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# PROBLEMS

## of Infectious and Parasitic Diseases

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**PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES**
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**CONTENTS**

# DIAGNOSIS OF TUBERCULOSIS - 5 YEARS EXPERIENCE AFTER THE ESTABISHMENT OF THE TB NATIONAL REFERENCE LABORATORY, NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES

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**SUMMARY:**
**Today, 5 years after the establishment of the National Reference laboratory of Tuberculosis as a part of the Microbiology Department of the National Center of Infectious and Parasitic Diseases, it is a modern laboratory with as well trained staff as other TB NRL in EU. TB NRL, NCIPD provided for first time in Bulgaria new, modern and rapid microbiological methods for diagnosis of tuberculosis. TB NRL, NCIPD covered the whole TB laboratory network in the country through an External Quality Assessment scheme and training of the personnel of all TB laboratories in Bulgaria.**

*Key words: Tuberculosis, NCIPD*

Nowadays, thousands of years after the earliest evidence of one of the oldest diseases in the world, Tuberculosis remains a problem.

The WHO estimated that during 2008 in worldwide aspect 9.4 millions was the numbers of the new TB cases and 1.8 millions was the deaths caused by TB.

The frequency of TB cases in EU and WHO European Region in 2008, according WHO's estimates, was:

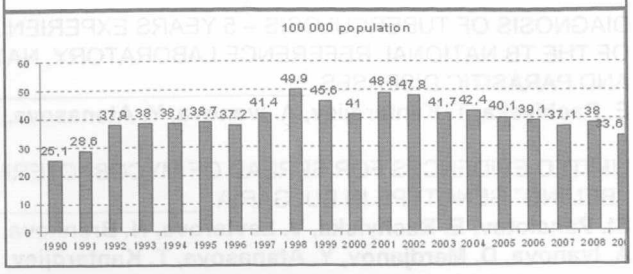
52.5/100 000 – in 50 of WHO European Region's countries and Liechtenstein (without Monaco, San Marino and Austria). It was reported 26% decline to 2007 (54/100 000)

16.7/100 000 – in the 26-th EU countries (except Austria) and 2 courtiers of EEC. The highest prevalence of TB was in: Romania – 115.1, Bulgaria – 41.2 and Baltic Republics – 33-67/100 000. In 2009 Bulgaria has been included in the list of the 27th countries with high prevalence of MDR-TB and XDR-TB.

97.5/100 000 – in the countries, outside the boundaries of the EU. The highest prevalence is in Kazakhstan – 204.9, Moldova – 160.7, Russian Federation –152, Georgia – 135.5, Kyrgyzstan – 131.6, Tajikistan – 117, Azerbaijan –115.4/100 000.

The new cases are presented in Table1 during the last 20 years according to the National Center of Health Information in Bulgaria. There is a tendency of stabilization in 2003 and decreasing afterwards.

**Table 1**: New cases of active TB disease in Bulgaria, 1990-2009




The microbiological examination is one of the five components of the recommended international strategy DOTS, which are:

Political commitment with raised and sustainable funding

Detection of the new cases by bacterial survey with guaranteed quality

Standardized treatment, observation and assistance of the patients

Effective scheme of medicine supply and distribution

Monitoring, assessment and impact measurement

In 2005 was established the National Referent Laboratory of Tuberculosis, in the structure of the National Center of Infectious and Parasitic Diseases.

Nowadays, five years later, TB NRL is a modern European laboratory with qualified staff and wide field of activities, such as each NRL TB of Tuberculosis in EU community.

Since 2006 NRL has taken successful participation in the schemes for external quality control conducted by INSTAND, Düsseldorf, Germany. The certificates insure the accuracy and the reliability of the results that TB NRL gives, in particular: microscopic examination, culture, drug susceptibility testing, PCR and Mycobacterium species identification.

Since 2007 TB NRL has been included in the scheme of WHO's external quality control for first line drugs susceptibility testing by SRL, Italy. After successful participation in the 3-th and the 4th tour of the control, TB NRL has been certified and recognized by WHO.

Guarantee for the quality of TB diagnosis is also the laboratory accreditation by Bulgarian Accreditation Service in 2008, according to ISO/IEC 17025.

Since its establishment, NRL has initiated External quality control of the microscopic examination for AFB twice a year for every TB laboratory in Bulgaria. It is planned this external control to be extended in the end of 2010 with control of culture examination and DST in the country.

In 2009 also started external quality assessment of TB laboratories by "on site evaluation" and "blinded rechecking" for AFB staining. This activity was introduced for first time in Bulgaria and accomplished not only in 2009, but also in 2010 with the support of the Global Fund for fight HIV/AIDS, tuberculosis and malaria, R6. Under the guidance of TB NRL each of the 35-th laboratories in the country has been visited once a year. The purpose is evaluation of the diagnostic and the work conditions, documented in the check lists.

The representative samples taken from microscopic slides of all laboratories were monitored by so-called "blinded rechecking" and an independent assessment about the preparation and reporting of results was made.

Over the last five years in TB NRL have been trained 108 people in training courses, funded by Swiss Agency of development and cooperation and Global Fund.

The trained laboratory specialists were from the TB laboratory network of 35 laboratories in: 7 specialized hospitals for active treatment of lung diseases, 11 multi profile hospitals, 12 dispensaries for lung diseases, 4 specialized hospitals

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for prolonged treatment of lung diseases
and 1 State Psychiatric Hospital with a ward for patients with active tuberculosis.
That means: in each TB laboratory in Bulgaria there is now more then one trained laboratory technician or doctor at least or one of them has been trained twice for the recent five years.
Since 2006 TB NRL, NCIPD has been leading unit in the country in the field of TB diagnostic:
Smear microscopy: Ziehl- Neelsen, Kinyoun and fluorescent microscopy
Culture examination: conventional solid culture, (Lowenstain-Jensen) and non-conventional liquid culture with fully automated system Bactec MGIT 960, with non-radiometric detection of Mycobacteria. There is a modern fluorescents oxygen sensor in the silicone membrane of the bottom of each MGIT (Mycobacteria Growth Indicator Tube). The metabolite active mycobacteria consume the oxygen, which allows measurement of the fluorescence by UV light. Bactec MGIT 960 System permits a 24-hour monitoring of the samples. The applied Epi-center allows automatic remembering of each measurement, graphic image and data preservation. The automatic system is used for both cultivating and drug susceptibility testing for first-line drugs. DST results are obtained in 13 days.

**Drug susceptibility testing:**
The drug susceptibility of M.tuberculosis complex to first-line drugs is important for:
Assessment of treatment regimen
Control of the treatment efficiency
Making the disease's prognosis
Conduction of epidemiological monitoring of M.tuberculosis resistance for the country by areas, for EU and worldwide measure.
WHO recommends four main standardized and worldwide spread in the international practice assays for DST which are:
Absolute concentration method
Resistant ratio method
Proportion method
Automated systems Bactec 460 TB or the non radiometric Bactec MGIT 960 System
The choice of the assay is usually based on the country laboratory's traditions. In each country it has to be used one of the recommended methods, because of: the effective epidemiological supervision of drug resistance of M.tuberculosis strains, result comparison and valuation of treatment efficiency
Traditionally nitrate-reducing method has been used in Bulgaria up to now. It is based on the fact that 97% of the mycobacteria reduce nitrates to nitrites (indirect way for DST of mycobacteria via their biochemical characteristic).
In 2006 TB NRL, NCIPD introduced for first time in Bulgaria automated non radio metric system Bactec MGIT 960. All of suspected or confirmed MDR strains from all over the country are reconfirmed in TB NRL, NCIPD. Only the patients with conformed MDR strains in the TB NRL are included in the cohort of MDRs for therapy course wit second line drugs (SLD)

**3.1. Molecular genetic methods for drug susceptibility detection – Line probe assay for the detection of potential multidrug resistant tuberculosis MDR and extremely multidrug resistant tuberculosis XDR TB**

The quick molecular assays for MDR detection was introduced in TB NRL for the first time in Bulgaria and the XDR detection by the same method – two years later.
DNA-STRIP®, is a special technology that reveals M.tuberculosis complex and its definite chromosome mutations in smear-positive clinical specimens and/or solid/liquid culture in 48 hours. The whole procedure is in four steps (Figure 1):
DNA extraction directly from specimens or decontaminated smear-positive specimens or solid/liquid culture.
Amplification
Reversed hybridization which includes: chemical denaturation of the amplification products, hybridization of single-straned, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction.
Interpretation – a template ensures the easy and fast interpretation of the banding pattern obtained

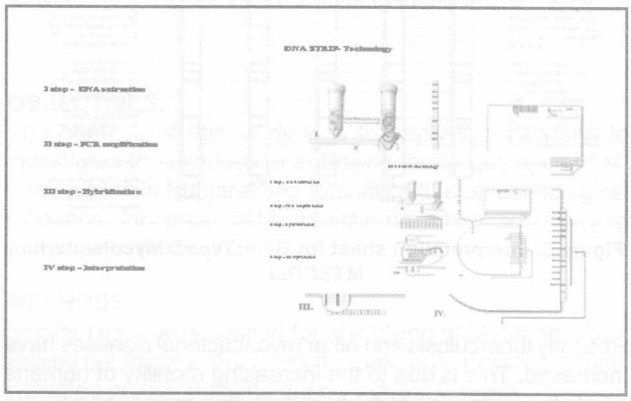

**Figure 1: DNA-STRIP® technology**

Line probe assay for the detection of potential multidrug resistant tuberculosis MDR – Figure 2:
GenoType MTBDRplus test is based on the DNA-STRIP® technology. The GenoType® MTBDRplus test allows for the detection of M.tuberculosis complex and simultaneously its resistance to rifampicin and/or isoniazid by mutations is the rpoB and katG/ihnA (high/low isoniazid resistance) gene, respectively.
The MTBDRplus detect from both positive cultures and smear positive pulmonary specimens. This test should not be used to detect mycobacteria directly from smear-negative materials.

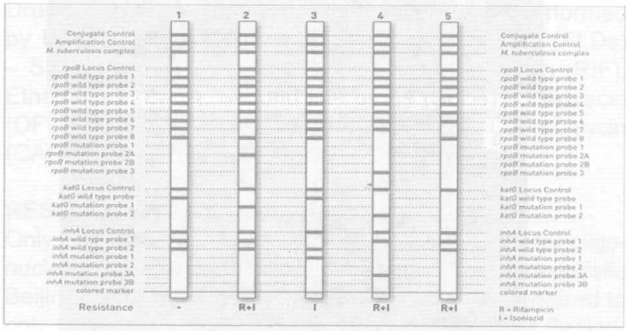


**Figure 2. Interpretation sheet for Geno Type® Mycobacterium MTBDRPlus**

Line probe assay for the detection of potential extremely multidrug resistant tuberculosis XDR TB – Figure 3:
The GenoType® MTBDRsl detects M.tuberculosis complex and simultaneously its resistance to fluoroquinolones (e.g. ofloxacin and moxifloxacin) and/or aminoglycosides/cyclic peptides (injectable antibiotics as capreomycin, viomycin/ kanamycin, amikacin) and/or ethambutol. The MTBDRplus and MTBDRsl detect from both positive cultures and smear

positive pulmonary specimens.
This test should not be used to detect mycobacteria directly from smear-negative materials.
The identification of resistance to fluoroquinolones is enabled by the detection of the most significant mutations of the gyrA gene (coding for the A-subunit of the DNA-gyrase). For detection of resistance to aminoglycosides/cyclic peptides,the 16S r RNA gene (rrs) and for detection of resistance to ethambutol the embB gene (which,together with the genes embA and embC, codes for the arabinosyl transferase)are examined.
Species identification

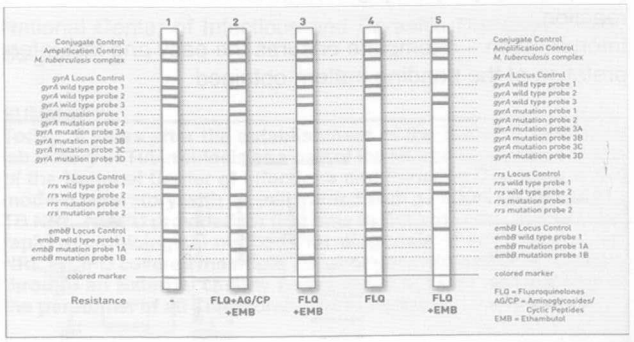


**Figure 3. Interpretation sheet for GenoType® Mycobacterium MTBDRsl**

Recently tuberculosis and other mycobacterial diseases have increased. This is due to the increasing mobility of humans and to the increasing number of immunosuppressed patients who are particularly susceptible to atypical mycobacterial (NTM or MOTT) infections. Another reason is that these diseases are better diagnosed.
Nowadays using of the molecular methods does not replace the conventional diagnostic of tuberculosis but it helps out for earlier and more accurate diagnostic.
TB NRL has introduced for species identification the following recommended by WHO molecular methods for in vitro diagnostic:
Chromatographic immunoassays for a qualitative detection of Mycobacterium tuberculosis complex – Figure 4:

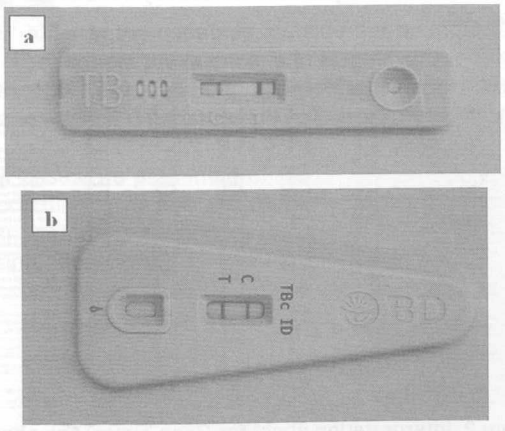


**Figure 4. Chromatographic immunoassays for the qualitative detection of Mycobacterium tuberculosis complex/ NTM from positive liquid culture**

Line probe assay for the differentiation of the Mycobacterium tuberculosis complex – Figure 5
Line probe assay for the identification of species from the genus
Mycobacterium: about 30 different of the most common mycobacteria and additional species Mycobacteria found in cultured samples(solid/liquid) - Figure 6 and Figure 7

