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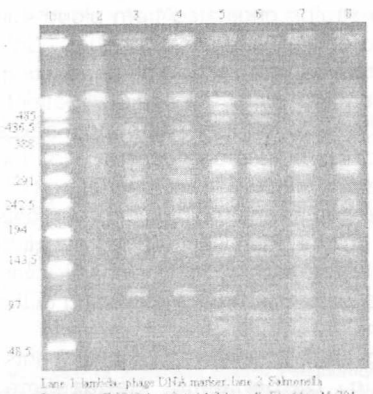
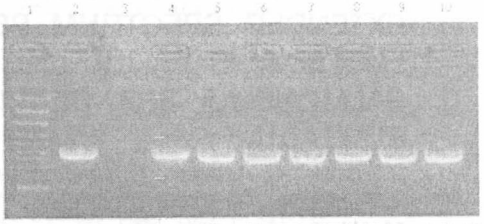
Figure 1 PFGE typing of *Salmonella* Brandenburg strains



Lane 1: lambda- phage DNA marker, lane 2: Salmonella Brandenburg H 9612, lane 3 and 4 Salmonella Blockley № 721, 734, lane 5 Salmonella Brandenburg № 852, lane 6 Salmonella Brandenburg № 853, lane 7 Salmonella Brandenburg № 854, lane 8, Salmonella Brandenburg № 857

Figure 2 PCR revealing *blaCTX-M-3* gene in *Salmonella* Brandenburg strains



Lane 1: molecular size marker 100 bp DNA Ladder Plus, lane 2 *Salmonella Infantis* № 672 (CTX-M-3 positive), lane 3 water (negative control), lane 4 Salmonella Brandenburg № 852, lane 5 Salmonella Brandenburg № 853, lane 6 Salmonella Brandenburg № 854, lane 7 Salmonella Brandenburg № 855, lane 8 Salmonella Brandenburg № 857, lane 9 Salmonella Brandenburg № 858, lane 10 Salmonella Brandenburg № 859

the presence of ESBLs pause serious therapeutic problems especially for infants and children. The source of the studied cluster caused by Salmonella Brandenburg in Bulgaria remained unknown. However, in another countries this Salmonella serotype was found to be closely related with infection in sheep (8). Sheep farming is traditional in Bulgaria. That makes the infection in sheep a possible source for human disease. Salmonella Brandenburg is a rare serotype in Bulgaria, but it ranked fourth after Salmonella Enteritidis, Salmonella Typhimurium and Salmonella Infantis in 2005 (9). Cases of human salmonellosis due to Salmonella Brandenburg have been reported in Belgium, Finland, Estonia, Greece. Few reports are available in literature about the resistance mechanisms among salmonellae belonging to this serotype. SHV-producing Salmonella.Brandenburg have been detected in Greece (10). In Bulgaria SHV extended-spectrum beta- lactamases were discovered mainly among Salmonella Corvallis and CTX-M-3 enzymes among Salmonella.Enteritidis and Salmonella Isangi (11).

In conclusion, this is a first occasion of a cluster caused by a unique genetic variant of a CTX-M-3- producing Salmonella Brandenburg in Bulgaria. ESBLs-producing salmonellae are widely distributed among the major and minor serotypes causing human disease in our country.

REFERENCES

1. Popoff MY, Le Minor L. Antigenic formulas of the Salmonella serovars, 8th ed. WHO Collaborating Centre for Reference and Research on Salmonella, Paris, France, 2001; p 20.
2. Jarlier V, Nicolas M, Fournier G, Philippon A. Extended broad-spectrum b-lactamase conferring transferable resistance to newer b-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis 1988; 10: 867-878.
3. Baraniak A, Fiett J, Hryniewicz W, Nordmann P, and Gniadkowski M Ceftazidime-hydrolysing CTX-M-15 extended-spectrum b-lactamase (ESBL) in Poland. J Antimicrob Chemother 2002;50(3): 393
4. Ribot E, Wierzba F, Angulo F, Barrett TSalmonella enterica serotype Typhimurium DT104 isolated from humans, United States, 1985, 1990,and Emerg Infect Dis 2002; 8:387-391.
5. Petrov M, Hadjieva N, Kantardjiev T, Velinov T, Bachvarova A. Surveillance of antimicrobial resistance in Bulgaria- a synopsis from BulSTAR 2003. Eurosurveillance 2005; 10 (6): 79-82.
6. Walther-Rasmussen J, Hoiby N. Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum b- lactamases. Can J Microbiol 2004; 50:137-165
7. Asseva G, Petrov P,Ivanov I, Kantardjiev T Surveillance of human salmonellosis in Bulgaria, 1999-2004: trends,shifts and resistance to antimicrobial agents. Eurosurveillance 2006; 11(5): 97-100
8. Kerslake JI, Perkins NR. Salmonella Brandenburg: case-control survey in sheep in New Zealand. NZ Vet J 54 2006 (3):125-31
9. http://thor.dfvf.dk/pls/portal/GSS. COUNTRY_DATA_SET_REP.show
10. Politi L, Tassios PT, Lambiri M, Kansouzidou A, Pasiotou M, Vatopoulos AC Repeated occurrence of diverse Extended-spectrum b-lactamases in minor serotypes of food-borne Salmonella enterica subsp.enterica. J Clin Microbiol 2005; 43 (7): 3453-3456.
11. Archambault M, Petrov P, Hendriksen RS, Asseva G, Bangtrakulnonth A, Hasman H, Aarestrup FM. Molecular Characterization and Occurrence of Extended-spectrum b-lactamase resistance genes among Salmonella enterica serovar Corvallis from Thailand, Bulgaria and Denmark. Microbial Drug Resistance 2006; 12 (3): 192-198.

HAPTOGLOBIN AS A MARKER FOR LIVER INJURY IN HEROIN ADDICTS

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SUMMARY:
The aim of the study is to evaluate the level of haptoglobin as a part of immune reactivity in heroin addicts. In the study are included 35 heroin abusers between 16 and 43 years old. The level of transaminases (ASAT and ALAT) have been investigated and the quantity of haptoglobin in the patient sera have been determined by Manchini's radial diffusion test in agar/agarose gel using immunoplates by "Immunotest", Sofia. In 15 (43%) of the studied patients we found statistically significant elevated level of transaminases above the referent values. The haptoglobin was also significantly increased (p<0,05) in 10 (28,8%) of heroin abusers. Having in mind the normal level of haptoglobin in humans - 0,3 to 2g/l - we found in our studied patients its value between 1,58 - 4,8 g/l what suggests that heroin may disturb the liver function provoking increased level of transaminases and haptoglobin. In conclusion we may say that haptoglobin could be used as a marker for liver disorders in heroin addicts.

Key words: heroin, immune reactivity, transaminases, haptoglobin, hepatitis C

Suppression or stimulation of liver enzymes from hazardous chemical substances or immunomodulators as therapeutic agents, leads to disturbance in normal biotransformation in liver. It leads to accumulation of acute metabolites, abnormal toxicity and pathological production of interleukins.

Haptoglobin is alfa-1 glycoprotein. It structure is similar to the structure of immunoglobuline. Haptoglobin is produce in liver and is spesific connected by protein globin in hemoglobin. The level of haptoglobin increases in cases of inflamation, parallely by alfa-1 antitripsin, orozomucoid, C3. It is normalized after acute inflamation was passing.

The aim of the study was to determine the level of haptoglobin as a part of immune reactivity in a group of heroin addicted patients.

MATERIALS AND METHODS

The study group included 35 heroin addicts (duration of the abuse with range between 1 to 6 years) hospitalized in Clinic of Toxicology, Emergency Hospital "N.I.Pirogov", Sofia with acute exogenous intoxications. The age of the patients was between 16 and 43 years. The relation males: females was 3:1. The level of enzymes ALAT and ASAT have been investigated and the quantity of haptoglobin in the patient sera were determined by Manchini's radial diffusion test in agar/agarose gel using immunoplates by "Immunotest", Sofia.

Statistic analysis of data was accomplish by program SPSS 10.00.

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ABBREVIATIONS USED IN THIS PAPER: HCV - Hepatitis C Virus

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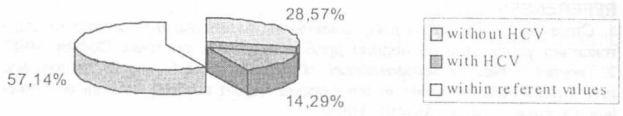

fig.1. Percentage of the patients with an increase level of transaminases

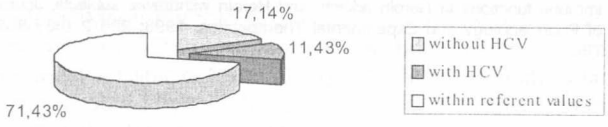

fig.2. Percentage of the patients with an increase level of haptoglobin

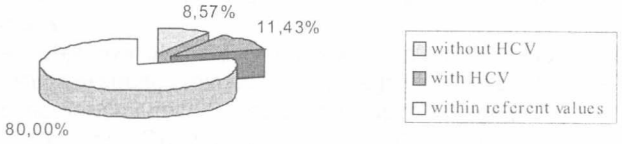

fig.3. Percentage of the patients with an increase level of transaminases and haptoglobin

RESULTS AND DISCUSSIONS

In 43% of the studied patients we observed changes of the liver metabolism - an increase of the level of enzymes ALAT and ASAT. Five of the studied heroin abusers were carriers of HCV. The mean level of transaminases in four of these five patients is higher then the mean level of enzymes to the persons without HCV, but no statistically significant difference was found in relation to the associated hepatitis C virus infection. The distribution of the studied patients in terms of the level of transaminases were presented on fig.1.

Rather interesting is the finding that in 10 or 28,8% of the cases the level of haptoglobin is substantially increased. Its level is statistically significant higher ($p<0.05$) in comparison with the normal referent values. Having in mind the normal level of haptoglobin in humans - 0,3 to 2g/l - we found in our studied patients its value between 1,58 - 4,8 g/l.

The same picture we have seen in the studied heroin abusers carriers of HCV. In four of them / 80% / its level is statistically significant higher ($p<0.05$) in comparison with the normal referent values.

The increased haptoglobin level is an index for liver disorders what correlates well with considerable incidence of damaged liver function in the studied patients. (fig. 2.)

In 7 (20%) from investigated 35 heroin abusers were established parallel increased level of transaminases and haptoglobin. 4 or 57% of them were carriers of HCV(fig.3).

From 20 patients with normal value of transaminases in 3 of them were established increased level of haptoglobin. This 3 patients were without HCV and were with long duration of the heroin abuse with range 3-6 years. Probably the registered changes of the level of haptoglobin were a result of long-standing heroin abuse and its toxic effect.

CONCLUSION

As a result of our study we might conclude that heroin destroy the liver function provoking increased level of transaminases and haptoglobin. We may say that haptoglobin could be used as a marker for liver disorders in heroin addicts.

REFERENSES
1. Стоянов Ст., И. Алтънкова, Химически вещества в околната среда - токсично увреждане на черния дроб и имунната система, София, 1999;
2. Tennant F, Moll D; Seroprevalence of hepatitis A, B, C and D markers and liver function abnormalities in intravenous heroin addicts; Journal of Addictive Diseases. 14/3 (35-49) 1995;
3. Dalekos GN, et al. Immunologic and viral markers in the circulation of anti-HIV negative heroin addicts. Eur J Clin Invest 1993; 23: 219 - 225.
4. Govitrapong P; Suttitum T; Kotchabhakdi N; Uneklabh T: Alternations of immune functions in Heroin addicts and Heroin withdrawal subjects; Journal of Pharmacology and Experimental Therapeutics. 1998; 286/2 (883-889);
5. Huang Y S; Yang Z C; Liu X S; Chen F M; He B B; Li A; Crowther RS: Serial experimental and clinical studies on the pathogenesis of multiple organ dysfunction syndrome (MODS) in severe burns. Burns. 1998; 24/8 (706-716);
6. Kreek M J; Immune function in heroin addicts and former heroin addicts in treatment. pre- and post- AIDS epidemic; NIDA research monograph, (1990) 96 192-219, Ref: 71.
7. Wong JB, McQuillan GM, McHutchinson JG, et al. Estimating future hepatitis C morbidity, mortality and costs in the United states. Am J Public health 2000; 90: 1562 - 1569.
8. World health Organization. Hepatitis C fact Sheet 164, 2000.

DIAGNOSTIC STUDIES ON THE ETIOLOGICAL ROLE OF RESPIRATORY SYNCYTIAL VIRUS AND INFLUENZA VIRUSES IN HOSPITALIZED CHILDREN

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SUMMARY

Respiratory syncytial virus (RSV) role as causative agent of severe respiratory system diseases in young children, is a priority problem world-wide. Influenza viruses annual epidemic incidence also has an impact over the morbidity rate in this age group. In the period October 2005 - April 2006 in the Laboratory of Influenza and Acute Respiratory Diseases (ARD) comparative studies have been conducted for RSV and influenza viruses on 359 samples as a total originating from hospitalized children up to the age of 5. There is no parallelism between the virologic and serologic investigations in one and the same children. In the virologic investigation [isolation, rapid immunosorbent tests and immunofluorescence assay (IFA)] of 174 samples 14 (8,04%) have proved RSV positive, and 10 (5,7%) have been positive for influenza viruses type A. In the ELISA IgG testings of 185 single serum pairs, 42 (22,7%) RSV positive, and 52 (28,1%) influenza viruses type A positive results have been obtained. The results obtained confirm the reference literature data for the RSV and influenza viruses concurrent circulation among the age group indicated. The present survey enrich the diagnostic studies as regards the etiological role of these viruses in childhood by contemporary data, and promotes (initiates) competent collaboration between virologists and pediatricians.

Key words: hospitalized children, RSV, influenza viruses, diagnostics

INTRODUCTION

Despite the great variety of viral etiological agents of acute respiratory diseases, respiratory syncytial virus (RSV) is of principal role in childhood. Influenza viruses annual epidemic incidence also has an impact over the morbidity rate in this age group. RSV role as etiologic agent of severe respiratory system diseases in young children, is a priority problem world-wide. According to reference literature data, RSV outbreaks coincide with the influenza viruses epidemic incidence. /11,12/ The prematurely born infants, as well as those with chronic pulmonary diseases or congenital heart diseases are among the high-risk groups, in which the diseases take particularly severe course.

Among the European Influenza Surveillance Scheme (EISS) priorities for 2003, diagnostic studies on respiratory syncytial viral infection have been included as well. This is determined by the fact that, together with the influenza disease, the RSV infection represents a serious problem of great social and economic importance, requiring the development of adequate scheme for control./9/ EISS underlines the importance of data collection regarding the incidence of this infection, likewise, for the separate European countries, that would enrich the knowledge in this field.

In 2002 the Laboratory of Influenza and Acute Respiratory Diseases (ARD) to the National Center of Infectious and Parasitic Diseases participated in an international control, organized by EISS, including investigation of a diagnostic panel, the latter comprising RSV likewise, besides influenza viruses. Since 2003, in connection with EISS requirements, the laboratory set itself the task of introducing contemporary methods for the diagnostics of this virus as well.

In 2004 -2005 we participated in a project on the subject: "Determination the role of infection caused by respiratory syncytial virus /RSV/ among high risk born infants", a joint one with the Clinic of Neonatology, Medical University Sofia and sponsored by the Medical University. With this project we laid the beginnings of a contemporary diagnostic scheme for the investigations in this field, and the beginnings of the collaboration between virologists and pediatricians.

An objective of the present work is a study of RSV and influenza viruses role as etiologic agents of respiratory diseases among hospitalized children up to the age of 5, tested in the period October 2005 - April 2006 with the application of a complex of virologic and serologic methods.

MATERIALS AND METHODS:

Nasopharyngeal swabs and bronchoalveolar aspirates. The materials have been taken up to the fifth day from the beginning of the disease using standard methodology. /6, 8, 10/

Viral agents isolation. Two laboratory models are used for influenza viruses isolation (chicken embryos and MDCK cell line), and for RSV isolation - HEp-2 cell line. /8/

Rapid immunosorbent tests for viral antigens determination. Directigen Flu A+B and Directigen RSV are used. /7/

Modification of direct immunofluorescence assay (IFA) with preliminary multiplication of the viruses from the original materials on cell lines for 48 h in microvolume on chamber-slides. /6/

ELISA IgM и IgG for influenza viruses and ELISA IgM and IgG for RSV. Original kits of IBL Germany are used with fixed specific antigens on the solid carrier. The testing of the sera from patients is made in compliance with the reaction stages, indicated by the manufacturer.

RESULTS AND DISCUSSION

In the last years in the Laboratory of Influenza and Acute Respiratory Diseases (ARD) a variety of diagnostic methods for influenza viruses diagnostics, different modifications of the techniques listed, as well as new contemporary methods for viral antigens and nucleic acids identification, is applied. /8/ The necessity of precise respiratory diseases diagnostics, especially in childhood, imposes complex approach as regards RSV as well.

In the period October 2005 - April 2006 for the parallel RSV and influenza viruses determination a total of 359 samples from hospitalized children up to the age of 5 have been investigated virologically and serologically. On Table 1 is shown that the samples investigated are of children with acute respiratory disease (ARD) symptoms, the predominating symptomatology originating from the lower respiratory tract.

Table 1. Clinical diagnosis of the investigated children

Diagnosis	Number of investigated children	
	Virologically	Serologically
Bronchiolitis acuta	26	20
Pneumonia	65	73
Bronchiolitis acuta + pneumonia	10	7
Tracheobronchitis acuta	3	0
Acute Respiratory Diseases	70	85
Total sample number	174	185

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ABBREVIATIONS USED IN THIS PAPER: RSV-Respiratory syncytial Virus; ARD-Acute Respiratory Diseases

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Table 2. Viral isolation and detection of RSV and influenza viruses among children up to the age of 5 for the period 10.2005 - 04.2006

Place for sample obtaining	Number investigated samples	CPE /Isolation		Directigen Months X, XI, XII		IFA Months X, I, II, III, IV	
		RSV	Influenza viruses	RSV	Influenza viruses	RSV	Influenza viruses
Cardiological hospital - Sofia	33	8/0	5/0	1	3	0	5
II city hospital-Sofia	141	8/0	6/0	3	0	10	2
Total sample number	174	16/0	11/0	4	3	10	7

In view of viruses isolation on cell cultures, the nasopharyngeal swabs and bronchoalveolar aspirates from the ill children have been taken up to the fifth day from the beginning of the disease, two types of cell cultures /MDCK and Hep-2/ being inoculated by them. For influenza viruses isolation chicken embryos are used as well. In RSV and influenza viruses determination the rapid immunosorbent tests (Directigen RSV and Flu A+B) and IFA have been applied, following the samples inoculation on cell cultures for 48 h. On Table 2 are indicated the results obtained from the viral isolation and detection, and on Figure 1 their graphic presentation. The poorer virologic determination of the causative agents among the group indicated is demonstrated. In the investigation of a total of 174 samples, 14 (8,04%) have been RSV positive, and 10 (5,7%) influenza viruses type A positive. The positive results were received using two viral detection methods - Directigen in the beginning of the period, and IFA in the rest part of the period. Unfortunately, despite the passages performed, isolation of RSV and influenza viruses from the hospitalized children has not been achieved. We consider that the reason for the lack of isolation among the age group tested is related to the impossibility of the performance of adequate number of passages on the materials demonstrating cytopathic effect (CPE), because of the presence of collateral (lateral) antibiotic-resistant microflora.