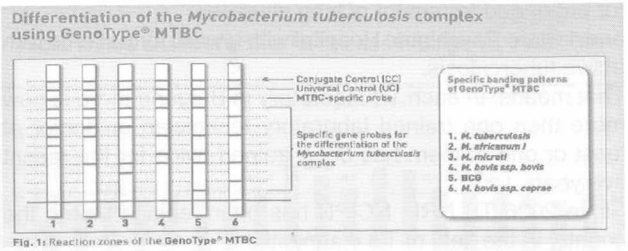


**Figure 5. Interpretation sheet for Geno Type® Mycobacterium MTBC**

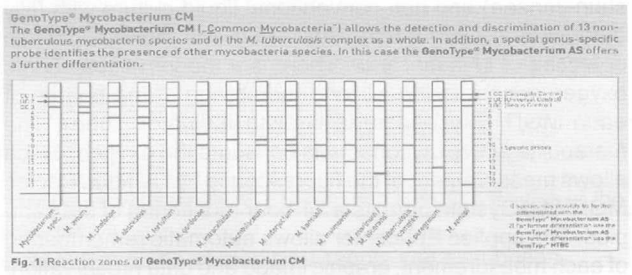


**Figure 6. Interpretation sheet for Geno Type® Mycobacterium CM**

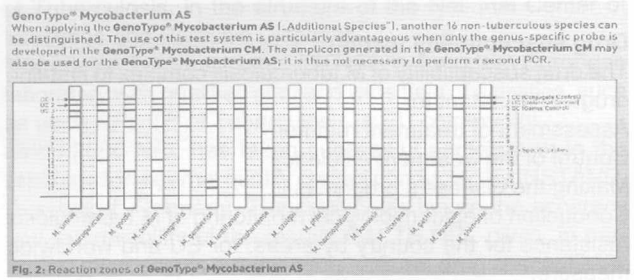


**Figure 7. Interpretation sheet for Geno Type® Mycobacterium CM AS**

All of these modern methods for species identification and drug susceptibility detection are with high sensitivity and specificity and give the physicians a possibility to get in the shortest time a reliable result.
Introducing of liquid media and the Line probe assay for the identification of Mycobacteria and the detection of potential multidrug resistant tuberculosis MDR and extremely multidrug resistant tuberculosis XDR TB in TB NRL, NCIPD initiate a new approach of microbiological diagnosis of tuberculosis in Bulgaria.

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# LIMITED EVIDENCES FOR SPREAD OF MYCOBACTERIUM TUBERCULOSIS "BEIJING" GENOTYPE IN BULGARIA

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**SUMMARY:**
**In 1995, IS6110 DNA fingerprinting identified in Mycobacterium tuberculosis isolates from China, a genetically closely related group of bacteria - the Beijing genotype family. Strains of this genotype family dominate in Southeast Asia and are globally spread. This genotype is associated with higher pathogenicity and multidrug resistance. In this study we show that Beijing genotype of Mycobacterium tuberculosis is not diffused in Bulgaria as evidenced by spoligotyping of M. tuberculosis sensitive and multidrug resistant strains collected across the country during the last 6 years. Only two strains with the characteristic Beijing spoligotype were identified. Our results suggest, that in Bulgaria, although the active exchange contacts with countries where Beijing genotype is predominant we do not observe wide spread of Beijing genotype, as observed in other West European and Central Asian countries. We suppose that genetic and environmental factors should be investigated to prove unpredisposition of the local population to this globally diffused Mycobacterium tuberculosis genotype.**

*Key words: DNA, Tuberculosis*

## INTRODUCTION

Beijing genotype of M. tuberculosis was firstly recognized in 1995 (1). It is reported to account for 86% of the tuberculosis isolates from Beijing, China (1), and a high proportion of isolates from Mongolia, Thailand and South Korea (2). Beijing genotype strains have also been associated with the transmission of drug-resistant tuberculosis in Germany (3), Cuba (4), Estonia (5) and Russia (6). In the United States, a highly drug resistant strain, strain W, also belongs to the Beijing family (7).

Spoligotyping is a PCR-based reference method widely applied for typing of M. tuberculosis strains. The method identifies strain-dependent polymorphisms in the M. tuberculosis short direct repeat (DR) chromosomal region, which consists of identical 36-bp DRs interspersed with 35- to 41-bp nonrepetitive spacer sequences. Spoligotyping is the method of choice when identifying M. tuberculosis Beijing genotype. The Beijing spoligotype gives characteristic absence of spacers from 1 to 34 and gives hybridization signal with all of the last nine spacers from 35 to 43 (Figure 1).

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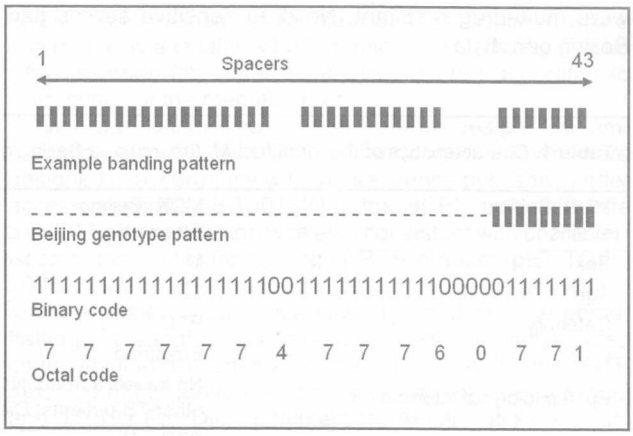


**Figure 1. Spoligotyping patterns**

## OBJECTIVES:

The purpose of this study was to apply spoligotyping to investigate the distribution of Beijing family genotype of M. tuberculosis in Bulgaria and to analyze the epidemiological evidences for spread of Mycobacterium tuberculosis Beijing genotype in the country.

## METHODS:

Spoligotyping was applied for identifying of M. tuberculosis Beijing genotype in drug sensitive and drug resistant strains collected across Bulgaria during the last 6 years.

A commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) was used as described by the manufacturer. Briefly, the amplified DNA was hybridized to a membrane covalently precoated with a set of 43 spacer oligonucleotides derived from the spacer sequences of M. tuberculosis H37Rv and M. bovis P3. Both M. tuberculosis and M. bovis reference strains were included in each test as positive controls. 20 ng of M. tuberculosis chromosomal DNA was PCR amplified with primers DRa (5'-CCG AGA GGG GAC GGA AAC-3') and DRb (5'-GGT TTT GGG TCT GAC GAC-3'). DRb primer is labeled with biotin. The resulting PCR products were then hybridized with the precoated membrane, and the final image was detected with an enhanced chemiluminescence system.

Data analysis was performed by using the MIRU-VNTR plus software (http://www.miru-vntrplus.org/)

Drug susceptibility tests of all of the strains were performed by Bactec MGIT 960 System for first line drugs (FLDs) – Streptomicin (STR), Isoniazid (INH), Rifampicin (RIF), Ethambutol (EMB). Second line drugs (SLDs) – Ofloxacin (OFL), Amikacin (AMK), Kanamycin (KAN) and Capreomycin (CAP) were performed of the MDR strains

## RESULTS:

Only strains that hybridized to all of the last nine spacer oligonucleotides (spacers 35 to 43) were defined as characteristic Beijing genotype strains, whereas strains that hybridized to only some of the last nine spacers are defined as Beijing-like genotype strains. Such strains were not observed in our study. Strains of all other spoligotypes were defined as non-Beijing genotype strains. We analyzed a total of 366 M. tuberculosis strains collected during the last 6 years from all over the country. Out of 229 drugs sensitive and 137 MDR strains analyzed only two M. tuberculosis strains were identified as Beijing genotype. This represents 0,6% of Beijing genotype among all isolates. The two identified M. tuberculosis Beijing genotypes had spoligotype pattern 000000000003771. 24 loci MIRU-VNTR profile for one of the strains was 244233352634425153353823. All strains

were multi-drug resistant. No drug sensitive strains had Beijing genotype.

to M. tuberculosis Beijing genotype, it does suggest that associations between host and parasite populations are sufficiently stable for such resistance/adaptation to evolve.

**Table 1.** Characteristics of the identified *M. tuberculosis* Beijing genotype strains from Bulgaria

| MDR, Beijing strains | MDR, Beijing Strains No 1 | MDR, Beijing Strains No 2 |
|---|---|---|
| Sex | M | M |
| Age | 32 | 42 |
| Category | PTC | PTC |
| Oigin | Bulgarian | Bulgarian of Armenian origin |
| Epidemiological information | No travels abroad; No data for contacts with other TB patients; Died in 2008 | No information for contacts with other TB patients; Died 2009 |
| First Line Drugs | STR – R<br>INH – R<br>RIF – R<br>EMB –R | OFL – S<br>AMK –S<br>KAN – S<br>CAP – S |
| Second Line Drugs | STR – R<br>INH – R<br>RIF – R<br>EMB –R | OFL – S<br>AMK – S<br>KAN – S<br>CAP – S |
| Spolygotype | 000 000 000 003 771 | 000 000 000 003 771 |
| 24 loci VNTR type | NA | 24423335263442515335382 3 |

## CONCLUSIONS:

366 M. tuberculosis strains have been investigated by us during the last 6 years. The collected strains originate from all over the country. We identified two MDR M. tuberculosis Beijing genotype strains. Among the drug sensitive strains Beijing genotype was not identified. The prevalence of the Beijing genotype in Bulgaria is less than 0,6% among all isolates. Balkan countries report scanty spread of the M. tuberculosis Beijing genotype in their countries (8, 9).

We suppose that Beijing genotype was recently introduced in the Balkan Peninsula. This is supported by the fact that in the Balkan countries Beijing genotype is not or rarely detected and most often is carried over from recent migrations. In the near past (1960s-80s) China and Albania had tight political, commercial and cultural relations. The Albanian isolates with Beijing genotype are MDR strains. They originate from a city where a steel factory was built by Chinese specialists (8). The Russian immigrant identified with M. tuberculisis Beijing genotype in Croatia is supposed to have been infected by her mother suffering from tuberculosis for which is confirmed to have caught the TB infection in Russia (personal communication). The Slovenian case of MTB Beijing genotype originates from a Chinese woman with lymph node tuberculosis (personal communication). The two Bulgarian MTB isolates are MDR strains. Two of the patients recently died. The patients lived geographically distant from each other. One of the patients is of Armenian origins, but his relatives immigrated in Bulgaria some 80 years ago. SpolDB4 database reports high prevalence of Beijing genotype among M. tuberculosis strains isolated from Yerevan. There are no evidences for spread of Mycobacterium tuberculosis Beijing genotype in Romania and Serbia. There are no data for Macedonia.

Natural resistance of the local population to Beijing genotype could be supposed. Environmental and cultural factors preventing adaptation of the Beijing genotype to the population could be supposed too. Although our results do not provide direct evidence for resistance of the Bulgarian population

Continuous monitoring should be performed to monitor the evolution of the M. tuberculosis Beijing genotype in Bulgaria and the Balkan Peninsula.

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# CLONING AND EXPRESSION OF OSP (OUTER SURFACE PROTEIN) C FROM BORRELIA BURGDORFERI SENSU STRICTO

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**SUMMARY:**
**One of major outer surface proteins of Borrelia burgdorferi was cloned and expressed. OspC C is a major immunodominant antigen and induces a strong immune response during the early stages of Lyme disease. Specific antibodies against OspC are detectable on 20-30 day after infection. Our main purpose was cloning and expression of this protein as a first step in developing more specific and sensitive ELISA and immunoblot tests for diagnosis of the disease. The ospC gene was amplified in PCR reaction with specific primers. The product was inserted in pET TOPO® vector. This construct was used for transformation of E.coli BL 21 strain. Expression of the protein was stimulated by IPTG. Production of the specific recombinant protein was detected by immunoblot with anti-OspC monoclonal antibody.**

*Key words: Lyme borreliosis, recombinant protein, Borrelia burgdorferi, OspC*

## INTRODUCTION

Lyme borreliosis is a common multisystem disorder. Clinical manifestations are not specific, except the erythema migrans in early Lyme borreliosis. Reliable diagnosis is very important to ensure adequate treatment of patients and to exclude this diagnosis in patients with other illnesses. Currently, a routine laboratory diagnostic is based mainly on serological techniques (2,3).

Many enzyme-linked immunosorbent assays (ELISA) and Western blot tests with various recombinant antigens have been investigated, searching for optimal range of sensitivity and specificity. Various proteins from Borrelia burgdorferi sensu lato complex have been studied (4-10). The bacteria express many antigens, which may differ in vectors and hosts (14,15,16). Some of them are expressed only in vivo in infected hosts (17). Others are not highly specific, but are strong imunnogens, like flagellin. It is clear now, that better diagnostic results could be achieved if optimal combination of specific antigens would be used (1).

OspC is basic structure protein from outer surface membrane of bacteria. This protein is expressed during the tick bite and is responsible for dissemination of spirochaetes and establishment of infection in patients. OspC is major antigen in early stages of Lyme borreliosis (11,12). It induces strong early IgM immune response against Borrelia burgdorferi, Borrelia afzelii and Borrelia garinii (13). That is why, OspC is one of the most studied and perspective for investigations in this field.

The object of this study is cloning, expression and purification of OspC recombinant protein from B. burgdorferi.

## MATERIALS AND METHODS

DNA isolation from Borrelia burgdorferi. Genome DNA from B. burgdorferi sensu stricto, stain B31 was isolated with a commercially available kit (Genomic Prep Cells and Tissue DNA Isolation kit, Amersham Biosciences) according to instructions of the manufacturer.

Polymerase Chain Reaction and primer design. For amplification of ospC gene from B. burgdorferi, primers were designed in accordance with the sequence published under accession number BBU01894 in the NCBI nucleotide data base. The primer design was also consistent with characteristics of the vector for cloning of PCR product (pET TOPO 200/D, Invitrogen). The primer sequences were as follows: forward primer caccatgtgcaattagttggc and reverse primer ttattgtggcagaaagtccaa. The reaction mixture for polymerase chain reaction contained 20 ng genomic DNA, 200nM dNTP mix, 200 nM of forward and reverse primers, 2 mM MgCl2 and 1.25 U Pfu DNA polymerase. Amplification protocol was as follows: initial DNA denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 45s, primer annealing at 50°C for 45 s, extension of product at 72° C for 2,5 min and final extension of product at 72°C for 5 min, (Thermal Cycler2720, Applied Biosystems).