Samples, taken after the fifth day from the beginning of the disease have been tested serologically by the ELISA IgM and IgG method. The results of these investigations are shown on Table 3 and Figure 2. In the testings of 185 single serum samples 42 (22,7%) RSV positive results, and 52 (28,1%) influenza viruses type A positive results, have been obtained. The positive results refer to the determination of late IgG antibodies. In 33 children IgG antibodies both for RSV, and influenza viruses have been determined concurrently, which confirms the fact that the respiratory morbidity in early childhood is due to more than one etiologic agent. Only in 4 children early IgM influenza viruses type A antibodies have been determined. The great number IgG antibodies positive samples most probably is due to the late sera sampling. They present a confirmation of pulling through the illness in the course of the period indicated, but cannot be admitted as confirmation of an etiological diagnosis of the disease, having provoked the hospitalization.

Figures 1 and 2 demonstrate the similar course of the two curves, reflecting the RSV and influenza viruses positive results, which confirms the reference literature data for the coincidence of the epidemic waves of influenza and RSV./ 11/

For the period indicated there is no parallelism between the virologic and serologic investigations in one and the same children, i.e. the samples tested by the two methods originate from different patients. The latter does not permit a complete interpretation to be made at the present stage of the results achieved for the concrete patient.

Studies over the RSV infection distribution in the country in the past have been conducted by a number of authors, e.g. Mandulov and Gatcheva /5/; Dobrev, Mihailov and Karaivanova /4/; Arnaudova-Todorova and al. /1/; Velitchkova, E. /2/. In serologic studies conducted in the 60-ies and in the 80-ies, the Bulgarian authors confirm RSV incidence from 12% to 22,9% in young children. The virologic investigations in the country during the period indicated determine RSV in much lower percentages, from 1.5% to 2.3%. These studies refer to the RSV etiology solely, and are performed on the grounds mainly of classical methods for determination of a raise in the antibodies titer by the complement fixation test (CFT) in double serum samples, immunofluorescence assay (IFA) with polyclonal specific antisera and virus isolation on cell cultures.

In the last years D. Dimova employed for the first time rapid immunosorbent tests in the studies on the respiratory syncytial virus infection in early childhood and conducted comparative serologic studies for influenza viruses and RSV. In these investigations higher percentages of the influenza viruses positive results have been determined compared to (versus) RSV (45,5% : 28,8%) through the application of classical serologic methods - HIT and CFT. /3/

The diagnostic scheme proposed by us comprises the employment of a contemporary complex of methods for viruses' virologic and serologic determination. The Reverse Transcriptase -Polymerase Chain Reaction (RT-PCR), a routine method in influenza viruses diagnostics, is coming into the practice world-wide for RSV infection diagnostics as well. The advantage of this method is its poor affection

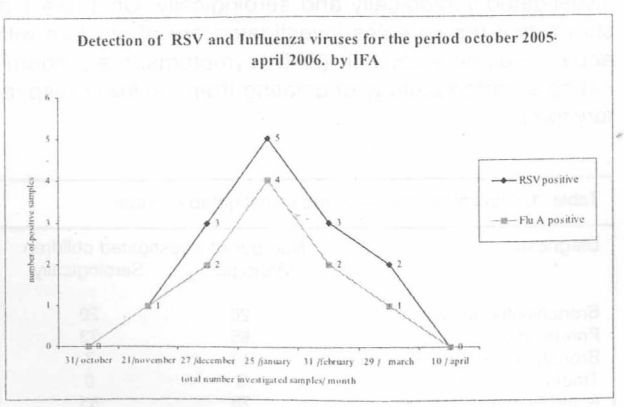

Fig. № 1

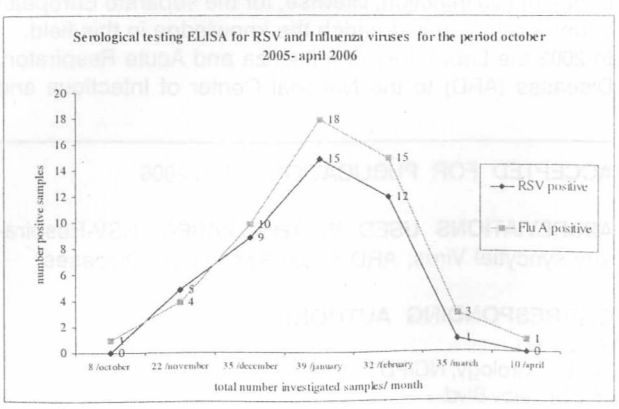

Fig. № 2

Table 3. Serological testing of sera by ELISA IgM and IgG from children up to the age of 5 for the period 10.2005 - 04.2006

Place for sample obtaining	Number investigated samples		Serologically detected samples by ELISA IgM		Serologically detected samples by ELISA IgG
		RSV	Influenza viruses	RSV	Influenza viruses
Cardiological hospital - Sofia	85	0	1	1016	
II city hospital - Sofia	100	0	3	32	36
Total sample number	185	0	4	42	52

by viable viruses presence in the original materials, the latter being of essential importance for the successful application of the other methods. RT-PCR is introduced in our laboratory practice for influenza diagnostics, but because of its high price, it is applied mainly for purposes of research. The method has been used successfully for RSV outbreak etiology detection in the nursing home in Pazardjik city in 2005./8/

The present study initiates useful and necessary for the country diagnostic studies regarding influenza viruses and RSV incidence in childhood, as well as competent collaboration between virologists and pediatricians. The results obtained confirm the reference literature data for the RSV and influenza viruses concurrent circulation among the age group indicated. Because of the frequently obscured, and to a great extent analogous clinical manifestations, the diagnostic differentiation of the latter is of particular importance, especially in connection with the therapy and prophylaxis of these diseases.

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REFERENCES

1. Арнаудова-Тодорова В., Шапкарева К., Василева М. Участие на респираторно-синцитиалните инфекции при дихателни заболявания у деца; Педиатрия 1970;IX(1):16-22.)
2. Величкова, Е. София; 1970 докторска дисертация.
3. Димова Д. Докторска дисертация, София 2005г
4. Добрев, И., Караиванова Г., Михайлов А. Етиология на острите респираторни заболявания през периоа 1976-1978 г. у нас; Епидемиология, микробиология и инфекциозни болести 1980;XVII(1):40-9.;
5. Мандулов В. и Гачева М. Инфекции причинени от респираторно-синцитиални вируси Епидемиология, микробиология и инфекциозни болести 1967;IV(3):225-30,)
6. Павлова С., Хаджиолова Т., Коцева Р., Младенова З., Дундарова Д. Модификация на имунофлуоресцентния метод за доказване на грипни вируси в клетъчни култури, сп. Инфектология Vol. XLI бр.3, 2004г, стр. 28-31
7. Павлова Сл., Хаджиолова Т., Коцева Р; Приложение на експресен имуноензимен метод Directigen Flu A+B и Directigen RSV за определяне на антигените на грипните вируси и RSV; Сборник II Национален Конгрес по Имунология, София, 2003г
8. Хаджиолова Т., докторска дисертация, София 2006г.
9. David M. Whiley et al., Detection of Human Respiratory Virus in samples by LightCycler Reverse Transcriptase PCR, J of Clinical Microbiology, Dec.2002, p.4418-4422
10. Hadzhiolova T., Pavlova S., Kotseva R. Possibilities for laboratory diagnosis of respiratory syncytial virus. Biotechnology and Biotechnological equipment] 2/ 2005/19, pp.67-71
11. Hector S.; Izurieta Influenza end the rares of hospitalization for respiratoriy disease among infants and young children The New England Journal of Medicine; 232-239;2000/
12. M. van Woensel J. B., C van Aelderen W. M.; Viral lower respiratory tract infection in infants and young children; BMJ 2003;327;36-40

USE OF NEURAMINIDASE INHIBITORS AND RESISTANCE IN PERSPECTIVE

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SUMMARY

Of the four antiviral drugs currently available for the treatment of influenza A infection (two neuraminidase inhibitors and two M2 ion channel inhibitors), only the neuraminidase inhibitors oseltamivir and zanamivir are also active against influenza B. All drugs are most effective if started within a few hours of the onset of symptoms and are generally licensed for use within 48 hours of the first symptoms. The neuraminidase inhibitors, oseltamivir and zanamivir, have fewer side effects than the M2 ion channel inhibitors rimantadine and amantadine, and drug resistance seems to develop less frequently. Numerous treatment studies in healthy adults have shown that neuraminidase inhibitors, when taken within 36 to 48 hours after the onset of symptoms, decrease the symptomatic illness by one or two days (Hayden 1997, Monto 1999, Treanor 2000, Cooper 2003, Whitley 2001, Aoki 2003). Early initiation of treatment is decisive for treatment efficacy (Aoki 2003, Kawai 2005). When started within the first 12 hours following the onset of fever, neuraminidase inhibitors shortened the illness by more than three days, in comparison to treatment that was started at 48 hours. The duration of fever, severity of symptoms, and time to return to normal activity also correlated with the time of initiation of antiviral intervention. The introduction of neuraminidase inhibitors was an important step for the more efficient control of human influenza infection. Today, neuraminidase inhibitors are the only drugs effective against recently isolated highly pathogenic avian influenza viruses in humans.

Key words: Neuraminidase Inhibitors, Influenza Viruses

Of the four antiviral drugs currently available for the treatment of influenza A infection (two neuraminidase inhibitors and two M2 ion channel inhibitors), only the neuraminidase inhibitors oseltamivir and zanamivir are also active against influenza B. All drugs are most effective if started within a few hours of the onset of symptoms and are generally licensed for use within 48 hours of the first symptoms. They can modify the severity of illness, as well as reducing the intensity of influenza symptoms and decreasing the duration of illness by about 1 to 3 days. Treatment success is a variable of the time between the onset of symptoms and the beginning of antiviral treatment. (Kamps 2006)

The antiviral properties of the older anti-influenza drugs, amantadine and the structurally-related molecule rimantadine target influenza A protein - the M2 ion channel. Function of this channel is required for viral penetration, uncoating and final assembly of the progeny viruses (Aoki 1998). A similar ion channel, the NB protein, is present in influenza B viruses, but not all of its function is blocked by amantadine or rimantadine.

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Because the influenza M2 protein may contain natural mutations that are resistant to the action of amantadine or rimantadine, the reported frequency of resistance during clinical trials with these compounds is significantly higher than that observed with neuraminidase inhibitors (NAIs) (30% of treated patients may have amantadine- and rimantadine-resistant viruses compared with just under 2% for some NAI drug (Hayden 1992, Monto 1992). Mutations in rimantadine-resistant virus are not associated with any detectable loss in viral function - both infectivity and pathogenicity of resistant virus in animals are indistinguishable from wild type (Hayden 1992). Virus resistant to M2 inhibitors is readily transmissible between humans; this is supported by reports of failure to control outbreaks of influenza in families and nursing homes where amantadine or rimantadine prophylaxis and treatment have been used simultaneously (Hayden 1989, Mast 1991).

The neuraminidase inhibitors, oseltamivir and zanamivir, have fewer side effects than the M2 ion channel inhibitors rimantadine and amantadine, and drug resistance seems to develop less frequently. These drugs introduced in 1999 and 2000 interfere with the normal function of the influenza neuraminidase by mimicking sialic acid, the natural substrate of the neuraminidase (Varghese 1992, Varghese 1995). The viral neuraminidase is responsible for cleaving sialic acid residues on newly formed virions, playing an essential role in their release and facilitating virus spread within the respiratory tract. The enzyme may contribute to viral pathogenicity and may induce cellular apoptosis and release of pro-inflammatory cytokines (Calfee 1998). When exposed to neuraminidase inhibitors, the influenza virions aggregate on the surface of the host cell, limiting the extent of infection within the mucosal secretions (McNicholl 2001) and reducing viral infectivity. Experimental evidence further suggests that influenza neuraminidase may be essential at the early stage of virus invasion of the ciliated epithelium of human airways (Matrosovich 2004). The design of the neuraminidase inhibitors is a result of the analysis of the three dimensional structure of influenza neuraminidase which disclosed the location and structure of the catalytic site (Colman 1983).

Numerous treatment studies in healthy adults have shown that neuraminidase inhibitors, when taken within 36 to 48 hours after the onset of symptoms, decrease the symptomatic illness by one or two days (Hayden 1997, Monto 1999, Treanor 2000, Cooper 2003, Whitley 2001, Aoki 2003). Early initiation of treatment is decisive for treatment efficacy (Aoki 2003, Kawai 2005). When started within the first 12 hours following the onset of fever, neuraminidase inhibitors shortened the illness by more than three days, in comparison to treatment that was started at 48 hours. The duration of fever, severity of symptoms, and time to return to normal activity also correlated with the time of initiation of antiviral intervention.

Oseltamivir (fig. 1a) is indicated for the treatment of uncomplicated acute illness due to influenza infection in patients aged 1 year and older who have been symptomatic for no more than 2 days. The recommended duration of treatment with oseltamivir is 5 days (but may be longer in severe H5N1 infection). Tamiflu(r) is currently the drug of choice for the treatment of human H5N1 influenza. Prevention trials have shown that oseltamivir administered prophylactically reduces the risk of developing influenza by 60-90 % when given at the start of the influenza outbreak (Monto 1999b, Cooper 2003). When administered prophylactically to household contacts of an influenza index case, protective efficacy against clinical influenza was generally > 80 % (Welliver 2001). Oseltamivir is generally well-tolerated. A 7-day course is indicated for the prophylaxis of influenza in

Table 1. Influenza mutant viruses resistant to neuraminidase inhibitors

Mutation	Drug	Reduction (-fold) in NA sensitivity	Reduction (-fold) in animal nfectivity	Source	Virus	References
Glu119Gly	Zanamivir	> 200	≤ 4	In vitro	A/H1N9/	(Blick1995, Gubareva 1996)
Glu119Gly	Zanamivir	> 200		In vitro	B	(Blick1995)
Glu119Ala	Zanamivir	> 300		In vitro	A/H4N2/	(Gubareva 1997)
Glu119Asp	Zanamivir	> 2500		In vitro	A/H4N2/	(Bantia 2000)
Glu119Val	Oseltamivir	> 200 - 400	> 100 - 1000	Clinical	A/H3N2/	
Arg292Lys	Oseltamivir	50- ~ 85 000	> 100 - 1000	In vitro and clinical	A/H3N2/	
Arg292Lys	Zanamivir	~ 10 - 30	~ 400	In vitro	A/H4N2/	(Bantia 2000)
Arg292Lys	RWJ-270201	~ 10 - 20		In vitro	A/H2N2/	(Ives 2000)
Arg152Lys	Zanamivir	> 1000	~ 60	Clinical	B	(Staschke 1995)
His274Tyr	Oseltamivir	> 70	> 100 - 1000	In vitro and clinical	A/H1N1/	

NA = neuraminidase

the age group: ≥ 13 years. Transient gastrointestinal disturbance (nausea, vomiting) is the major adverse effect of oseltamivir. Rarely, with oseltamivir, serious skin/hypersensitivity reactions may occur, and patients should, therefore, be cautioned to stop taking oseltamivir and contact their healthcare providers if they develop a severe rash or allergic symptoms (FDA 2005).

Zanamivir (fig. 1b) is indicated for the treatment of uncomplicated acute illness due to influenza infection in patients aged 7 years and older and who have been symptomatic for no more than 2 days. With the exception of two countries, zanamivir has not been licensed for prophylactic use. The treatment duration is usually 5 days. Zanamivir is delivered by inhalation and is well tolerated; however, children, especially those under 8 years old, are usually unable to use the delivery system appropriately and elderly people may have difficulties, too (Diggory 2001). Bronchospasm and a decline in lung function have been reported in some patients with underlying pulmonary conditions, such as asthma or chronic obstructive pulmonary disease. Zanamivir is therefore not generally recommended for the treatment of patients with underlying airways disease, and should also be discontinued in patients who develop bronchospasm or who have a decline in respiratory function (Relenza 2003).

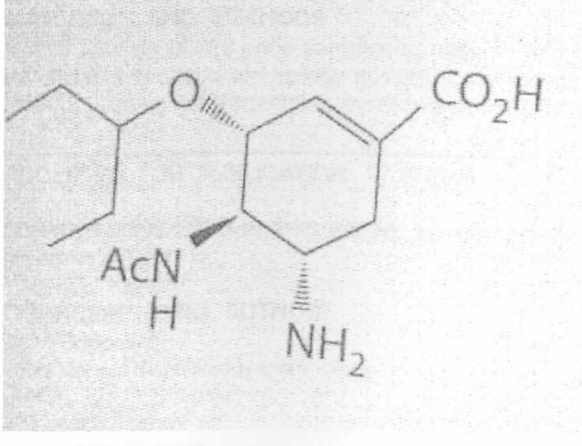
According to recent investigations resistant mutants appear rarely in some laboratory experiments and clinical trial after treatment of NA inhibitors. For example, during the clinical trials of oseltamivir, considerable persistence (repeated swabbing of the nose and throat during treatment) was needed to identify resistant virus in patient's specimens. Resistant viruses arose late in the 5-day treatment period (not before the forth day of treatment and usually later), and were often present in a mixture with wild-type virus where wild-type predominated. A summary of influenza mutant viruses resistant to NAIs is presented in Table 1.

Resistance to NAIs arises through point mutations in the viral RNA leading to a single amino acid change in or close to the active site of the NA enzyme (Mendel 1998). Three different single mutations have been identified in influenza A viruses collected from adults and children treated with oseltamivir and the other NAIs.

* The predominant mutation in the viral NA giving resistance in H3N2 virus is Arg292Lys (Covington 2000). The same mutation was also observed in laboratory experiments conducted with zanamivir (McKimm-Breschkin 2000).
* A lower incidence of NA mutation Glu119Val has also been seen following oseltamivir treatment of patients with influenza A H3N2 (Covington 2000). Different mutations at position 119 have also been observed in laboratory experiments conducted with zanamivir (McKimm-Breschkin 2000).
* A His274Tyr NA mutation has been described in individuals experimentally infected with influenza A H1N1 virus and treated with oseltamivir (Gubareva 2000).
* No mutations have been identified in oseltamivir-treated patients with influenza B (Hayden 2000).
* One zanamivir-induced mutation (Arg152Lys) has been identified in a day-12 isolate from an immunosuppressed child infected with influenza B (Gubareva 1998).

Studies in animal models of influenza have supported the clinical trial data, indicating that oseltamivir carboxylate-resistant viruses are significantly less virulent than wild-type virus. Mutant viruses show reduced infectivity, and

a. Oseltamivir carboxylate (Tamiflu)



b. Zanamivir (Relenza)

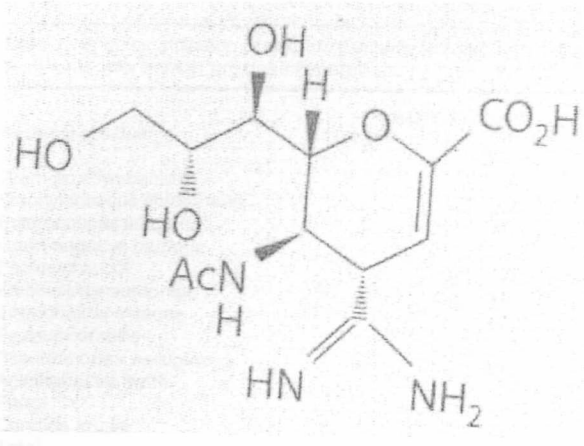


Figure 1. Chemical structure of neuraminidase inhibitors

early studies suggest that they also have reduced pathogenicity and transmissibility (Mendel 1998). All three mutants isolated from individuals treated with oseltamivir have reduced infectivity/replicative ability by at least 100- to 1000-fold in mice and ferrets (Ives 2000, Carr 2000). This might be expected as the mutations are in the otherwise highly conserved active site and hence would be expected to impair NA function and viral fitness.