The presence of PCR product about 860 kb was confirmed with electrophoresis on 1,5% agarose gel.

**Cloning and transformation.**

Before cloning, the PCR product was purified from agarose gel using DNA Extraction Kit (Fermentas). The cloning reaction was performed for 1 hour and contained 4 µl DNA, 1 µl vector (pET TOPO 200/D, Invitrogen) and 1 µl buffer (supplied with the vector from Invitrogen). The prepared vector constructs were used for transformation of chemically competent E.coli cells (stain pMOS Blue®, Fermentas). Positive transformants were selected on LB plates containing 50 µg/ml kanamycin (Sigma).

To confirm correct orientation of inserted gene plasmid DNA was isolated from transformants (illustra plasmid Prep Mini Spin Kit, Amersham Biosciences) and used as template for Polymerase chain reaction (PCR). The primer pair was: Forward primer – taatacgactcactataggg (supplied with vector, Invitrogen) and Reverse primer for ospC gene ttattgtggcagaaagtccaa. The running protocol and reaction mixture were identical with already mentioned upwards. The product with molecular weight about 1000 bp was visualized in agarose gel electrophoresis (figure 1).

**Expression of recombinant protein.**

DNA constructs were used for transformation of E.coli (DE3, Invitrogen). For expression of recombinant OspC protein, transformed cells were inducted with 0.1 M IPTG (isopropyl-β-D-thiogalactopyranoside) and cultivated overnight in LB medium at 37°C. The cultures were centrifuged and the cells were lysed in lysis buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole, pH 7.8). The lysates were analysed in SDS-PAGE with a 12% running gel and 4% stacking gel. Uninducted bacterial lysates were used as negative control. The gel was stained with Coomassie blue.

**Purification.**

Recombinant proteins were purified with His-select Ni-affinity Gel (Sigma), according to manufacturer instructions.

**Immunoblot confirmation.**

Purified proteins were loaded on polyacrylamide gel and after SDS-PAGE were transferred to nitrocellulose membrane. Membrane was blocked with 3% skim milk in TBST buffer for 2 hours at room temperature. Monoclonal anti-OspC antibody was diluted 1:50 and incubated with shaking overnight. The secondary anti-mouse IgG antibody conjugated with HRP was diluted 1:800 and incubated for 1 hour at room temperature. Substrate for enzyme reaction was 0,05% 3,3`-diaminobenzidine with 0,1% hydrogen peroxide.

## RESULTS AND DISCUSSION

OspC gene was amplified by PCR. For this purpose, specific primers were designed as follows: Forward primer caccatgtgcaattagttggc and Reverse primer ttattgtggcagaaagtccaa, where cacc is a lieder sequence for direct cloning of PCR

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product. The atg and tta in both primers are first (for methionine amino acid) and last (stop) codons, necessary for the expression of recombinant protein. Finally, the primers were constructed to provide cloning in open read frame for expression of protein of interest.

After purification of the PCR product, it was cloned into a vector pET TOPO 200/D. Competent E.coli cells were transformed with this construct.

To confirm the presence of insect in the vector, before second transformation, plasmid DNA from all positive clones was isolated and characterised by specific PCR reaction.

Expression of recombinant OspC protein was inducted by adding IPTG (isopropyl-β-D-thiogalactopyranoside) into media. Cell cultures were centrifuged and pellets lysed and load on polyacrylamide gel. After SDS-PAGE, the suggested recombinant protein with molecular mass about 25-26 kDa, was visualised by staining with Coomassie blue.

Presence of the specific recombinant protein was confirmed by immunoblot with anti-OspC monoclonal antibody.

OspC is a major antigen, involved in early Ig M immune response in patients with Lyme borreliosis.

The purified antigen could be used for developing ELISA test for screening the immune response in early stage of Lyme borreliosis when antibodies are barely detectable. Also, it can be useful in clarifying the nature of immune response

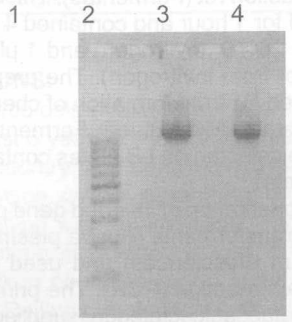


**Figure 1 Agarose gel electrophoresis. After first transformation, plasmid DNA from positive clones was isolated and used as a template in PCR reaction to confirm the presence of correctly orientated gene ospC. (1 – DNA Lader, Gene O'Ruler, Fermentas, 3 and 5 – fragment of vector with gene for OspC about 1000 kb in length, 2 and 4 – plasmids with no inserts of the gene)**

in different groups of patients. According to the data in the US, 62% of patients with erythema migrans have antibodies against OspC (10). In Europe this percentage is lower – 44%, because of heterogeneity of the causative species in B. burgdorferi sensu lato complex (18). We do not have actual data for Bulgaria.

Introducing second generation ELISA and immunoblot tests with recombinant antigens is a step to improve serological diagnostic. Because of their high specificity, they reduce possibility for false positive results due to non-specific cross-reactions. In a combination with other antigens, it could be applied in diagnostic tests for Lyme borreliosis.

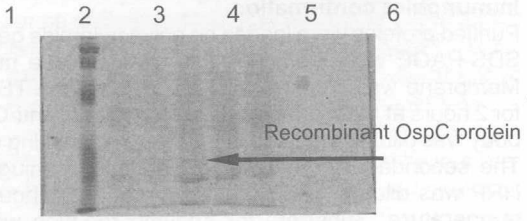


**Figure 2 SDS-PAGE. The cells lysates were analysed in SDS-PAGE with a 12% running gel and 4% stacking gel. Uninducted bacterial lysates were used as negative control. The gel was stained with Coomassie blue. (1 – Kaleidoscope Prestained Standarts, Bio-Rad, 2 and 3 – uninducted lysates, 4, 5, 6 – IPTG inducted E. coli cultures. The suggested recombinant protein is with molecular mass about 25-26 kDa.**

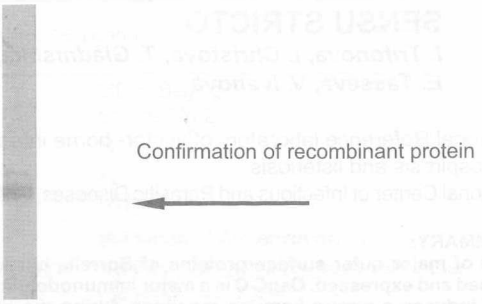


**Figure 3 Immunoblot confirmation. Presence of the specific recombinant protein was confirmed by immunoblot with anti-OspC monoclonal antibody.**

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# HUMAN SERUM REACTIVITY AGAINST BORRELIA OSPC, OSPA, FLAB AND VLSE PROTEIN ANTIGENS IN EARLY AND DISSEMINATED LYME BORRELIOSIS

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**SUMMARY:**
**Reliable laboratory diagnostic of Lyme disease is still a non solved problem. We tested 116 serum samples from patients with Lyme disease by ELISA tests based on one or more Borrelia antigens in different combinations. Four Borrelia proteins – OspC, FlaB, VlsE and OspA, were cloned, expressed and applied as antigens. The aim of this study was to compare humoral immune response against these proteins in the corse of infection. In the group of patients with erythema migrans, reaction to at least one antigen was found in 48/68 (71%). Of them, IgM class antibodies were found in 28/48 (58%) of the patients and IgG antibodies were detected in 40/48 (83%). Among patients with disseminated Lyme disease, a total of 75% (36/48) were found positive, IgM antibodies only were detected in 72% (26/36) of the patients and IgG antibodies in 83% (30/36) of the patients. The most commonly detectable antigen by IgG antibodies and quite often by IgM antibodies in patients with early and disseminated Lyme borreliosis was VlsE. OspC and FlaB antigens were recognized in similar amounts of serum samples from early Lyme borreliosis. FlaB was more often found in disseminated than in early stage of the disease. OspA was detectable at low rate in all patients despite the stage of the disease. We concluded that no single antigen could ensure appropriate diagnosis. Hence, a combination of antigens to obtain optimal balance in specificity and sensitivity is required.**

*Key words: Lyme borreliosis, recombinant proteins, Borrelia burgdorferi, ELISA*

## INTRODUCTION

Lyme disease is a multisystem infection, transmitted by a tick bite and caused by spirochetes from Borrelia burgdorferi sensu lato complex. In Europe, three of them most frequently are isolated as a causative agent of Lyme borreliosis – B. burgdorferi sensu stricto, B. afzelii and B. garinii.

In the beginning of illness symptoms, if appear, are nonspecific, flu-like. The only typical manifestation is a skin rash – erythema migrans. Erythema migrans does not occur in all cases. Months or years after the onset in untreated or inadequately treated patients, infection can affect various systems and organs– joints, central or peripheral nervous system, heart, skin, rarely eyes. Those nonspecific manifestations require reliable laboratory diagnostic. This is especially important to ensure appropriate antibiotic treatment (1,2).

Laboratory diagnosis of Lyme borreliosis is still a non solved problem (3). Routine laboratory diagnostic of Lyme disease is mainly based on immunoenzyme methods – ELISA and immunoblot (4,5,6). The center for disease control and prevention of infections (CDC) recommends two-tier testing protocol for serodiagnosis (7). First, serum samples from suspected patients are screened for presence of specific antibodies by ELISA tests. Second, all positive or borderline from the first testing samples are further tested by immunoblot techniques for confirmation.

Borrelia burgdorferi expresses a wide range of different antigens in vector and vertebrate host (8). Some of them are expressed only during infection in vivo (9). A lot of antigenic variations are found among the species and strains in B. burgdorferi s. l. complex (10,11). Some antigens, like flagellin, are not very specific but are strong immunogens. Obviously, better results could be achieved by optimal combination of specific antigens (1,12). Efforts are concentrated on research of protein antigens that may increase sensitivity and specificity of serodiagnostic tests in every stage of Lyme disease. Appropriate proteins should be expressed in significant quantities during infection and be strong immunogens. In additions, they should have conserved gene sequences among the species and the strains in B. burgdorferi s. l. complex in different geographic regions.

The two major outer surface proteins of B. burgdorferi – OspC and OspA have been extensively studied as antigens.

Outer surface protein C (OspC) is one of the most immunodominant antigens in early Lyme borreliosis (8,13-15). OspC expression starts during the tick feeding and is associated with migration of borrelia from tick's midgut to salivary glands and with establishment of infection in vertebrate hosts. Immune system of the host produces specific antibodies and their selective pressure suppresses bacteria with OspC epitops (12,16-19). It was shown that in a mouse model of Lyme disease, B. burgdorferi express OspC for a short period of time – first one to three weeks after infection while bacteria circulate in blood stream. Then, ospC gene is downregulated but specific anti-OspC IgM antibodies remain detectable (7,8,10,18,20). Presence of strong IgM immune response against OspC in early Lyme disease is crucial for focusing on this antigen in almost all studies investigating new tests for serodiagnosis (21).

During ospC gene expression, ospA gene is downregulated. OspA is expressed while B. burgdorferi is in a tick midgut and is downregulated during the tick bite. OspA (MM 31 kDa) is not detected in significant quantity at the onset of Lyme borreliosis. In most cases, antibodies against OspA are detectable in patients with disseminated infection. Expression of OspA is established during acute inflammatory process. It is not clear whether upregulation of gene activity is a response to the inflammation in the host or the presence of the protein is required for progression of chronic infection (22).

Flagellin B (FlaB) is constitutively expressed in B. burgdorferi. The FlaB protein builds the central part of Borrelia flagellum (6). Earlier studies gave shown that FlaB (MM 41 kDa), is major immunodominant antigen. A strong immune response against FlaB is detectable in early and late Lyme borreliosis (21,23). Unfortunately, besides immunogenic capacity, FlaB demonstrates high level cross-reactivity. Studies have shown that inner part of FlaB molecule possesses invariable, species specific and high immundominant region with lower cross-reactivity.

VlsE (Vmp-like sequence) antigen is expressed on Borrelia surface. This protein is strong immunogen. Antibodies against VlsE are detectable in all stages of Lyme borreliosis, including the early stages, when antibody responses to multiple borrelial antigens are not yet fully developed. Interestingly, anti-VlsE antibodies are usually IgG class (13). The vlsE gene is localized on a linear plasmid lp 28. It is established in a murine model that recombinant events occur between homologue sequences on silent cassettes

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of the gene and the cassette part in the expression site of vlsE gene. These recombination events generate antigenic variations (13,24).
In Europe, laboratory diagnostic of Lyme borreliosis is quite difficult due to known heterogeneity among different species and strains in B. burgdorferi sensu lato complex. Enzyme-linked immunosorbent assays (ELISA) and Western blot tests with recombinant antigens improve specificity of serological diagnostics.

## MATERIALS AND METHODS

**Serum samples**. A panel of 116 serum samples collected from patients suspected for infection with Borrelia burgdorferi was tested. Samples were divided into two groups, depending on the patient symptoms. The first group consisted of 68 serum samples from patients with physician-diagnosed erythema migrans. The second group contained 48 serum samples from patients with symptoms of disseminated and late Lyme borreliosis.
**ELISA** test for determination of IgM and IgG-class antibodies against Borrelia burgdorferi in human serum. (NovaTec, Immunodiagnostica GmbH). The wells were coated with recombinant OspC (B. burgdorferi s.s. and B. garinii), p100 and p18 (B. afzelii) and p41i (B.garinii) antigens for detection of IgG antibodies and with recombinant OspC (B. afzelii and B.garinii) and p41i (B. garini) for detection of IgM antibodies. Serum samples were processed according to the instructions of the manufacturer.
**Recombinant antigens**. Four recombinant proteins from Borrelia burgdorferi were used as antigens – separately or in different combinations – OspC, OspA, VlsE and FlaB. The proteins were cloned in pET TOPO 200/D vector (Invitrogen) and expressed in E.coli BL 21 strain (Invitrogen). The antigens were coated to 96-cell microplates (Costar) incubated overnight at 4°C. All antigens were used in concentration 10 µg/ml. Serum samples were diluted in PBS/0,05% Tween 20/0,5% bovine serum albumin (BSA) to final dilution 1:200, 100 µl were added to the wells after washing of unbound antigens and incubated 60 minutes at 37°C. After washing procedure, 100 µl of rabbit anti-human IgM or IgG HRP (horse-radish peroxidase) conjugate (Dako) were added to the wells. Plates were again incubated 60 minutes at 37°C. After washing procedure, 100 µl substrate (3% hydrogen peroxide) and a chromogen (o-phenylendiamin dihydrochloride) were added. The reaction was stopped with 1N HCl. Presence of colored product is measured spectrophotometrically at 492 nm wavelength.
**Negative controls**. Serum samples from 10 healthy blood donors served as negative controls.
**Calculation of the results**. A cut-off value was determined to evaluate extinction data from ELISA rider and to avoid false positive and false negative results in the interpretation. A panel of 10 serum samples from healthy blood donors was tested and the average of their extinctions /x/ and sum with 3 standard deviations (SD) were calculated.