The introduction of neuraminidase inhibitors was an important step for the more efficient control of human influenza infection. Today, neuraminidase inhibitors are the only drugs effective against recently isolated highly pathogenic avian influenza viruses in humans. In the case of a future pandemic, antiviral drugs may play an important role in the early phase, when vaccines against the new strain are not yet available or as long as the available vaccine is in short supply.

REFERENCES:

1. Aoki FY, Macleod MD, Paggiaro P, et al. Early administration of oral oseltamivir increases the benefits of influenza treatment. J Antimicrob Chemother 2003; 51: 123-9.
2. Aoki FY. Amantadine and rimantadine. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of influenza. Oxford: Blackwell Science, 1998: 457 - 476.
3. Bantia S, Ananth S, Horn L, et al. Generation and characterization of a mutant of influenza A virus selected with the neuraminidase inhibitor RWJ-270201, Antiviral Res, 2000; 46: A60.
4. Blick TJ, Tiong T, Sahasrabudhe A, et al. Generation and characterization of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en, Virology, 1995; 214: 457 - 484.
5. Calfee DP, Hayden FG. New approaches to influenza chemotherapy. Neuraminidase inhibitors, Drugs, 1998; 56: 537 - 553.
6. Carr J, Ives J, Roberts N, et al. Virological assessment in vitro and in vivo of an influenza H1N1 virus with a H274Y mutation in the neuraminidase gene, Antiviral Res, 2000; 46: A59
7. Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature 1983; 303: 41-4.
8. Cooper NJ, Sutton AJ, Abrams KR, Wailoo A, Turner D, Nicholson KG. Effectiveness of neuraminidase inhibitors in treatment and prevention of influenza A and B: systematic review and meta-analyses of randomised controlled trials. BMJ 2003; 326: 1235.
9. Covington E, Mendel DB, Escarpe P, et al. Phenotypic and genotypic assay of influenza virus neuraminidase indicates a low incidence of viral drug resistance during treatment with oseltamivir [#P-326], J Clin Virol, 2000; 18: 253.
10. Diggory P, Fernandez C, Humphrey A, Jones V, Murphy M. Comparison of elderly people?s technique in using two dry powder inhalers to deliver zanamivir: randomised controlled trial. BMJ 2001; 322: 577-9.
11. FDA . Food & Drug Administration. FDA Approves Tamiflu for Prevention of Influenza in Children Under Age 12. 2005
12. Gubareva LV, Bethell RC, Hart GJ, et al. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en, J Virol, 1996; 70: 1818 - 1827
13. Gubareva LV, Matrosovich MN, Brenner MK, et al. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus, J Infect Dis, 1998; 178: 1257 - 1262.
14. Gubareva LV, Robinson MJ, Bethell RC, et al. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en, J Virol, 1997; 71: 3385 - 3390.
15. Gubareva LV, Tai CY, Mendel DB, et al. Oseltamivir treatment of experimental influenza A/Texas/36/91 (H1N1) virus infection in humans: selection of a novel neuraminidase variant, Antiviral Res, 2000; 46: A59.
16. Hayden FG, Belshe RB, Clover RD, et al. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families, N Engl J Med, 1989; 321: 1696 - 1702.
17. Hayden FG, Hay AJ. Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine, Curr Top Microbiol Immunol, 1992; 176: 119 - 130.
18. Hayden FG, Jennings L, Robson R, et al. Oral oseltamivir in human experimental influenza B infection, Antiviral Ther, 2000; 5: 205 - 213.
19. Hayden FG, Osterhaus AD, Treanor JJ, et al. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenzavirus infections. N Engl J Med 1997; 337: 874-80.
20. Ives J, Carr J, Roberts N, et al. An oseltamivir treatment selected influenza A/N2 virus with a R292K mutation in the neuraminidase gene has reduced infectivity in vivo [abstract #P-321], J Clin Virol, 2000; 18: 251
21. Ives J, Carr J, Roberts N, et al. An oseltamivir treatment-selected influenza A/Wuhan/359/95 virus with E119V mutation in the neuraminidase gene has reduced infectivity in vivo [#P-330], J Clin Virol, 2000; 18: 255.
22. Kamps B, Hoffmann C, Preiser W. Influenza Report 2006. Flying Publisher
23. Kawai N, Ikematsu H, Iwaki N, et al. Factors influencing the effectiveness of oseltamivir and amantadine for the treatment of influenza: a multicenter study from Japan of the 2002-2003 influenza season. Clin Infect Dis 2005; 40: 1309-16.
24. Mast EE, Harmon MW, Gravenstein S, et al. Emergence and possible resistance of amantadine-resistant viruses during nursing home outbreaks of influenza A (H3N2), Am J Epidemiol, 1991; 134: 988 - 997.
25. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J Virol 2004; 78: 12665-7
26. McKimm-Breschkin JL. Resistance of influenza viruses to neuraminidase inhibitors - a review, Antiviral Res, 2000; 47: 1 - 17.
27. McNicholl IR, McNicholl JJ. Neuraminidase inhibitors: zanamivir and oseltamivir. Ann Pharmacother 2001; 35: 57-70.
28. Mendel DB, Sidwell RW. Influenza virus resistance to neuraminidase inhibitors, Drug Resist Updates, 1998; 1: 184 - 189
29. Monto AS, Fleming DM, Henry D, et al. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza A and B virus infections. J Infect Dis 1999; 180: 254-61.
30. Monto AS, Robinson DP, Herlocher ML, Hinson JM Jr, Elliott MJ, Crisp A. Zanamivir in the prevention of influenza among healthy adults: a randomized controlled trial. JAMA 1999; 282: 31-5.
31. Monto AS. Implications of viral resistance to amantadine in control of influenza A, Clin Infect Dis, 1992; 15: 362 - 367.
32. Relenza (zanamivir for inhalation). Research Triangle Park, NC: GlaxoSmithKline, 2003
33. Staschke KA, Colacino JM, Baxter AJ, et al. Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2en, Virology, 1995; 214: 642 - 646.
34. Treanor JJ, Hayden FG, Vrooman PS, et al. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. US Oral Neuraminidase Study Group. JAMA 2000; 283: 1016-24.
35. Varghese JN, Epa VC, Colman PM. Three-dimensional structure of the complex of 4- guanidino-Neu5Ac2en and influenza virus neuraminidase. Protein Sci 1995; 4: 1081-7.
36. Varghese JN, McKimm-Breschkin JL, Caldwell JB, Kortt AA, Colman PM. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins 1992; 14: 327-32.
37. Welliver R, Monto AS, Carewicz O, et al. Effectiveness of oseltamivir in preventing influenza in household contacts: a randomized controlled trial. JAMA 2001; 285: 748-54.
38. Whitley RJ, Hayden FG, Reisinger KS, et al. Oral oseltamivir treatment of influenza in children. Pediatr Infect Dis J 2001; 20: 127-33.

ANTIMICROBIAL SUSCEPTIBILITY OF URINE PATHOGENES ISOLATED FROM PATIENTS WITH BENIGN PROSTATIC HYPERPLASIA TREATED WITH UROSTIM

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SUMMARY

Benign prostatic hyperplasia (BPH) is one of the most frequent reasons for recurring infections of the urinary tract. To determine the antibiotic susceptibility of microorganisms isolated from urine of patients with BPH and the results of immunotherapy with urostim. A total number of 126 urine samples of patients with BPH treated in the Clinic of Urology during 2005, were examined. Isolated strains were identified by conventional methods. E. coli and K. pneumoniae strains were screened for ESBLs production. Susceptibility to antibiotics was determined by using disc diffusion method of Bauer-Kirby, according to NCCLS. Immunotherapy with urostim for a 3-month period was applied to 36 patients and the urine was monitored on a monthly basis. The patients were divided into two groups: Group I - 12 patients with asymptomatic bacteriuria treated with urostim only, and Group II - 24 patients with symptomatic bacteriuria treated with urostim and antibiotic. Fifty strains were isolated from 40 patients with significant bacteriuria and pyuria. The most frequently isolated microorganisms were: E. coli (42%), K. pneumoniae (18%) and E. faecalis (12%). Producers of ESBLs among E. coli strains were 61.9%, and among K. pneumoniae - 77.8%. The E. coli and K. pneumoniae strains remained susceptible to imipenem and meropenem. The E. faecalis strains were susceptible to vancomycin and teicoplanin. After conducted treatment liquidation of the uroinfection were registered in 10 (83.3%) patients from Group I and in 18 (75.0%) patients from Group II. The bacteriuria remained persistent in 2 (16.7%) patients from Group I and in 6 (25%) patients from Group II. The immunotherapy with urostim does not exclude treatment with antibiotics in order to achieve highest therapeutic results

Key words: Benign prostatic hyperplasia (BPH), antimicrobial susceptibility, urostim.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is one of the most frequent reasons for recurring infections of the urinary tract (6).

AIMS

To determine the antibiotic susceptibility of microorganisms isolated from urine of patients with BPH and the results of immunotherapy with urostim.

MATERIALS AND METHODS

A total number of 126 urine samples of patients with BPH treated in the Clinic of Urology in 2005, were examined. Isolated strains were identified by conventional methods. E. coli and K. pneumoniae strains were screened for ESBLs production. Susceptibility to antibiotics was determined by using disc diffusion method of Bauer-Kirby, according to NCCLS. Immunotherapy with urostim was applied to 36 patients for a 3-month period and the urine was monitored on a monthly basis.

The patients were divided into two groups: Group I - 12 patients with asymptomatic bacteriuria treated with urostim only, and Group II - 24 patients with symptomatic bacteriuria treated with urostim and antibiotic.

RESULTS

Fifty strains of uropathogens were isolated from 40 patients with significant bacteriuria and pyuria. The species affiliation of the isolated microorganisms is presented in table 1. The most frequently isolated microorganisms were: E. coli (42.0%), K. pneumoniae (18.0%), and E. faecalis (12.0%), followed by E .cloacae (8.0%), P. mirabilis (6.0%), S. epidermidis (6.0%), P. aeruginosa (4.0%), A. lwoffii (2.0%), and C. krusei (2.0%).