Cut-off value = x + 3 SD
Samples with extinction values higher than calculated cut-off value were accepted as positive, those between the x + 3 SD and x + 2 SD values were interpreted as borderline or gray zone, and samples with extinctions lower than the cut-off value were accepted as negative.

## RESULTS AND DISCUSION

Antibody response against 4 particular antigens, OspC, OspA, FlaB and VlsE, was studied in patients with early localized (erythema migrans) and disseminated Lyme borreliosis. In the group of patients with erythema migrans, reaction to at least one antigen was found in 48/68 (71%). Of them, IgM class antibodies were found in 28/48 (58%) of the patients and IgG antibodies were detected in 40/48 (83%). IgM antibodies in early Lyme borreliosis were detectable against OspC, FlaB and VlsE in similar amount of samples – respectively in 11, 14 and 12 of the samples. Anti-OspA IgM antibodies prevalence was less, found in only 8 of all tested samples. IgG class antibodies were detectable against all tested antigens in early Lyme disease. The major antigen found in this study to be a target of the humoral immune response was VlsE. Anti-VlsE antibodies were detected in 34/68 (50%) of the tested samples. Anti-OspC antibodies were found in 12/68 (18%) of the serum samples, anti-FlaB in 8/68 in (12%) and anti-OspA in 4/68 (6%). The results are presented in figure 1. Analysis of the results revealed that a total of 59% (40/68) of the tested samples from this study group showed anti-VlsE reactivity, 28% (19/68) of the samples showed anti-OspC reactivity, 28% (19/68) anti-FlaB reactivity and 18% (15/68) anti-OspA reactivity.
Among patients with disseminated Lyme borreliosis, 36/48 (75%) of samples were found positive for at least one studied Borrelia antigen. IgM class antibodies were detected in 26/36 (72%) and IgG antibodies in 30/36 (83%) of them. Data from specific IgM antibody response revealed that the predominant antibodies were against VlsE and FlaB antigens, detected in 14 and 12 samples respectively. Anti-OspC and anti-OspA antibodies were found each in 6 samples. Analysis of IgG antibody response gave similar results. Specific IgG antibodies against VlsE antigen were founded in 24 samples; anti-FlaB antibodies were detected in 12 samples, anti-OspC antibodies were found in 8 and no anti-OspA antibodies were detected. The most commonly detectable antigen in patients with disseminated Lyme borreliosis was VlsE. A total of 32/48 (67%) of the investigated serum samples showed specific anti-VlsE reactivity. Reactivity to the other studied antigens varied from 46% (22/48) to FlaB to 25% (12/48) to OspC and 13% (6/48) to OspA (fig.2).
The results were compared with those obtained by a commercially available kit with recombinant Borrelia antigens and currently used as a routine test in our laboratory (Table 1). Generally, routine ELISA test detected much more frequently IgM and less frequently IgG antibodies in both Lyme borreliosis stages than did testing with our 4 different antigens. The main reason for that could be due to the strong IgG immune response against VlsE and the fact that this antigen is not included in the commercial kit. Overall, routine ELISA showed higher sensitivity at very early stage but lower sensitivity at disseminated Lyme borreliosis than combination of our 4 antigens showed. Analysis of the results of all samples together revealed similar mean sensitivity of both tests (about 70%).
In Europe, similar studies have shown lower reactivity (30 to 44%) of IgM antibodies to recombinant OspC in serum samples from patients with early Lyme borreliosis (25,26). Padula et al. detected IgM antibodies in 62% of the patients with erythema migrans in USA (19). One of limitation factors to use recombinant OspC for serologic diagnosis is known heterogeneity of the molecule among different Borrelia isolates. In Europe, this is further complicated by the fact that species from Borrelia burgdorferi sensu lato complex commonly involved in pathogenesis of Lyme borreliosis are at least three – B. burgdorferi sensu stricto, B. garinii and B. afzelii.
Studies on whole recombinant FlaB and on only the inner more conservative part of FlaB as antigens for serologic diagnostic in patients in different stages of Borrelia infection reported that the inner part of the molecule is more specific, but gives lower sensitivity (7,27).
Rauer et al. tested serum samples from patients with early

Lyme disease with OspC and FlaB antigens together (15). Presence of specific IgM antibodies is founded in 46% of the samples. The same sensitivity is reported in studies with whole-cell antigen.

Recombinant VlsE antigen has shown high level sensitivity in patients with early and disseminated Lyme disease. (13,24,28-31).

In conclusion, four Borrelia proteins were cloned, expressed and tested as antigens in an attempt to investigate their relative significance in antibody response. The most commonly detectable antigen by IgG antibodies and quite often by IgM antibodies in patients with early and disseminated Lyme borreliosis was VlsE. OspC was the most commonly recognized antigen by IgM antibodies in early Lyme borreliosis. OspA was detectable at low rate in all patients despite the stage of the disease. FlaB was more often found in disseminated than in early stage of the disease. It was shown by the results of this and other studies that no single Borrelia antigen could assure appropriate laboratory diagnosis of Lyme borreliosis. Efforts of scientists to reveal best combination of antigens for serologic assays continue. It is difficult but very important to find maximal balance in specificity and sensitivity, first to avoid false positive results due to cross-reactivity and second to ensure reliable and fast laboratory diagnosis in all Lyme borreliosis stages.

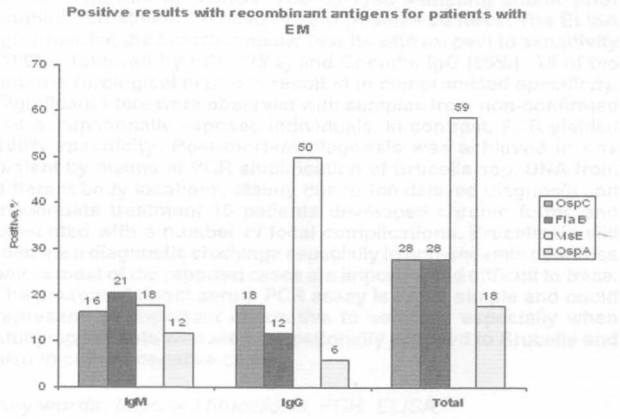


**Fig. 1 Early localized Lyme borreliosis (erythema migrans). Serum sample reactivity in ELISA with 4 recombinant Borrelia antigens – OspC, OspA, FlaB and VlsE. Results are given separately for IgM and IgG antibodies as well as for both classes in tested serum samples.**

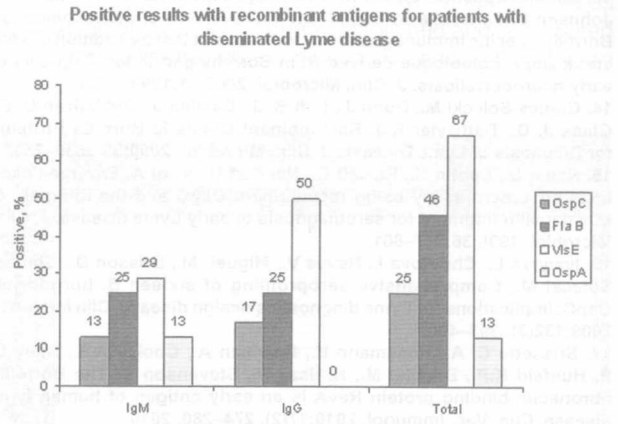


**Fig. 2. Disseminated Lyme borreliosis. Serum sample reactivity in ELISA with 4 recombinant Borrelia antigens – OspC, OspA, FlaB and VlsE. Results are given separately for IgM and IgG antibodies as well as for both classes in tested serum samples.**

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**Table. 1** Comparison of the results from routine ELISA test with recombinant antigens to in-house developed ELISA tests with 4 different Borrelia antigens – OspC, OspA, FlaB and VlsE.

| Lyme borreliosis stages | Routine ELISA test | ELISA with 4 antigens | Routine ELISA test | ELISA with 4 antigens | Routine ELISA test | ELISA with 4 antigens |
|---|---|---|---|---|---|---|
| | IgM-positive results | | IgG-positive results | | Total positive results | |
| Erythema migrans (n=68) | 37 | 28 | 6 | 40 | 39 | 48 |
| Disseminated Lyme disease (n=48) | 26 | 18 | 30 | 32 | 48 | 36 |
| Total (n=116) | 63 | 46 | 36 | 72 | 87 | 84 |

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# CLINICAL MANAGEMENT OF TRAVEL-ASSOCIATED BRUCELLOSIS CASES IN BULGARIA

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**SUMMARY:**
**Bulgaria is free from brucellosis and no resident human cases have been documented for the last 50 years. In 2005 a relatively large number of cases with brucellosis compatible symptoms were reported and investigated. This work describes the epidemiological, clinical and diagnostic findings in these cases. The study also illustrates a simple technique for treatment of serum prior to PCR without the need for DNA purification. A total of 63 individuals were clinically and laboratory studied for brucellosis by applying a number of methods including: epidemiological investigations, hematological and microbiological methods- blood cultures, Rose Bengal, SAT, Coombs IgG, ELISA IgM and IgG. A novel direct PCR assay with serum samples was adapted and evaluated as a rapid confirmative test. This approach excludes any DNA purification steps resulting in significantly shorter turnaround time. In addition, PCR was successfully applied in the post-mortem diagnosis in one patient. Combining multiple diagnostic methods a total of 21 out of the 63 people were confirmed to be infected with Brucella spp. and classified as imported cases. The epidemiological data corroborated that 20 out of the 21 confirmed brucellosis patients had been working in sheep-breeding farms in endemic region in Greece and one in Cyprus. The delayed sampling and/or prior antibiotic treatment resulted in zero positive cultures. The ELISA IgG provided the best diagnostic results with respect to sensitivity (100%), followed by PCR (95%) and Coombs IgG (90%). All of the applied serological methods resulted in compromised specificity. Significant titers were observed with samples from non-confirmed but occupationally exposed individuals. In contrast, PCR yielded 100% specificity. Post-mortem diagnosis was achieved in one patient by means of PCR amplification of Brucella spp. DNA from different body locations. Mainly due to the delayed diagnosis and inadequate treatment 15 patients developed chronic forms and presented with a number of focal complications. Brucellosis still remains a diagnostic challenge especially in non-endemic countries where most of the reported cases are imported and difficult to trace. The presented direct serum PCR assay is rapid, simple and could represent an important alternative to serology especially when studying patients who are occupationally exposed to Brucella and also in culture negative cases.**

***Key words:*** *Imported brucellosis, PCR, ELISA,*

## INTRODUCTION

Brucellosis is a worldwide healthcare problem and has an enormous overall economic impact. It is assessed that human brucellosis is greatly underreported especially in non-endemic countries due to lack of or weak diagnosis and failures in the reporting systems. Prevention of human disease requires control and eradication of the disease in animals (13).

Since the eradication of bovine and goat brucellosis in 1958, Bulgaria is officially free of human disease (1, 6, 13). During the 1950s a veterinarian surveillance system as well as vaccination programs had been established and contributed significantly to the low level of human incidence (<0.3) (Figure 1) (1, 6, 13).

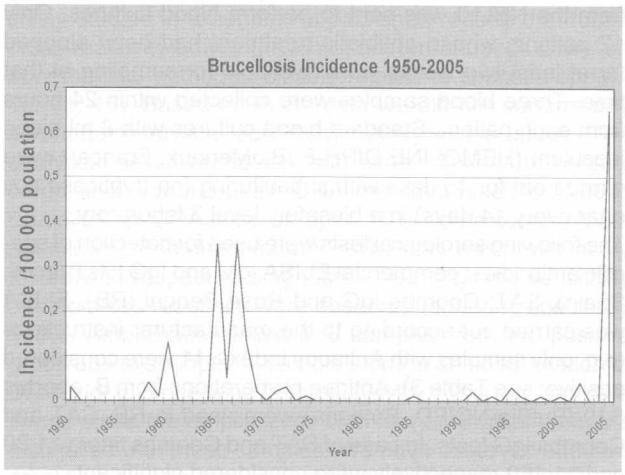


**Figure 1. Human brucellosis incidence in Bulgaria for the period 1950 - 2005**

Nearly all the human cases for the period 1958-2005 had been imported or associated with imported animals or food products. In contrast, all neighboring countries (except Romania) report an incidence as high as four to 15 per 100 000 population posing the Balkan Peninsula as a major endemic region (1, 6, 13).

At the beginning of 2005 a large number of patients with brucellosis compatible symptoms were reported and subsequently hospitalized. The epidemiological investigation corroborated that all of these patients except one (who stayed in Cyprus) had been residing as seasonal farm workers in Xanthi region, a recognized endemic area in Northern Greece. The current study describes the clinical, epidemiological and laboratory findings of 21 newly reported, travel-associated cases of brucellosis in Bulgaria. In addition, it demonstrates a novel diagnostic serum PCR assay that proved highly specific and sensitive and can be easily introduced in the clinical laboratory setting.

### Patients and methods

A total of 63 individuals were included in this study. The anamnestic data were collected by examination according to standard epidemiological questionnaires. All studied individuals originated from a common region in Eastern Bulgaria – the town of Sliven and several villages in its vicinity. For the period 2001-2005 a total of forty five individuals had worked or resided in four sheep-breeding farms in the region of Xanthi (Northern Greece). All forty five individuals had stayed there for at least six months and 75% of them had been there for multiple times, while one of them had been previously hospitalized and treated in the Xanthi General Hospital with confirmed brucellosis. Seventeen individuals were their relatives who had taken short visits in Greece. One more patient had worked in an unknown region of Cyprus.

The initial examinations of the 63 individuals were performed at the Infectious Diseases Ward of "Dr. Ivan Seliminski" Multi-profile Hospital, Sliven. Clinical data analysis included: medical histories, physical examinations, complete blood cell counts, peripheral blood smears, erythrocyte sedimentation rates, blood chemistry profiles, abdominal echography and chest radiographs. Serum samples were sent to the National Reference Laboratory of High-risk Infections (NRLHI-NCIPD) for confirmation of diagnosis. Twenty-one out of the 63 indi

### ABBREVIATIONS USED IN THIS ARTICLE:

PCR – Polymerase Chain Reaction
ELISA- Enzyme-Linked Immunosorbent Assay
SAT – Serum tube Agglutionation Test

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viduals were confirmed as brucellosis patients. All confirmed cases were hospitalized in the same hospital for treatment. No blood cultures were performed on patients' admission at the local hospital and antibiotic treatment was started immediately after serological confirmation. Later, a team from the NRLHI was sent to perform blood cultures. Only 12 patients whose antibiotic treatment had been stopped for at least two weeks were available for sampling at that time. Three blood samples were collected within 24 hours from each patient. Standard blood cultures with 8 ml blood inoculum (HEMOLINE DIPH-F /BioMerieux, France/) were carried out for 45 days with subculturing (on trypticase soy agar every 14 days) in a biosafety level 3 laboratory.

The following serological tests were used for detection of specific antibodies: commercial ELISA IgM and IgG kits (Vircell, Spain), SAT, Coombs IgG and Rose Bengal (RB). ELISA was carried out according to the manufacturer instructions (e.g. only samples with Antibody Index ≥ 11 were considered positive; see Table 3). Antigen preparations from B. abortus S19 (BulBio-NCIPD, Bulgaria) were used in RB, SAT and Coombs IgG tests. In case of SAT and Coombs titters ≥1:80 and ≥1:160 respectively were considered significant.

The most common clinical symptoms and signs were: fever and adynamics (21 cases/100%); myalgias and hepatomegaly (18 cases/ 85.7%); headache, sweating and joint aches (17 cases/ 80.9%)(Table 1).

Based on the clinical presentation and the duration of symptoms, six patients were classified as acute (duration - up to 12 months) and 15 patients as chronic cases (more than 12 months).

**Table 1**. Clinical data for the 21 confirmed brucellosis patients.

| Clinical and laboratory findings | Number of patients (total 21) |
|---|---|
| Undulant fever | 21 (100%) |
| Adynamics | 21 (100 %) |
| Hepatomegally | 18 (85.7%) |
| Myalgia | 18 (85.7%) |
| Headache | 17 (80.9 %) |
| Loss of appetite | 16 (76.2 %) |
| Increased perspiration | 15 (71.4%) |
| Arthralgia | 15 (71.4 %) |
| Elevated erythrocyte sedimentation rate | 14 (66.7 %) |
| Splenomegally | 12 (57.1%) |
| Spondilitis | 11 (52.4%) |
| Increased Alanine- Aminotransferase activity (ALT) | 11 (52.4%) |
| Cough | 7 (33.3%) |
| Leucopenia | 5 (23.8 %) |
| Lymphopathy | 4 (19.0 %) |
| Orchitis | 4 (19.0%) |
| Ear decrease | 1 (4.8%) |
| Rash syndrome | 1 (4.8%) |

**Case definition and antibiotic treatment**

A total of 150 serum samples from 63 suspect individuals were examined. A case of brucellosis was defined as patient with compatible clinical picture and persistent high titters or a rise in titters between the S1 (taken on admission prior to antibiotic therapy) and S2 (3 weeks after S1) or S3 (4 weeks after S2) (1). All others were classified as clinically healthy. All the patients had already been treated for at least 10 days with doxycycline before confirmation of the diagnosis at the National Reference laboratory. Systemic aetiotropic treatment was carried out with doxycycline and rifampicin for 6 weeks followed by ciprofloxacin for another 3 weeks. Additional symptomatic therapy was administered with non-steroid anti-inflammatory drugs, hepatoprotectors and vitamins.

**Direct Serum Polymerase Chain Reaction**

A simple and rapid PCR assay was modified and optimized in a way that avoids any DNA purification steps and at the same time effectively amplifies Brucella spp. DNA in 5 µl serum of patients. Preliminary microwave treatment was applied for inactivation of PCR inhibitors in serum and to release DNA suitable for amplification (12). We used the primers B4/B5 published by Baily et al. amplifying 223bp DNA fragment from the Brucella genus-specific bscp-31 gene (4). Briefly, 5 µl serum was placed on the bottom of 0.5 ml thin-wall PCR tubes. A series of experiments were conducted in order to determine the optimal irradiation time in the microwave oven (Samsung CE2714). Eight minutes at 850W was found to be optimal in respect to amplification efficiency (data not shown). After irradiation the tubes were immediately placed on ice. Thirty-five microliters of PCR mix was then added to a final volume 40 µl. The optimized concentrations of the PCR components were as follows : 0.45 µM B4/B5 primers (4), 175µM dNTP (GE Healthcare Life Sciences), 1x PCR buffer (50mM KCl, 20mM Tris-HCl pH 8.4, Invitrogen Inc.), 1.75 mM MgCl2, 0.1 µg/µl non-acetylated Bovine Serum Albumine (Sigma-Aldrich), 1.25U Taq polymerase (Invitrogen Inc.). The thermal cycling was carried out in a GeneAmp 9700 thermal cycler (PE-Applied Biosystems) with initial denaturation at 930C for 4 min and 45 cycles of 25s at 920C, 20s at 600C (annealing), 30s at 720C (extension) and a final extension for 4min at 720C. The amplicons (20 µl) were separated on 2% agarose gels and stained with ethidium bromide. In all experiments 1pg of B. abortus S19 DNA was used as positive PCR control whereas ddH2O and healthy serum were processed as negative controls. All standard precautions for DNA contamination were strictly kept.

The analytical sensitivity was tested with serial dilutions of serum spiked with Brucella DNA. Briefly, 1µl containing 1.6 µg B. abortus S19 genomic DNA was mixed with 99 µl healthy serum to a concentration of 16 ng/µl. Serial tenfold dilutions was made with the same serum to 1.6 fg/µl DNA. Five microlitters from all dillutions were processed to PCR as described above. The diagnostic specificity and sensitivity was tested with 100 serum samples from bacteriologically confirmed acute or relapsing brucellosis patients (kindly provided by The Refik Saydam National Institute of Hygiene, Turkey) and with 40 samples from patients with another etiology (yersiniosis, tuberculosis etc.) and 40 healthy control sera. The diagnostic yield of the serum PCR for the 21 confirmed cases was calculated by taking ELISA IgG as reference method.

DNA was isolated from necropsy samples by a standard phenol-chloroform procedure and the PCR conditions were the same as in the case of serum samples (3).

## RESULTS

In the beginning of 2005 due to a brucellosis outbreak situation, a total of 63 epidemiologically related individuals were laboratory and clinically studied. From these, 21 were laboratory confirmed. All patients originate from a common region in Eastern Bulgaria (vicinity of Sliven) with no previous incidence of brucellosis.

**Table 2**. Age and sex distribution of the patients

| Age distribution (years) | Male | Female |
|---|---|---|
| < 20 | none | 1 (4.8%) |
| 20-29 | 4 (19%) | 2 (9.5%) |
| 30-39 | 2 (9.5%) | 1 (4.8%) |
| 40-49 | 6 (28.6%) | 1 (4.8%) |
| 50-59 | 3 (14.3%) | 1 (4.8%) |
| Mean 38.5 | Total 15 | Total 6 |

Twenty out of 21 confirmed brucellosis patients (age 19-57) (Table 2.) worked or resided at four, sheep-breeding farms in Northern Greece during 2001-2005 and the onset of symptoms was on-site. Most of the patients (males between 40-50 years) report that they had been involved in the growing and delivering of animals that might be ill. A lot of animals had suffered with mastitis or other severe disorders that had overall high lethality. Abortions and stillbirth were exceptionally frequent among the herds. The workers report for occasional consumption of animal borne foods (e.g. unpasteurized milk, cheese, meat etc.) but the close contact and handling of infected animals on a daily basis could represent a route for airborne transmission (14).

No systemic prophylaxis or treatments were carried out during their stays abroad. Howsoever 13 patients report that they had undergone short courses of unspecified antibiotic treatment that was not prescribed by a physician but rather given by the employers supposedly as prophylaxis. One patient was hospitalized and treated for brucellosis in the Xanthi General Hospital but without improvement. After their return in Bulgaria all patients were examined, hospitalized and studied clinically, paraclinically and epidemiologically. According to the gathered epidemiological and forensic data all laboratory confirmed cases were classified as imported or travel-associated. One patient who had worked in Cyprus was also classified as imported as the epidemiological data confirmed that he had not been in Bulgaria for two years and the symptoms appeared six months prior to his return. Six patients were with acute forms and duration of symptoms for up to 12 months whereas in 15 patients the symptoms have lasted for more than a year (chronic forms) before hospitalization and diagnosis. The clinical analyses indicate that the course of the disease goes as systemic illness that affects various organs and systems. The instrumental examination revealed the following disorders: hepatomegaly in 18 cases (85.7%), bronchitis in four patients (19.0%) and left-lobe pneumonia in one patient (4.8%), double n. auricularis neuritis in one patient (4.8%). These clinical signs and focal complications correlate with other studies in the literature (2, 8, 9, 14).

All patients experienced the initial clinical symptoms when they were abroad and the blood cultures performed in 12 patients were all negative probably due to delayed sampling and/or prior antibiotic treatment in Greece and Bulgaria. Therefore, the diagnosis relied mainly on the serological data and the clinical picture. The obtained overall sensitivity of the tests was as follows: ELISA IgG - 21/21 (100 %), ELISA IgM 8/21 (38%), SAT - 8/21(38 %), Coombs IgG- 19/21 (90.4 %), Rose Bengal 3/21(14.2 %), PCR 20/21(95.2%).

and an occupational exposure to Brucella antigens is highly probable (14). The polymerase chain reaction (PCR) was positive in 20 out of the 21 confirmed cases, and negative in all 42 healthy individuals (specificity 100%).

The analytical specificity of the B4/B5 primers was extensively studied in a number of published reports that found cross-reactivity only with Ochrobactrum anthropii DNA, an opportunistic pathogen closely related to Brucella spp.(5, 10, 11, 15). Based on these data we did not perform any additional analytical specificity testing in this study. The analytical sensitivity tested with serial dilutions of healthy serum spiked with Brucella DNA was determined to be 80 fg Brucella spp. DNA per test (appr. 16 genome copies) (data not shown). The direct serum PCR assay was preliminarily tested with respect to diagnostic specificity and sensitivity and no cross-reactions were observed rendering specificity 100%. Amplification failed in 6 samples from the positive group due to suboptimal sample storage and degradation of DNA prior to processing (sensitivity 94%). This fact was confirmed since the amplification with human β-globin primers also failed with these samples. We have observed effective amplification with sera stored for up to 1 year at -200C and we speculate that the method would be appropriate for older samples too (12).

The epidemiological investigation pointed us to two patients apart from the study group who used to work in the affected farms and already had died at the time of investigation. In one of them a lung carcinoma was determined as a cause of death and the other one was with unclear etiology. Necropsy samples from these patients were tested by culture and PCR. The cultures were negative but *Brucella* specific DNA was amplified from the heart, liver and knee joint samples from one patient suggesting a possible sepsis and endocarditis.

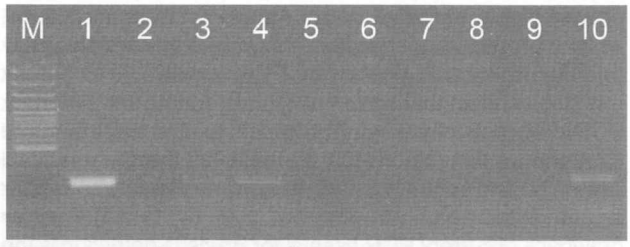


**Figure 2. bscp31 (223bp) PCR analysis of necropsy samples from one patient**
**Lane M- DNA ladder 100bp (Fermentas); lane 1- positive control (1pg B. abortus S19 DNA); lane 2 – negative control (ddH2O); lane 3 – knee joint; lane 4- heart ; lane 5- left lung; lane 6- right lung; lane 7- kidney; lane 8- spleen; lane 9 - bone marrow; lane 10- liver**

**Table 3.** Serological and PCR results for the sixty-three individuals studied.

| Method (Cut-off value) | Patients with acute brucellosis (total n=6) | | | Patients with chronic brucellosis (total n=15) | | | Healthy individuals (total n=42) |
|---|---|---|---|---|---|---|---|
| | S1a | S2b | S3 c | S1 | S2 | S3 | S1, S2 or S3 |
| ELISA IgG (Ab Index d ≥11) | 2 (33.3 %) | 5 (83.3%) | 6 (100 %) | 15 (100 %) | 14 (93.3 %) | 14 (93.3 %) | 2 (4.8 %) |
| ELISA IgM (Ab Index ≥11) | 5 (83.3%) | 5 (83.3%) | 4 (66.7 %) | 3 (20 %) | 2 (13.3 %) | 2 (13.3 %) | 1 (2.4 %) |
| SAT (≥1:80) | 2 (33.3 %) | 2 (33.3 %) | 3 (50 %) | 5 (33.3%) | 5 (33.3%) | 5 (33.3%) | 5 (11.9 %) |
| Coombs Test (≥1:160) | 2 (33.3 %) | 4 (66.7 %) | 4 (66.7 %) | 14 (93.3 %) | 15 (100 %) | 13 (86.7 %) | 3 (7.1 %) |
| Rose Bengal test | 1 (16.7 %) | 1 (16.7 %) | 1 (16.7 %) | 2 (13.3 %) | 2 (13.3 %) | 2 (13.3 %) | 2 (4.8 %) |
| PCR | 6 (100 %) | 6 (100 %) | 6 (100 %) | 14 (93.3 %) | 13 (86.7 %) | 14 (93.3 %) | 0 (0%) |

The specificity of the serological tests was more or less compromised. Significant titters were observed occasionally with samples from the remaining 42 healthy cases (Table 3). This observation was not unexpected since most of the individuals worked as farmers abroad (or their family members)

(Figure 2). Only bone material was available for processing from the second patient where PCR was negative.