Producers of ESBLs among - E.coli strains were - 13 (61.9%), and among K. pneumoniae - 7 (77.8%).

The resulys from the antimicrobial susceptibility testing of the most frequently isolated microorganisms are presented in fig. 1, 2 and 3. E. coli strains (fig. 1) were highly resistant to ampicillin, nalidix acid (90.5% and 90.5%); to cephalosporins II and III generations (61.9% and 61.9%). This could be explained by their ESBLs production. High levels of resistant strains were observed for gentamicin, TMP/SMZ (66.7% and 66.7% respectively); and ciprofloxacin (71.4%). In support of our study are the results from other authors [4, 5, 7]. All E.coli strains were susceptibility to carbapenems and amikacin which allows their application in cases of multiple resistance to the other antimicrobials. Imepenem and meropenem remain highly active to ESBLs E.coli strains [9].

Strains of E. coli, causative agent of ESBL showed high level of resistance towards Amoxicillin/Clavulanate and towards Cephalosporins from 2nd and 3rd generation

K. pneumoniae strains (fig. 2) were highly resistant to ampicillin, nalidix acid (100% and 100% respectively); to cephalosporins II and III generations (77.8% and 77.8% respectively). This could be explained by their ESBLs production. High level percentage of resistant strains was observed for gentamicin, ciprofloxacin and TMP/SMZ (77.8%, 77.8% and 77.8% respectively). Comparatively low percentage of resistant strains was registered for amikacin (22.2%). All K. pneumoniae strains were susceptible to imipenem and meropenem.

The isolated strains of E. faecalis (fig. 3) had high level resistance to ciprofloxacin (100%); comparatively lower to ampicillin (33.4%). All E. faecalis strains were susceptible

Table 1. Species affiliation of microorganisms, isolated from urine of patients with benign prostatic hyperplasia.

Bacterial species	Isolated strains Number	%
Gram-positive bacteria		
Staphylococcus epidermidis	3	6.0
Enterococcus faecalis	6	12.0
Gram-negative bacteria		
Escherichia coli	21	42.0
Klebsiella pneumoniae	9	18.0
Enterobacter cloacae	4	8.0
Proteus mirabilis	3	6.0
Pseudomonas aeruginosa	2	6.0
Acinetobacter lwoffii	1	2.0
Fungi		
Candida krusei	1	2.0
Total	50	100.0

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ABBREVIATIONS USED IN THIS PAPER: Benign prostatic hyperplasia - BPH

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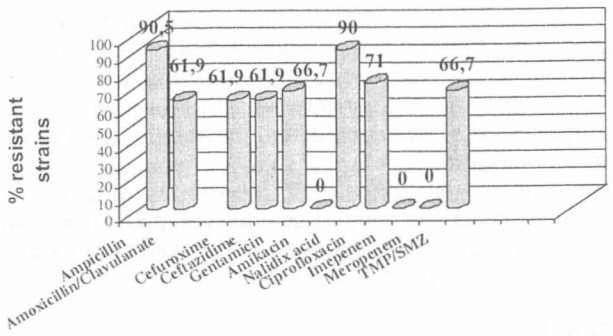

Fig. 1. Antimicrobial resistance of E. coli, isolated from urine of patients with benign prostatic hyperplasia

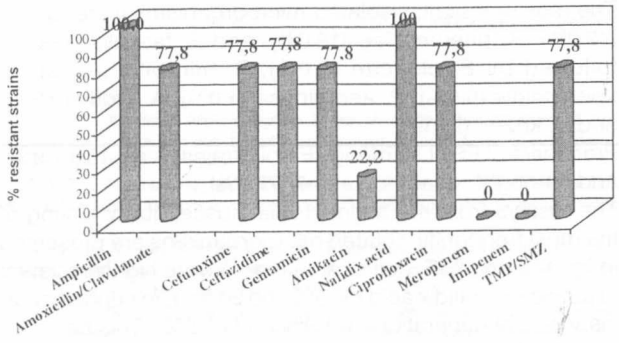

Fig. 2. Antimicrobial resistance of K. pneumoniae, isolated from urine of patients with benign prostatic hyperplasia

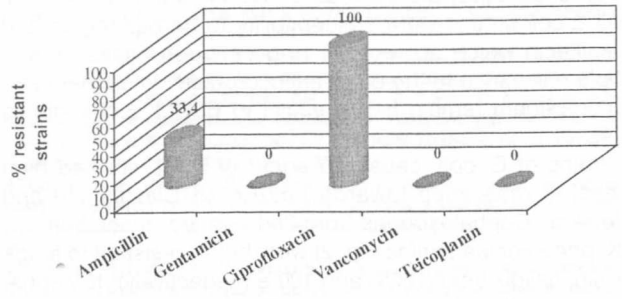

Fig. 3. Antimicrobial resistance of E. faecalis, isolated from urine of patients with benign prostatic hyperplasia.

to, vancomycin, teicoplanin and gentamicin.

Immunotherapy with urostim for a 3-month period was applied to 36 patients and the urine was monitored on a monthly basis.

After conducted treatment liquidation of the uroinfection were registered in 10 (83.3%) patients from Group I and in 18 (75.0%) patients from Group II. The bacteriuria remained persistent in 2 (16.7%) patients from Group I and in 6 (25%) patients from Group II.

CONCLUSIONS

Current knowledge of bacterial adhesion and colonization of organs of excretory system stresses on the necessity to affect the macro and microorganism together in the process of complex treatment of uroinfection in patients with BPH. Presence of multiple resistant strains towards the tested antibiotics gives possibility for combined therapy with an antibiotic and polybacterial immunostimulator Urostim (NCIPD Sofia, Bulgaria), which has wide spectrum effect over most frequent causative agents of uroinfection [1,2,3,]

The most frequently isolated microorganisms were E.coli (42.0%), K. pneumoniae (18.0%),and E. faecalis (12.0%). According to Winokur, et al. (9) the frequently of uropathogenic strains E. coli isolated from Europa were 49.1%, Raveh et al. (8) - E.coli 48.0%, K. pneumoniae (11.0%), P. aeruginosa (9.0%) et Enteroccocus spp.(8.0%).

In Europe for patients with UTI, the frequency of isolation of E.coli is 49.1% [9]. It correlates with the results obtained by authors from Israel regarding most frequently isolated organisms - E.coli (48.0%), K. pneumoniae (11.0%), Enteroccocus spp. (8.0%) [8]. All E. coli strains were susceptibble to carbapenems and amikacin; K.pneumoniae strains - to imipenem and meropenem and all E. faecalis strains were susceptible to vancomycin, teicoplanin and gentamicin. After conducted treatment liquidation of the uroinfection was registered in 10 (83.3%) patients from Group I and in 18 (75.0%) patients from Group II. The bacteriuria remained persistent in 2 (16.7%) patients from Group I and in 6 (25%) patients from Group II.

Presence of effect with cure of uro-infection was established in 10(83.3%) of patients administered with urostim and in 18(75.0%) of patients with urostim plus antibiotic.

The immunotherapy with urostim does not exclude treatment with antibiotics in order to achieve highest therapeutic results.

REFERENCE

1. Митов И., П. Ненков, Й.Цветанов.Полибактериален препарат за перорална имунотерапия и имунопрофилактика на неспецифичните инфекциозни заболявания на урогениталния тракт. Епид.,микроб., инф. болести, 4,1991, 44-50

2. Пенчева П., Б. Зозиков. Полибактериалната ваксина уростим при комплексното лечение на уроинфекциите. Инфектология, 4, 1992, 26-27.

3. Стратев С., В. Григорова, П. Панайотов. Предоперативна имунопрофилактика на раневата и уроинфекцията при урологично болни с уростим. Хирургия, 5, 1993, 9-10.

4. Daza, R., J. Gutierrez, G. Piedrola. Antibiotic susceptibility of bacterial strains isolated from patients with community-acquired urinary tract infections. - International J. of Antimicrobial Agents, 18, 2001, 211-215.

5. Fluit, A. C., F. J. Schmitz, J. Verhoef. European SENTRY Participants. Multi-resistance to antimicrobials agents for the ten most frequently isolate bacterial pathogens. - International J. of Antimicrobial Agents, 18, 2001, 147-160.

6. Garraway, W. M. at al. High prevalence of benign prostatic hypertrophy in the community. Lancet, 338, 1991, 469-471.

7. Lautenbach, E., et al. Epidemiological investigation of fluoroquinolone resistance in infections due to extended-spectrum (-lactamase-producing Escherichia coli and Klebsiella pneumoniae. - Clin. Infect. Dis., 33, 2001, 1288-1294.

8. Raveh, D., et al. Use of time-trend analysis in the design of empirical antimicrobials treatment of urinary tract infections. - Eur. J. Clin. Microbiol. Infect. Dis., 22, 2003, 158-164.

9. Winokur, L. P., et al. Variations in the prevalence of strains expressing an extended-spectrum (-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. - Clin. Infect. Dis., 32 (Suppl. 2), 2001, S94-S103.

A RAPID METHOD FOR DIAGNOSIS OF RARE FORMS OF TULAREMIA - ANALYSIS OF SWABS

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SUMMARY

The current report describes some of our findings during the tularemia outbreak 1997-2005 in Bulgaria. 285 cases were serologically and clinically confirmed for tularemia. Herein we describe the laboratory findings and the diagnosis of oculoglandular forms of infection. Oculoglandular tularemia was diagnosed in four patients by culture, immunofluorescent antibody analysis and polymerase chain reaction assay. Histological methods were applied for characterization of ocular tularemia granuloma. Three F. tularensis strains were isolated and characterized. One strain was isolated from an ocular swab specimen obtained from a seronegative patient. We report for the first time a successful application of diagnostic PCR performed directly on ocular swab specimen. We also describe the histological picture of a conjunctival granuloma in the course of infection. The proposed strategies are not invasive and could represent a new approach for resolving rare and hard-to-diagnose oculoglandular forms of tularemia.

Key Words: Francisella tularensis, Parinaud's syndrome, PCR analysis of eye swab, histology of conjunctival granuloma

INTRODUCTION

Tularemia is a zoonotic disease caused by the gram-negative bacterium Francisella tularensis. It affects a variety of mammalian species, mostly humans, hares and rodents [1]. The microbe survives in protozoa [2]. The Francisellaceae family is in the y subclass of class Proteobacteria and range over closely related microorganisms incorporated within a single genus Francisella with two species F. tularensis and F. philomiragia [3]. F. tularensis with its four subspecies (subsp. tularensis, subsp. holarctica, subsp. mediasiatica and subsp. novicida) is the causative agent of tularemia disease in North America, Europe, Asia and recently in Australia [4]. F. tularensis subsp. tularensis is highly virulent (infectious dose less than 10 CPU) for various mammals including human and is considered by the Centers for Disease Control and Prevention (CDC) a category A potential bio-weapon agent. The rate of mortality in untreated patients can be as high as 30-60% [4]. The genetic basis of its high virulence and pathogenicity is currently unknown. The second species F. philomiragia is an opportunistic pathogen, rarely causing disease particularly in immunocompromised patients and often associated with water.

Depending on the route of entry, tularemia occurs in several clinical forms: ulceroglandular, glandular, oculoglandular, oropharyngeal, intestinal, pneumonic and typhoidal. The oculoglandular form of tularemia is one of most rare - usually approximately 1-4 percent of all cases [5, 6]. Humans may acquire this disease form by direct contact with infected material (contamination by hand or aerosol impaction) and by bites from vectors such as ticks, flies, mosquitoes etc. The intracellular bacterium appears to enter and replicate in macrophages via cytoplasm-B-insensitive pathway and without triggering a respiratory burst [7]. We discuss herein four patients with Parinaud syndrome during the outbreak. Parinaud oculoglandular syndrom is a rare medical condition characterized by granulomatous conjunctivitis, associated with homolateral cervical and/or pre- or retroauricular lymph adenopathy [8]. It has been associated with several infectious diseases [9].

METHODS

All tularemia cases were diagnosed according to the case definitions of the CDC [10]. Clinically relevant information was gathered by interview, referral to hospitals, and questionnaires and sent to general practitioners in the region for submission to the reference centers for epidemiological analysis. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study and the overall study was in accordance with the Declaration of Helsinki. Serum samples for serological assays for specific anti-FranciseHa antibodies were collected from all patients 1 to 4 weeks after the onset of the disease (SI). We obtained second and third samples after 14 days (S2) and 3 months later respectively (S3). All samples were tested with a serum tube agglutination kit (BulBio- NCIPD, Bulgaria). Serial dilutions of sera, starting from 1:20, and antigen prepared from F. tularensis strain Srebarna 19, were mixed in tubes. After overnight incubation at 37°C, the agglutination was evaluated by unaided eye. Serum specimens with 1:80 or higher or at least fourfold titer rise in the convalescent phase were interpreted as positive results (11,12).

Biopsy specimens from cervical lymph nodes (2 patients), conjunctival swab (1 patient), surgically extracted conjunctival granuloma (1 patient), and blood samples (4 patients) were collected for culture and PCR detection.

Half of the volume from each specimen suspension was cultured on modified Thayer-Martin agar plates (containing Gc medium base, hemoglobin and IsoVitaLex [4] at 37°C with 5%CO_2 in air) and the other half was processed for PCR. All manipulations were carried out in a biosafety level 3 cabinet. DNA from biopsy specimens/aspirates (500 µl), blood samples (1 ml) or swabs was isolated following standard phenol-chloroform protocol [13] with slight modifications. 100 ng (5 µl) DNA was submitted to PCR (20 µl volume) with tul4 and RD1 F. tularensis specific primers [14,15].

Direct inimunofluorescence assay (IFA) with FITC-conjugated anti-Francisella sera (BulBio-NCIPD Bulgaria) was used to detect F. tularensis antigens. Preparations (smears) from ocular lymph nodule (patient 3) and smears were finally counterstained with 0.25% Evans blue. The histological techniques were as follows: briefly, 4% p-formaldehyde was used for fixation and 4 µm paraffin embedded tissue sections were microscopically examined.

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ABBREVIATIONS USED IN THIS PAPER: PCR - polymerase chain reaction

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FOOTNOTES:

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Table 1. Diagnostic findings in observed Parinaud patients

	Isolation from eye	Isolation Ly node	PCR eye	PCR Ly node	PCR blood	SI Titer	S2 Titer	S3 Titer	Histology
1	-	+	NA	+	NA	1:320	1:640	1:1280	NA
2	-	+	NA	+	NA	1:160	1:320	1:640	NA
3	-	NA	+(Nodule)	NA	+	-	1:160	1:640	+
4	+	NA	+(Swab)	NA	+	<1:80	<1:80	<1:80	NA

RESULTS

A typical clinical picture of oculoglandular tularemia was observed in all patients.(fig. 1)

Apart from the Parinaud syndrome, two patients exhibited signs of cervical lymphadenopathy. All lymph node and ocular samples from all patients were IFA positive. Except for one patient significant and/or rising agglutination titters were detected in all cases.

Surgically extracted ocular nodule with dimensions 5/2mm was white-yellow by color. We observed an area surrounded by stratified non-keratinized, squamous epithelium with regions of parakeratosis, submembrane region with manifested mononuclear infiltrations, pronounced vascularization and single Langhans cells. A granuloma with central necrosis was detected in the center of the nodule.

The systematic observations of all other diagnostic findings are summarized in table 1.

Direct isolation was achieved from cervical lymph node aspirates in two patients. We did not succeed with attempts for primary direct isolation of the causative agent from the ocular swab and blood specimens. However, F. tularensis was isolated from one patient by i.p. passage of ocular swab suspension (500 ul) in guinea pig. All animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals [17]. F. tularensis was detected by PCR in both types of ocular specimens - swab (patient 4) and surgically extracted nodule (patient 3). Blood samples from the same patients were tested for presence of F. tularensis specific DNA. A positive amplification was observed indicating bacteriaemia (fig.2). All isolates gave characteristic band (900 bp) for ssp. holarctica (RD1 primers- fig.2). In three cases, the consecutive serological samples showed marked titter dynamics, which further supported diagnosis. In Patient 4 seroconversion was not presented and she remained seronegative for 6 months (when the last sample was tested) after successful therapy. In this case there was clinical history of an extensive abdominal intervention shortly before the infection took place. This case was resolved by isolation of the causative agent and a positive PCR with ocular swab and blood specimens.

DISCUSSION:

A large number of studies discussing tularemia outbreaks in various regions of Northern Hemisphere and recently in Australia have been published in the last fifteen years [8]. There are several publications describing outbreaks in the Balkan Peninsula with significant healthcare importance. The first ocular case was reported in Bulgaria in 2003 [16]. Since the ocular forms are extremely rare and the overall index of suspicion is low, they often remain unresolved and underreported. The clinical picture of Parinaud syndrome is non-indicative for the particular causative agent and more often is associated with Bartonella henselae, Mycobacterium tuberculosis or Herpes infections.

To our knowledge the direct detection and/or isolation of F. tularensis from ocular swab material have not been described in the literature. In the last decade PCR assays have proved to be of great clinical and diagnostic value providing rapid and definitive results. Since special biosafety conditions are required for handling F. tularensis and the overall isolation rate is low (appr. 5%), cultures are not routinely performed in many laboratories. Specific antibodies are not detectable in the early stages of the disease or in immunocompromised patients. In these cases PCR assays either on ulcerous or ocular swabs are very useful tool for early confirmation of diagnosis and respectively a proper treatment.

Our diagnostic approach is not invasive and not traumatic but appears to be highly specific and sensitive. In addition, it is highly supportive in cases where no specific immune response is presented or it is delayed. This was demonstrated in one patient - No 4, having no positive serology. This patient had a history of recent serious abdominal sur-

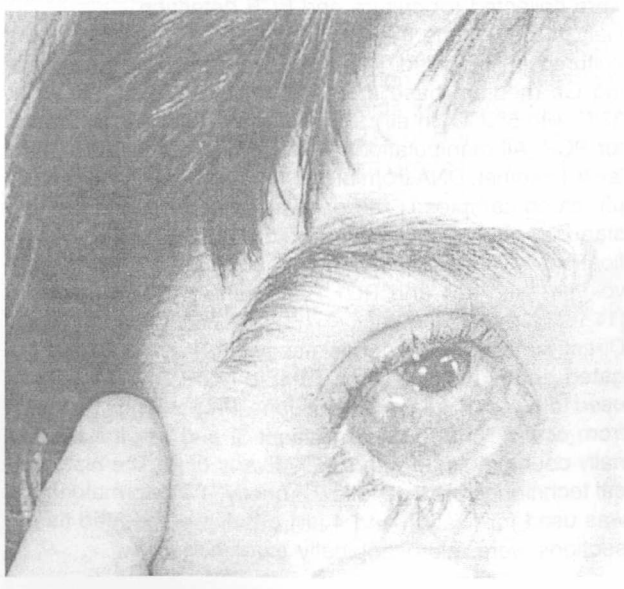

Fig. 1. Picture of a tularemia infected eye.