The duration of the symptoms for more than a year and the various focal complaints point out that at least 15 of our patients had been with chronic disease at the time of inves

tigation. At present, 12 patients are still with active chronic brucellosis and five are hospitalized with osteoauricular and other focal complications. Nine are asymptomatic with no detectable Brucella DNA in serum and decreasing titters but remain regularly examined for possible relapses.

## DISCUSSION

Human brucellosis became extremely rare in Bulgaria and most of the general practitioners and clinicians are unaware of the clinical presentation, which is mostly unspecific on the other hand. The outbreak character and the subsequent epidemiological investigation in our case were the main points that contributed to the recognition of the disease. All 21 patients originate from a common region in Eastern Bulgaria with no history of brucellosis cases. However many people and their families living there occasionally travel to Greece to work as seasonal farm workers in several animal-breeding farms. Many of these farms are situated in the area of Xanthi, a well-recognized endemic region.

On hospital admission, most of the patients were in overall bad shape with symptoms lasting for more than three to six months and in fifteen patients for more than a year. The initial clinical laboratory examinations, e.g. the hematological and instrumental results were non-indicative of brucellosis, as it is frequently reported in the literature (14). After the serological tests performed at the local hospital laboratory, samples were sent to the National Reference Laboratory of High-Risk Infections for confirmation. Since all attempts for Brucella isolation failed, the diagnosis relied mainly on clinical, serological data, while the polymerase chain reaction (PCR) was able to detect the vast majority of the confirmed cases with brucellosis. Despite their simple usage and sensitivity the serological methods yielded an unacceptable high rate of false-positive results, which hampered the diagnosis. In contrast, PCR proved highly sensitive both in acute and chronic cases, while it proved very specific, since all 42 epidemiologically related healthy individuals were PCR negative. Previously it has been shown that serum is an appropriate specimen for PCR diagnosis of human brucellosis and at least in acute cases better than blood (15). It is believed that the microbial DNA in human serum is usually in extremely low copy number and tends to be highly fragmented (5, 7, 10, 11, 15). A major drawback of current PCR assays for Brucella detection is the need for DNA purification that, in the case of the precipitation methods, is inevitably associated with significant DNA losses through the multiple steps of sample processing. On the other hand, the silica columns need to use 200-400 µl of patient's serum and they are relatively costly (11, 15). We have modified a simple, rapid and inexpensive PCR assay that avoids the DNA purification steps and effectively amplifies Brucella spp. DNA directly from five µl of patients' serum, and reduces the cost of the laboratory diagnosis. The primers B4/B5 used in this study have been widely exploited by other groups on various types of specimens and showed excellent specificity and sensitivity (4, 5, 10, 11, 15). An increasing number of reports demonstrate the value of serum as clinical specimen for the PCR detection of various infectious agents (7). This approach has some advantages in respect that the same samples used for serology can be readily applied in PCR without the need for additional sampling. The results are ready in only three hours, which could improve the overall diagnostic outcome. The serum contains less PCR inhibitors than blood decreasing the rate of false negative results owing to inhibition. The introduction of such PCR assays greatly facilitated the diagnosis of brucellosis especially with culture negative, serologically false-positive and chronic cases (11). However the multi-center standardization and validation of these methods with respect to specificity, sensitivity and reagents is still in progress and their introduction into the routine clinical practice is yet to be implemented.

This study shows that PCR is also applicable to retrospective and forensic analyses on necropsy specimens when brucellosis is suspected as a contribution to death. Apart from the study group, the deaths of two patients who also had been working at the affected farms were investigated. The PCR testing confirmed that one of these patients was infected with Brucella spp. although the cause of death was recognized as lung carcinoma. The infection of multiple organs including the heart suggests that brucellosis can easily be misdiagnosed and overlooked when underlying disorders are presented.

Despite that brucellosis had never been a significant health-care problem in Bulgaria the newly imported cases represent an indication for making certain changes in the current surveillance system. Much attention should be focused to the relatively large cohort of seasonal farm workers traveling abroad. We propose that all farmers working in brucellosis endemic countries be examined at least serologically and immediately upon arrival.

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# LEMIERRE'S SYNDROME AFTER AN OROPHARYNGEAL INFECTION: TWO CASES WITH ISOLATION OF ANAEROBES AND REVIEW OF THE LITERATURE

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**SUMMARY:**
**We present two cases /from a period of about an year – June 2008-July 2009/ with isolation of anaerobes in patients with an oropharyngeal infection that developed Lemierre's syndrome. Lemierre's syndrome is a severe illness caused by Gram-negative anaerobes such as Fusobacterium sp. and mainly F. necrophorum and F.nucleatum. The infection originates from the throat or other oropharyngeal infections and spreads via a septic thrombophlebitis of the internal jugular vein. The ensuing bacteraemia is complicated by infections of the lung /abscess or empyema/, central nervous system /meningitis/, joints and bones, liver, kidneys, etc. Although rare in our days which differs from the past, there is evidence of increasing its frequency in the last years, possibly associated with reduced use of antibiotic therapy for sore throats. The typical clinical picture is characteristic but many clinicians are unaware of the condition and diagnosis is often delayed with potentially fatal consequences. Early diagnosis and the adequate and prolonged antibiotic therapy are extremely important for reducing the mortality of this forgotten from the preantibiotic era disease.**

*Key words: Lemierre's syndrome, oropharyngeal infection, anaerobes*

## HISTORY

**Lemierre's syndrome** (or Lemierre's disease) is also known as postanginal sepsis and human necrobacillosis. Loeffler is the first to discover in 1884 that Fusobacterium necrophorum causes necrobacillosis and different necrotic infections in wild and domestic animals. The first reported case of human necrobacillosis is from 1900 when Courmond and Cade /12/ describe a patient who dies of an overwhelming sepsis after an acute tonsillitis and from the blood of whom anaerobic bacillus was isolated. Sepsis following from a throat infection was described by Scottmuller in 1918 as well /22/. However it was Andre Lemierre, in 1936, who published a series of 20 cases where throat infections were followed by identified anaerobic septicemia, of whom 18 patients died /16/ and describes the characteristic syndrome of the disease as an oropharyngeal infection with septic thrombophlebitis of the internal jugular vein.

In 1955, Alston /12/ reports 280 cases of necrobacillosis and divides them in 4 groups according the primary foci of the infection:1/ Infection starting in the throat, following a sore throat or otitis media and mastoiditis; 2/ primary infection in the skin or subcutaneous tissues; 3/in the female genital tract or primary foci in the alimentary tract and 4/ Empyema, with or without lung abscess.

Lemierre's syndrome was more common in the early 20th century before the discovery of penicillin and in the preantibiotic era with mortality rate of 90% /16/. It was rare and sparsely reported later, during the 1960s and 1970s because of the widespread use of penicillins in the treatment of throat infections /24/. During the last decades, the reduced use of antibiotics for sore throats may have increased the risk of this disease and it is becoming less rare with many cases being reported/1,2,5-7,9-11,12,15,18,19,21,23,26,28,29/. Improved anaerobic diagnostics, better clinical microbiological laboratories and blood culture methods on the whole have probably also raised the chances of diagnosing Lemierre's syndrome.

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## ETIOLOGY

Fubacterium necrophorum is the main causative agent in Lemierre's syndrome. 81% of the people with Lemierres's syndrome has been infected with Fusobacterium necrophorum, while in 11% of people it was caused by other Fusobacterium species /F. nucleatum, F. mortiferum, F. varium/ . Rarely Lemierre's syndrome is caused by other (usuallyGram-negative) bacteria, which include Bacteroides fragilis or other Bacteroides and Eikenella corrodens; Peptostreptococcus, Staphylooccus aureus, Enterococcus sp. and other nonhemolytic, microaerophilic and beta-hemolytic streptococci of groups A, B and C as well /11/. MRSA might also be an issue in Lemierre infections /4, 20/.

## PATHOPHYSIOLOGY

Fusobacterium necrophorum and other anaerobes are part of the normal microbial flora of the oropharynx, gastrointestinal tract and genitourinary tract of humans and animals. They do not normally invade intact mucosal surfaces /3,17,27/ . A reduced host defence in the pharyngeal mucosa by viral / infectious mononucleosis and Epstein-Barr virus/ or bacterial infection may predispose to Lemierre's syndrome /1,5,8,9/. Previous tonsillar and peritonsillar infections are common /52%/ in those infected with F.necrophorum /13/.

Among the anaerobes of the normal oral flora F.necrophorum and F.nucleatum are one of the most virulent species /10,25/. Unique to anaerobes, F.necrophorum has the capability of invading in previously healthy individuals, mainly due to its similar to aerobic Gram-negative rods lipopolysaccharide endotoxin and different exotoxins like hemolysin, heparinase, leucocidin and other proteolytic enzymes /12/. F. necrophorum produces hemaglutinin which causes platelet aggregation that can lead to diffuse intravascular coagulation and thrombocytopenia /24/.

During the primary /oropharyngeal/ infection, F. necrophorum colonizes the infected site and spreads to the parapharyngeal space. The bacteria then invade the peritonsillar blood vessels where they can spread to the internal jugular vein /6/ and cause the formation of a thrombus containing these bacteria. This septic thrombophlebitis can give rise to septic microemboli that disseminate to other parts of the body where they can form abscesses and septic infarctions. The first capillaries that the emboli encounter where they can nestle themselves are the pulmonary capillaries. As a consequence, the most frequently involved site of septic metastases are the lungs, followed by the joints (knee, hip, sternoclavicular joint, shoulder and elbow). In the lungs, the bacteria cause abscesses, nodulary and cavitary lesions. Pleural effusion is often present. Other sites involved in septic metastasis and abscess formation are the muscles and soft tissues, liver, spleen, kidneys and nervous system (intracranial abscesses, meningitis)/6/.

## SIGNS AND SYMPTOMS

Lemierre's syndrome, or postanginal sepsis syndrome, begins as an oropharyngeal infection resulting in internal jugu

lar vein /IJV/ thrombophlebitis and subsequent metastatic inefections in previously healthy persons without underlying diseases, mainly young patients. It is a rare complication of tonsillitis, pharyngitis, otitis media, mastoiditis, parotitis, sinusitis, odontogenic infections.
Signs and symptoms are quite variable and depend on the primary infection. Fever, oropharyngeal pain, neck swelling and trismus along the sternocleidomastoid muscle are the clinical manifestations of the septic thrombophelibitis of the / IJV/ as well as abdominal pain, nausea, vomiting which usually occur several days to 2 weeks after the initial symptoms. The ensuing bacteraemia is complicated by infections of the lung /abscess or empyema/, central nervous system / meningitis/, joints and bones /arthritis/, the liver, kidneys, mediastinitis, endocarditis, etc.

## DIAGNOSIS

Diagnosis of Lemierre's syndrome is based mainly on its typical clinical signs and symptoms. Thrombosis of the internal jugular vein can be diagnosed noninvasively with sonography. However, thrombi that have developed recently have low echogenicity and thus will not show up on ultrasound. A CT scan or MRI scan is more sensitive in displaying the thrombus. Chest X-ray and chest CT may show pleural effusion, nodules, infiltrates, abscesses and cavitations.
Laboratory investigations reveal signs of a bacterial infection with elevated C-reactive protein, erythrocyte sedimentation rate and white blood cells (notably neutrophils). Platelet count can be low or high. Liver function tests and renal function tests are often abnormal.
Isolation of F. necrophorum or other anaerobes at the site of the infection and positive anaerobic blood cultures prove the etiology of the infection.

## MICROBIOLOGY

We present 2 cases with pharyngeal abscesses, complications of a sore throat /acute tonsillitis/. The patients were young men at the age of 33 and 28. The specimens that we received to inoculate were aspirates - pus from the abscesses that were transported to the laboratory in a syringe and a transport medium of Stuart.
First case. We isolated anaerobes like Fusobacterium necrophorum, Bacteroides fragilis, Prevotella melaninogenica, Peptostreptococcus anaerobius, P.micros, P.magnus Bifidobacterium sp. From the aerobes it was only S.epidermidis and obviously it was an anaerobic infection with a leading anaerobic role.
**In the second case** the anaerobes were Fusobacterium nucleatum, Prevotella oralis, Peptostreptococcus sp. Aerobes - S.epidermidis and the conclusion is - an anaerobic infection with a leading role of the anaerobes. Blood cultures were negative.
**Identification /14/:** 1/ Gram stain of F.necrophorum – Gram-negative, very pleomorhic /from coccobacilli to long rods and filaments/ bacteria, with swollen and bizarre forms /Fig.1/ and F.nucleatum - Gram-negative thin rods with sharply pointed, tapering ends – a "needle-shaped" morphology /Fig.2/.
2/ Growth and morphology on Brucella blood agar . F.necrophorum – flat to convex, with an umbonate profile and irregular shape colonies, and a greening of the agar because of the hemolysis /Fig. 3/. F.nucleatum has dry, irregular, white breadcrumb-like colony morphology or speckled and translucent colonies. Both Fusobacterium sp. have a characteristic rancid odor due to butyric acid production.
3/ Tests for identification. Susceptibility to special-potency antibiotic discs /discs with a diagnostic use/ : Kanamycin /1000 mg/ - S; Vancomycin /5 mg/- R; Colistin /10 mg/ – S. They are indole-positive; lipase - positive /F.necrophorum/.