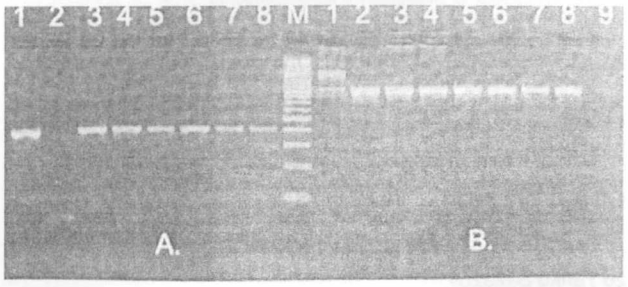

Fig.2. Polymerase chain reaction on clinical specimens with tul4 and RD1 primers

A: 1) F. tularensis ssp. holarctica strain LVS, 2) negative control - ddH2O, 3) lymph node -patient 1. 4) lymph node - patient 2., 5) blood- patient 3, 6) blood - patient 4, 7) ocular nodule patient 3, 8) ocular swab- patient 4, M - molecular size marker - 100-3000 bp B: 1) F. tularensis, ssp. tularensis strain Schu, 2) F. tularensis ssp. holarctica strain LVS, 3) lymph node- patient 1, 4) lymph node- patient 2, 5) blood- patient 3, 6) blood- patient 4, 7) ocular nodule - patient 3, 8) ocular swab- patient 4, 9) negative control - ddH_2O;

gical intervention resulting in significant level of immunosupression. Therefore we would recommend a broader acquisition and application of this methodology when oculoglandular tularemia is suspected.

REFERECES:

1. Dennis D, Inglesby T, Henderson D, Bartlett J, Ascher M, Eitzen E, et al.. Tularemia as a biological weapon: medical and public health management. JAMA 2001; 285:2763-73
2. Kantardjiev T, Velinov T. Interaction between protozoa and microorganisms of the genus Franacisella. Problems of Infectious Diseases 1995; 22: 34-35
3. Sjostedt A. Family XVII. Francisellaceae, genus I. Francisella, In D. J. Brenner (ed.), Bergeyњs manual of systematic bacteriology. Springer, New York, N.Y. 2003; 111-135.
4. Sandstrom G, Sjostedt A, Forsman M, Pavlovich N, Mishankin B. Characterization and classification of strains of Francisella tularensis isolated in the Central Asian focus of the Soviet Union and Japan. J. Clin. Microbiol 1992; 30:172-175
5. J. Erin Staples, Kristy A. Kubota, Linda G. Chalcraft, et al., Epidemiologic and
Molecular Analysis of Human Tularemia, United States, 1964-2004, Emerg. Infect Dis, 2006; Vol.12, No7, 1113-1118
6. Kantardjiev T, Ivanov I, Velinov T, Padeshki P, Popov B, Nenova R, et al. Tularemia outbreak, Bulgaria, 1997-2005. Emerg Infect Dis. 2006; Vol.12, 4: 678-680.
7. Fortier A. Life and death of an intracellular pathogen: Francisella tularensis and the macrophage. Immunol. Ser. 1994; 60: 349-361
8. Steinemann TL, Sheikholeslami MR, Brown HH, Bradsher RW. Oculoglandular tularemia. Arch Ophthalmol 1999; 117: 132-133.
9. Starck T, Madsen BW. Positive polymerase chain reaction and histology with borderline serology in Parinaudњs oculoglandular syndrome. Cornea. 2002; (6):625-7
10. Centers for Disease Control and Prevention Division of Public Health Surveillance and Informatics, Available from: http://www.cdc.gov/epo/dphsi/casedef/tularemia current.htm
11. Bevanger L, Maeland J, Naess A. Agglutinins and antibodies to Francisella tularensis outer membrane antigens in the early diagnosis of diseases during an outbreak of tularemia. J. Clin. Microbiol. 1988; 26: 433-437
12. Olsufjev N, Tularemia. Methods for laboratory diagnostics of tularemia. In: Olsufjev N, ed. Diagnostics of infections at high medical risk. Rostow University. 1970; p. 170-179
13. Ausbel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1995). In: Current Protocols in Molecular Biologyh. John Wiley and Sons, 2.4.1
14. Johansson A, Berglund L, Eriksson U, Goransson I, Wollin R, Forsman M, et. al.
Comparative analysis of PCR versus culture for diagnosis of ulceroglandular tularemia J Clin Microbiol. 2000 Jan;38(I):22-6.
15. Broekhuijsen M., Larsson P., Johanssonsen A., Bystrom M., Eriksson U., Larsson E.et al. Genome-wide DNA microarray analysis of Francisella tularensis strains demonstrates extensive genetic conservation within species but identifies regions that are unique to the highly virulent F. tulatemis subsp. tularensis. J. Clin. Microb. 2003; : 41:2924-31
16. Vassileva P., Petrow P., Kantardjiev T., Efremova, Padeshki P., Shandurkov D. Tularemia - First Cases of Oculoglandular Form of the Disease in Bulgaria. COMPTES RENDUS- ACADEMIE BULGARE DES SCIENCES Bibliographic details 2003, VOL 56; PART 7, p. 117-120
17. Guide for the Care and Use of Laboratory Animals. Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council, National Academy Press Washington, D.C.

In memory of H.T.Ricketts

SFG - RICKETTSIOSES IN BULGARIA

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SUMMARY

One hundred years ago, H.T. Ricketts laid the foundations of the research of tick spotted fevers (SFG-rickettsioses). The Mediterranean (Marseilles) spotted fever (MSF) was recognized and described for the first time in Bulgaria in 1948. We would like to report our studies and experience by dedicating them to H.T. Ricketts. From 1948 to 1970 (wave I period covering 22 years), a total of 240 cases had been recorded as sporadic cases or small outbreaks. Wave II of MSF began in 1993 by a sharp raise of the incidence. Until 2003, the number of cases was 20-25 times higher compared to the entire wave I 22-year period. 1,000-1,500 cases were recorded annually. The studies clarified that the key factors determining MSF case occurrence in Bulgaria were three: the stray population; the regular deacarization of farm animals; and the way of their breeding - concentrated in large animal breeding farms or in multiple small farms. Currently, a National Program for Combating and Prevention of Tick-borne Infections is being developed in Bulgaria, which is using also the experience accumulated in the MSF studies.

Key Words: Rickettsioses, spotted fever

One hundred years ago, H.T. Ricketts laid the foundations of the research of tick spotted fevers (SFG-rickettsioses). The classical studies of Rocky Mountain spotted fever conducted by him in Misula, Montana have not lost their significance hitherto, as well. [1]

The great health and social importance of this group of rickettsioses has become clear throughout the years. Such rickettsioses feature common worldwide distribution, variety of illnesses caused, variety of vectors, which are rather flexible and undergo alterations under the influence of various factors, expansion of known habitats and encompassing new ones, specificities of the course of epidemiology in modern conditions, globalisation, and environmental balance fluctuations.

The Mediterranean (Marseilles) spotted fever (MSF) was recognized and described for the first time in Bulgaria in 1948.[2] For a 50-year period, various peculiarities in MSF incidence and habitat dynamics have been observed as a result of the impact of a set of factors. These processes represent an interesting model for development of strategies for effective combat with SFG rickettsioses. We would like to report our studies and experience by dedicating them to H.T. Ricketts.

From 1948 to 1970 (wave I period covering 22 years), a total of 240 cases had been recorded as sporadic cases or small outbreaks. Their course had demonstrated the classical clinical picture and epidemiology of a benign SFG rickettsiosis. MSF had been recorded in endemic areas: Southern Bulgaria and along the Black Sea coast - habitats of the canine tick R.sanguineneus, which is considered as the principal reservoir and vector of the pathogen R.conorii. Following 1970, no MSF case had been reported for 22 years.

Wave II of MSF began in 1993 by a sharp raise of the incidence. Until 2003, the number of cases was 20-25 times higher compared to the entire wave I 22-year period. 1,000-1,500 cases were recorded annually. Wave II featured a line of specificities. MSF had a severer course; the number of complications was higher; rickettsiosis course differences were identified among Bulgaria's regions; all age groups were afflicted, and urban inhabitants to a greater extent; the risk factor was different; except the tick factor, of particular importance was contact with dogs; etc.

The above processes resulted from the impact of a complicated set of environmental and socioeconomic factors. For the period between both waves, systematic studies were conducted on the availability of natural and agricultural MSF foci in Bulgaria. It was identified that such foci existed in many areas, also beyond those known as endemic areas. Other ixodide tick genera were also identified as R.conorii carriers: D.marginatus, R. bursa. Other SFG rickettsiae species were discovered by genus-specific monoclonal antibodies; it was proved that in Bulgaria also existed other varieties of the SFG-rickettsioses, etc. Or, the natural prerequisites for MSF and other SFG- rickettsioses were available. [3]

The studies clarified that the key factors determining MSF case occurrence in Bulgaria were three: the stray population; the regular deacarization of farm animals; and the way of their breeding - concentrated in large animal breeding farms or in multiple small farms. Ultimately, all the above has determined the intensity of contact with natural and agricultural foci, and the transportation of vectors from the natural and agricultural foci to the populated localities.

During the 60's, in relation to the fight against rabies, control had been instituted on strays; organized deacarization of farm animals had begun in connection with veterinary medical problems; the majority of animals had been concentrated in the farms of the ex-cooperative farms. Thus, regardless of the available active foci, cases had declined and MSF had disappeared for a long time period. Reverse processes were observed following 1990: larger stray population, irregular deacarization of farm animals due to economic and other reasons, restitution of private farms, etc. Undoubtedly, many other factors of global significance were important.

For the entire period since the recognition and description of the first MSF case in 1948 so far, these systematic studies have illustrated very well the health and social significance of SFG rickettsioses, the problem complexity, the set of various factors, which determine their dynamics, as well as the effective combat strategies and the importance of the extensive population health education.

Currently, a National Program for Combating and Prevention of Tick-borne Infections is being developed in Bulgaria, which is using also the experience accumulated in the MSF studies.

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ABBREVIATIONS USED IN THIS PAPER: MSF - Mediterranean (Marseilles) spotted fever

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REFERENCES

1. Ricketts H.T. The study of "Rocky Mountain Spotted Fever" (tick fever) by means of animal inoculation. JAMA 1906; 735-36.
2. Vaptzarov I. An observational study of a small boutonneuse fever outbreak in the Plovdiv region. Zdr. Delo, 1948; 121-28.
3. Alexandrov E., B. Kamarinchev, D. Alexandrova, etc. Marseilles fever in Bulgaria - status and problems. Infectologia; 2001 ; 4 16-23.