## THERAPY

Surgical drainage of empyemas and abscesses and debridement of necrotic tissue is indicated to accompany appropriate antibiotic therapy.
F.necrophorum is known to be susceptible to penicillin, cephalosporins, tetracyclines, chloramphenicol, clindamycin, metronidazole, imipenem. Beta-lactam agents are drugs of choice for treatment. Beta-lactamase producing strains have rarely been reported but other anaerobic bacteria involved in the infection may be beta-lactamase-positive and the addition of beta-lacatamse inhibitors is obligatory then.
Most authors recommend a combined treatment with high-dose penicillin and metronidazole or monotherapy with clindamycin for a prolonged period of time – 2 -6 weeks /11/. The initial treatment is with intravenous penicillin, for example penicillin G at 1.2-3.0 g 4 times daily and iv or rectal metronidazole, 500 mg 3 times daily. Monotherapy with iv clindamycin at 0.6-0.9 g 3 times daily is an alternative treatment.
When the anaerobic infection is combined with an aerobic one, gentamicin is added to the therapy. In our cases it was ceftriaxone+metronidazole +gentamicin and there was a favourable outcome of the therapy.
Lemierre' syndrome is still known as the "forgotten disease" as many doctors are unaware of its existence, therefore often not even diagnosed . The mortality rate is now generally quoted as 15% once this illness is correctly diagnosed and cured with proper medical treatment, although one series of cases reported mortality as low as 6.4% /6/.
The early diagnosis and alertness to the possibility of Lemierre's syndrome in both clinicians and microbiologists as well as a prolonged and an adequate iv antibiotic treatment are very important to reduce the high mortality of this infection.

**Fig. 1 Gram stain of F.necrophorum – Gram-negative, very pleomorhic /from coccobacilli to long rods and filaments/ bacteria, with swollen and bizarre forms**

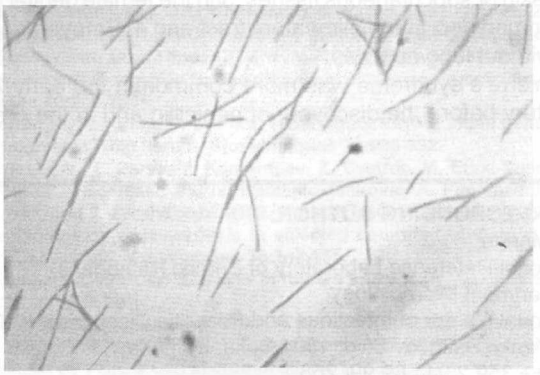

**Fig. 2 Gram stain of F.nucleatum - Gram-negative thin rods with sharply pointed, tapering ends – a "needle-shaped" morphology**

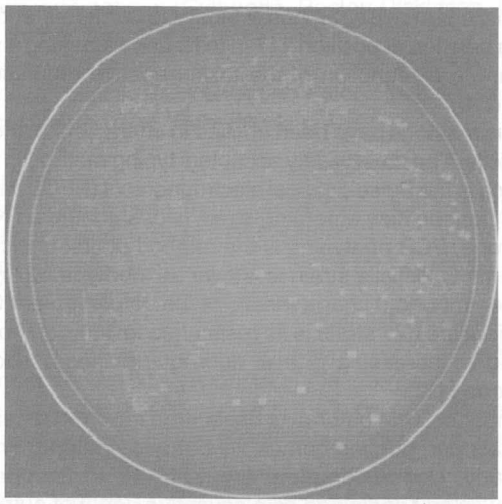

Fig. 3 Growth and colonial morphology of F.necrophorum on Brucella blood agar

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# FIRST CASES OF SEVERE HOSPITAL-ACQUIRED CLOSTRIDIUM DIFFICILE INFECTIONS IN SOFIA, BULGARIA

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**SUMMARY:**
**Background: Clostridium difficile has become a critically important pathogen worldwide, particularly in hospitalized patients. In 2008, the European Center for Disease Prevention and Control (ECDC) initiated a pan European surveillance study to collect epidemiological and microbiological data of C. difficile associated diseases (CDAD). Since Bulgaria has joint this study a number of CDAD cases were discovered. The objective of the present work is to report the first cases of CDAD occurring in Sofia, Bulgaria.**
**Material and Methods: For the period November 2008 - February 2009, thirty six fecal samples from patients with severe enterocolitis and previous antibiotic treatment have been investigated. The stool samples derived from 3 hospitals in Sofia. Strains were typed and further characterized for the presence of toxins A (TcdA), B (TcdB)_and binary toxins (CdtA and CdtB).**
**Results: No outbreaks were reported and the incidence of C. difficile infection (CDI) in the hospitals was 3.12 per 10,000 patient admissions (0.7 per 10,000 patient-days). Four patients with severe CDI were identified of which two patients died due to complications of the infection. Two of the isolates belonged to PCR ribotypes 017 (TcdA-; TcdB+; CdtA/B-), one was 046 (TcdA+; TcdB+) and one was 078 (TcdA+; TcdB+; CdtA/B+). All of these ribotypes have been reported to cause oubreaks worldwide.**
**Conclusion: These are the first well documented cases of CDI in Sofia, Bulgaria. Despite the fact that none of the Bulgarian isolates belonged to the hypervirulent C. difficile NAP1/BI/027, C. difficile ribotypes 078, 046 and 017 were found to be associated with a severe CDI.**

*Key words: Clostridium difficile, toxins, ribotypes, Bulgaria.*

## INTRODUCTION

Clostridium. difficile was first discovered by Hall and O`Toole in 1935 as a part of the normal intestinal flora of newborns. It was found in 1974 that C. difficile causes antibiotic-associated colitis by producing toxins with high lethality in mice. Since 2003 outbreaks associated with the emerging hypervirulent strain – C. difficile PCR ribotype 027 have been reported worldwide. The increased virulence of this strain is assumed to be associated with increased production of toxins A and B (1) . C. difficile PCR ribotype 027 was designated as a PCR ribotype 027 C. difficile NAP1/BI/027; North American pulsed-field gel electrophoresis (PFGE) type 1 (NAP1), restriction endonuclease analysis (REA) type BI and toxinotype III (1-6).

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C. difficile NAP1/BI/027 has a binary toxin genes and contains a deletion at position 117 in tcdC gene, causing a frame shift and a dysfunction of the toxin-negative regulation by TcdC (7-9).

Various methods have been applied to characterize and type C. difficile. The most widely accepted typing method in North America is the Pulse-field gel electrophoresis (PFGE) and for this region the new emerging strain is designated as NAP1. In Europe, PCR ribotyping is the method of first choice and the same C. difficile strain is known as ribotype 027 (10).

Another important characteristic of the hypervirulent C. difficile ribotype 027 strain is its resistance to fluoroquinolones leading to increasing rate of fluoroquinolones-associated diarrhoea (1,2).

A number of large outbreaks have been reported recently in hospitals and nursing homes in Canada, Finland, France, Germany, United Kingdom etc. (11-14). In Europe, the strain was first described as a causative agent of outbreaks in 2004 in England and shortly thereafter – in 2005 in The Netherlands (8). In 2008, the European Center for Disease Prevention and Control (ECDC) initiated a pan European surveillance study to collect epidemiological and microbiological data of C. difficile associated diseases (CDAD). Bulgaria also participated and continued the surveillance for two extra months (15).

The aim of the present work is to report on the initial results of the Bulgarian surveillance study.

## MATERIAL AND METHODS

Patients

For the period of four months, November 2008 - February 2009, thirty six fecal samples have been investigated at the National Reference Laboratory for Enteric Pathogens, at the National Center of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria. Three hospitals participated in the surveillance study. The incidence of CDI in the selected hospitals was 3.12 per 10,000 patient admissions (0.7 per 10,000 patient-days). Patients with enterocolitis were selected according to some of the following criteria:

Age over 2 years.
Antibiotic associated diarrhea.
Onset of diarrhea after the third day of hospital admission.
Patients admitted to hospital with diarrhea.
Patients with colonic endoscopy findings characteristic of C. difficile infection.
Negative laboratory results for Salmonella, Shigella, Campylobacter, Enteropathogenic Escherichia coli.
Patients with complications like: toxic megacolon, pseudomembanous colitis, ileus,
peritonitis.

Laboratory diagnosis

All 36 stool samples were tested for the presence of C. difficile toxins. Subsequently, positive tested stool samples were cultured for C. difficile. Determination of toxins in stool samples was performed using Immuno Card Toxins A&B- EIA (Meridian, Bioscience, USA) rapid test kit.

Culture method

Fecal samples were divided into two portions. The first portion was processed with the alcohol shock procedure. For that purpose 0.5 ml absolute CH2OH were mixed with 0.5 ml liquid feces, following incubation for 1 h at room temperature and culture on Brucella agar plates with 10% sheep blood. The second portion was directly cultured on Brucella agar plates with 10% sheep blood containing cefoxitin, cycloserine and amphotericin B. All plates were incubated 2-5 days at 37°C in anaerobic conditions.

Identification

Suspected colonies were identified as C. difficile by performing Gram stains and latex co-agglutination tests for detection

of a common antigen (Culturette, Becton Dickinson, USA).

PCR Ribotyping

The strains identified as C. difficile were ribotyped at the Clostridium difficile Reference Laboratory, Leiden University Medical Center, The Netherlands (14 ).

## RESULTS

The samples derived from 3 hospitals serving 1,800,000 inhabitants (incidence of CDI 2%000).

In total, 36 stool samples were tested for C. difficile toxins and 4 of them were toxin positive.

Toxin positive fecal samples were cultured and four C. difficile strains were isolated. All patients received antibiotic treatment previous to the onset of the diarrhea.

Table 1 summarizes the patients's clinical records and outcomes, laboratory results, antimicrobial therapeutic courses.

## DISCUSSION

Recent surveillance studies show that C. difficile NAP1/BI/027 associated infections have been increasing worldwide (4,12,16,17). In 2008, 16 European countries reported outbreaks and a number of sporadic cases (4,15). This prompted ECDC to perform a surveillance study in 34 European countries. CDI incidence varied across hospitals (mean: 5.5 per 10,000 patient-days per hospital; range: 0 to 36.3). Sixty-five different PCR ribotypes were found, among which 014/020 (16%), 001 (9%) and 078 (8%) were most prevalent. The prevalence of PCR ribotype 027 was 5% (15). Bulgaria also participated in this European surveillance study. Until that time the laboratory diagnosis of C. difficile infections was insufficient in Bulgaria and cases were rarely reported. An incidence of 3.12 per 10,000 patient admissions (0.7 per 10,000 patient-days) was found in 3 participating hospitals.

**Table1.** C. difficile isolates and clinical records of studied patients

| Lab No | Age,sex | Hospital | Clinical diagnosis | Previous antimicrobial therapy | Ribotype / toxin type of C. difficile | Presence of toxins | Outcome |
|---|---|---|---|---|---|---|---|
| 09 | 66, female | University Hospital, "Saint Anna", Sofia | Hepatic carcinoma Broncho-pneumonia, Enterocolitis | Ciprofloxacin, Ceftriaxon | 078/V | (A+/B+) Binary toxin (+) | lethal |
| 15 | 3, male | University Hospital, "Saint Anna", Sofia | Enterocolitis | Amoxicillin Cefalexine | 046/0 | (A+/B+) | recovered |
| 36 | 66, female | Hospital of Infectious Diseases "Prof. Kirov", Sofia | Neurological operation, Enterocolitis | Lincomycin | 017/VIII | (A-/B+) Binary toxin (-) | recovered |
| 181 | 75, female | Hospital of Infectious Diseases "Prof. Kirov", Sofia | Acute bronchitis, Pseudomembranous colitis with peritonitis | Ampicillin/ Sulbactam | 017/VIII | (A-/B+) Binary toxin (-) | lethal |

Two of the C. difficile isolates (laboratory numbers: 09 and 15 ) belonged to the 078 and 046 ribotypes respectively and were positive for toxins tcdA+, tcdB+ and the binary toxin cdt A/B+, while the other two (laboratory numbers: 36 and 181) were ribotype 017 and tcdA-, tcdB+.

The patient 09, a 66-year-old female, with previously diagnosed hepatic carcinoma, was admitted to hospital with a pneumonia and received ciprofloxacin and ceftriaxon for two weeks before the onset of the diarrhea. The outcome of this case was lethal. Another patient 181, a 75- year-old female, was admitted to hospital in a very compromised general condition. She suffered a severe form of acute bronchitis and has been treated with ampicillin/sulbactam for 10 days before the onset of the profuse diarrhea. This patient suddenly died and her laboratory diagnosis was established post mortem. The pathoanatomical conclusion stated that the main cause for her death were a number of complications: diffuse pseudomembranous colitis, diffuse fibrinous and purulent peritonitis, rectal and sygmoidal diverticulosis and purulent intramural abscesses in the bowl.

As demonstrated in Table 1, two patients, a 3-year- old male and a 66- year-old female, developed C. difficile associated infection after antibiotic treatment. The ribotyping has revealed that these two strains belonged to 046 and 017 ribotypes respectively. Both patients have successfully recovered after therapeutic course with metronidazole.

These isolates belonged to ribotypes 017, 046 and 078. Barbut et al. reported that the PCR ribotypes differed strikingly between hospitals and countries (10). A new emerging ribotype 078 C. difficile is recently found to be prevalent in Belgium, The Netherlands, Nothern Ireland, Scotland and Greece (18). Patients infected with type 078 were younger compared to those infected with type 027, but both groups demonstrated similar rates of severe diarrhea and mortality (19). One Bulgarian patient was also found to be infected with 078 and subsequently died. The complicated course of disease can be explained by the hyperproduction of virulence factors: toxin A, B and binary toxin by the microorganism and the immunocompromised status of the patient. Interestingly, Dutch authors have recently discovered that 078 was a predominant C. difficile strain in some farm animals, especially pigs, dairy calves and horses (19,20,21). Similar findings were reported by Spanish researchers (22). The same ribotype 078 has been detected in retail meat in North America (23). These facts suggest the possibility of animal-to-human transmission or, more likely, suggest a common source for human and animal strains.

C. difficile belonging to ribotypes 046 and 017 have been reported by a number of countries to be the most prevalent types both in nosocomial and community-aquired settings (24). The first outbreak due to C. difficile ribotype 017 was described in Canada in 1999 (11). It has been found that

the main characteristic of this strain is the lack of a part of the toxin A gene and the presence of a toxin B gene. Polish researchers reported that PCR ribotype 017 predominated in their country with approximately 40% of the C. difficile isolates (25). An outbreak of type 017 has also been reported in The Netherlands and data from the European surveillance study reveal that type 017 was also frequently found (3.7%) (26). Additionally, type 017 is more frequently found in Asia than other continents (27-29).

Metronidazole is the drug of choice in mild to moderate CDI and is preferred by clinicians, because it is less expensive and avoids the selection of vancomycin-resistant enterococci (30,31). In contrast, vancomycin is the drug of first choice for severe complicated cases of C. difficile NAP1/BI/027. Oral treatment with vancomycin reaches rapidly high local concentrations in the intestinal tract. Retrospectively, 3 of our patients were treated with metronidazole.

In conclusion, these are the first well documented cases of CDI in Sofia, Bulgaria. Despite the fact that none of the Bulgarian isolates belonged to the hypervirulent C. difficile NAP1/BI/027, C. difficile ribotypes 078, 046 and 017 were found to be associated with severe CDI.

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# COMPARISON OF DIFFERENT METHODS FOR DETECTION OF HELICOBACTER PYLORI INFECTION

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**SUMMARY:**
**Helicobacter pylori colonizes the human stomach and is associated with active chronic gastritis, peptic ulcers, gastric carcinoma and MALT-lymphomas. The different methods, used to detect H. pylori, are either invasive (histology, culture, PCR) or non-invasive (serology, urea breath test).**
**The aim of this study was to compare a non-invasive H. pylori antibody detection test with invasive ones - histological and bacteriological (examination of gastric biopsy samples with direct microscopy, rapid urease test and culture).**
**33 patients (17 male and 16 female) submitted to endoscopy because of gastrointestinal disorders were studied. Serums for serological examination was obtained from all patients.**
**30 (91%) of all patients were infected with H. pylori. H. pylori positive rates by microbiological methods were 88% (with diagnostic sensitivity /DS/ - 97%), by histological examination - 70% (DS - 77%) and by serology - 76% (DS - 83%). Diagnostic accuracy of different bacteriological methods was: for direct rapid urease test - 93%, direct microscopy examination - 63% and culture - 51%.**
**The combination between histological and microbiological methods was with highest efficiency for detection of Helicobacter pylori infection - 98%.**

*Key words: Helicobacter pylori, urease test*

## INTRODUCTION

Helicobacter pylori colonizes the human stomach and is associated with active chronic gastritis, peptic ulcers, gastric carcinoma and MALT-lymphomas. For that reason it is necessary to develop different tests for its accurate diagnosis and successful eradication. The different methods, used to detect H. pylori, are either invasive (histology, culture, PCR) or non-invasive (serology, urea breath test) (1,2, 9, 11,15,17, 20,22,23).

The aim of this study was to compare a non-invasive H. pylori antibody detection test and invasive ones - histological and bacteriological (examination of gastric biopsy samples with direct microscopy, rapid urease test and culture).

## MATERIALS AND METHODS

### Patients

Thirty three patients /mean age 42, range 23-69, 17 male and 16 female/ with different gastrointestinal disorders and with following diagnoses were tested: 26 with pangastritis, 13 - duodenal ulcers, 12 – esophagitis, 2 - gastric ulcers, 2 - erosive gastritis, 1 - MALT- lymphoma and 3 after treatment.

### Specimens

Sixty six biopsy specimens /33 from the antrum and 33 from the corpus/ were taken at routine endoscopy during investigation of upper gastrointestinal symptoms in the Clinic of Gastroenterology of "Queen Joanna" Hospital, Sofia. One ml serum for serological examination was obtained from all 33 patients.

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### Microbiology investigation

Transport of the specimens

Each tissue specimen was transported to the National Reference Anaerobic Laboratory /NCIPD/ in 0,5 ml sterile saline with 5% glucose within 1 to 3 h. All biopsies were homogenized by spreading.

Direct microscopy examination

Each biopsy was stained by the Gram method and examined for the presence of Gram-negative curved rods.

Direct rapid disk urease test

Rapid disk urease test /Tzvetika, Bulgaria/ was applied for all tissue specimens. A change of the colour of the disc from yellow to pink within 1 min to 1 h was indicated as a positive result.

Culture

All biopsies were cultured on non selective Mueller Hinton agar with 10% sheep blood and on selective one with the following antimicrobial agents: vancomycin - 10 mg/l, trimethoprim - 5 mg/l, nalidixic acid - 5 mg/l and griseofulvin - 5 mg/l (8,13,20).

Cultures were incubated for 4 to 7 days at 370 C in microaerophilic conditions in 5% O2 and 10% CO2. Gram - negative, spiral, microaerophilic bacteria that were positive for catalase, oxidase and urease were identified as H.pylori.

### Histology

66 gastric biopsies /33 from the antrum and 33 from the corpus/ were examined histologically. Formalin-fixed paraffin-embedded sections of all of them were stained with Gimsa and Gram methods.

The results were tested by the semi quantitative method of Chan (7).

Serology

From all patients 33 serums were obtained and tested for IgG antibody against H.pylori with immunodiagnostic test "Rapid H.p." /US Meds/.

## RESULTS

30 /91%/ of all patients were infected with H.pylori. Their endoscopical and histological diagnosis are given on Table 1. /Classification is according Marshall et al /14/ .

30 patients /91%/ had endoscopic pathology: 26 /79%/ were with pangastritis, 13 /40%/ - with duodenal ulcer, 12 /36%/ - with esophagitis, 2 /6%/ - with gastric ulcer, 6 /18%/ - with duodenitis, 1 /3%/ - with MALT lymphoma, ect. /The sum of the percentage is above 100% because there were patients with more than one diagnosis/.

Three of the patients /9%/ had normal endoscopy. They were tested after treatment for eradication of H.pylor and together with the remaining 30 patients had different degree of damage of their gastric mucosa, according to histological diagnosis /table 1/.

Table 2 summarised results for identification of H.pylori by microbiological, histological and serological methods. 19 patients were H.pylori positive by all three methods, 18 of them had gastric pathology, according their endoscopic and histological diagnosis. One of these positive patients /tested after treatment/ had normal endoscopy but he was diagnosed mild chronic gastritis. His treatment for eradication of H.pylori, probably, has been unsuccessful.

The microorganism was isolated by microbiological methods from 2 patients, but was not proved histologically and serologically. The clinical diagnosis of these patients /one with pangastritis and antral erosion and other with pangastritis and

**Table 1**: Endoscopic and histological diagnoses

| N of patients | Endoscopy | Histology |
|---|---|---|
| 5 | Pangastritis | ACG*, mild CCG** |
| 2 | Pangastritis, duodenal ulcer | ACG, moderdte CCG |
| 1 | Pangastritis, gastric ulcer | ACG, CCG |
| 5 | Pangastritis, duodenitis, esophagitis | ACG, mild CCG |
| 1 | Pangastritis, duodenitis, esophagitis, corpus with erosions | Severe active antral gastritis |
| 1 | Pangastritis, gastric ulcer, esophagitis | MALT Lymphoma |
| 5 | Pangastritis, duodenal ulcer, esophagitis | Mild ACG, mild CCG |
| 2 | Duodenal ulcer | Mild CCG |
| 3 | Normal endoscopy | Mild CCG |
| 1 | Pangastritis, antrum with erosions | ACG, mild CCG |
| 3 | Pangastritis, duodenal ulcer, antrum and corpus with erosions | Moderate ACG, CCG |
| 2 | Gastritis erosiva | ACG, mild CCG |
| 1 | Pangastritis, duodenal ulcer, esophagitis,corpus with erosions | ACG, CCG |
| 1 | Pangastritis, antrum with erosions | Active ACG |

*** ACG - Antral chronic gastritis, ** CCG - corpus chronic gastritis**

**Table 2**: Comparison of differend methods for identification of H.pylori

| N of patients | Microbiology | Histology | Serology |
|---|---|---|---|
| 19 | + | + | + |
| 2 | + | - | - |
| 1 | - | - | + |
| 4 | + | + | - |
| 4 | + | - | + |
| 3 | - | - | - |
| N of positive /%/ | 29 /88%/ | 23 /70%/ | 24/73%/ |
| Diagnostic sensitivity | 97% | 77% | 83% |

**Table 3**: Provement of H.pylori in the antrum and the corpus with invasive and non invasive methods

| N of patients | Antrum Direct mic-roscopy | Rapid urease | Culture | Histo-logy | Corpus Direct mic-roscopy | Rapid urease | Culture | Histo-logy | Sero-logy |
|---|---|---|---|---|---|---|---|---|---|
| 1 | - | ++++ | - | - | - | ++++ | - | - | + |
| 2 | - | ++ | + | - | - | + | - | - | + |
| 3 | + | ++++ | + | + | - | ++++ | + | + | ++ |
| 4 | + | ++ | + | ++ | + | ++ | + | ++ | ++ |
| 5 | + | ++ | + | + | + | ++ | + | - | + |
| 6 | - | ++++ | + | + | + | ++ | + | - | ++ |
| 7 | - | - | - | - | + | ++ | - | - | ++ |
| 8 | + | ++ | + | - | - | ++ | - | + | + |
| 9 | - | ++++ | + | - | - | ++++ | + | + | ++ |
| 10 | - | - | - | ++ | + | ++ | - | + | ++ |
| 11 | + | +++ | + | + | + | +++ | + | + | ++ |
| 12 | + | - | - | - | + | +++ | - | ++ | + |
| 13 | - | - | - | - | - | - | - | - | - |
| 14 | - | ++ | - | + | - | ++ | - | + | - |
| 15 | + | ++++ | - | + | - | + | - | + | + |
| 16 | - | + | + | + | - | + | - | + | + |
| 17 | - | - | - | - | - | - | - | - | + |
| 18 | - | ++ | - | + | + | ++ | + | + | ++ |
| 19 | - | ++ | - | + | - | ++ | - | + | ++ |
| 20 | - | - | - | - | - | ++ | - | + | + |
| 21 | - | + | - | - | + | +++ | - | ++ | + |
| 22 | - | +++ | - | - | - | +++ | - | - | - |
| 23 | - | +++ | - | - | - | + | - | ++ | + |
| 24 | - | +++ | + | +++ | + | ++ | - | +++ | ++ |
| 25 | - | ++ | - | - | - | - | + | - | + |
| 26 | - | - | - | - | - | - | - | - | - |
| 27 | + | ++ | - | - | - | - | - | - | ++ |
| 28 | +* | - | - | + | - | - | - | + | + |
| 29 | + | + | + | ++ | - | - | - | - | - |
| 30 | + | + | - | +++ | - | - | - | - | + |
| 31 | + | +++ | + | + | + | +++ | + | + | - |
| 32 | - | - | - | - | - | - | - | - | - |
| 33 | + | +++ | + | + | + | ++ | + | + | + |
| N posi-tive /%/ | 13 /39%/ | 24 /73%/ | 13 /39%/ | 17 /51%/ | 12 /36%/ | 23 /70%/ | 10 /30%/ | 19 /58%/ | 25 /76%/ |
| DS*/%/ | 43% | 80% | 43% | 57% | 40% | 77% | 33% | 63% | 83% |

*** DS Diagnostic sensitivity**

duodenal ulcer/, however, supposed the presence of H.pylori. One patient was negative for Helicobacter by microbiological and histological methods but he had antibody against bacteria. This patient was tested after successful treatment. The other treated patient was negative by all three methods. Four patients were microbiological and histological positive but had no antibody. These patients, probably had an initial infection when antibody against H.pylori were not yet formed.

We isolated H.pylori from 4 persons, they also had antibody but had negative histology.
Three of the patients were accepted to be truly negative for H.pylori, because the bacteria was not proven by the tree /invasive or non invasive/ methods, although they had mild chronic gastritis. We supposed that these patients had passing infection with H.pylori /one of them was tested after treatment/.
H.pylori was isolated by microbiological methods from 29 /88%/ patients /table 2/, by histology - from 23 /70%/ and by serology - from 24 /73%/. The diagnostic sensitivity /DS/ of the three methods /estimate on the base of 30 truly positive for H.pylori patients/ was: for microbiological methods - 97%, histology - 77% and serology - 83% /Table 2/. We obtained a high level of correlation between microbiological and histological methods on the one hand and between microbiological and serological on the other hand by applying the method of two alternative distributions with exact criteria of Fisher.
The results from the study of biopsy specimens from the antrum and from the corpus with invasive and non invasive techniques are given on table 3. The frequency of the provement of H.pylori in the biopsies from antrum – corpus was: for direct microscopy - 39-36%, for rapid disc urease test - 73-70%, for culture - 39-30%, histology - 51-58% and serology - 76%.
The comparison between different bacteriological methods gave priority for disc urease test. The test was rapid - reading the results between 1 min - 1h. Rapid disc urease test was positive in 28 patients, both for antrum and corpus, with diagnostic sensitivity - 93% and specificity - 100%, followed by direct microscopy - 19 positive /DS -63%/, and culture 15 positive /DS - 51%/.

## DISCUSSION

Our study confirmed the association between presence of H.pylori and gastroduodenal pathology. From all 33 tested patients 91% were infected. 9% had normal endoscopy, but their histology showed different degree of damage of their gastric mucosa. That revealed a past infection with H.pylori. All patients with pangastritis and duodenal ulcer were H.pylori positive. These findings are in agreement with the reports from the other authors who found H.pylori infection in 95-100% of the patients with the such pangastritis and duodenal ulcer /3, 5, 10, 24/.
H.pylori was reported in about 77% of patients with gastric ulcer and 100% with MALT - lymphoma /1, 2, 4, 12/. We proved this microorganism bacteriologically, serologically and histologically in 2 patients with gastric ulcer and 1 with MALT - lymphoma.
Some authors described the presence of the organism in the esophagus /4, 6, 10/. All of our patients /n=12/ with esophagitis were infected with H.pylori.
H.pylori was eradicated successfully in about 85-95% of the treated patients. Three of our patients were studied after treatment. One of them was still infected with H.pylori and the other two were cured.
Which is the best method for detection of H.pylori in gastric biopsies?
The "golden standard" is microbiology together with histological confirming. The combination between two methods gives 99,5% detection of the bacteria /9, 15, 16/. Our results for diagnostic sensitivity of the different methods used were: for microbiology - 97%, histology - 77% and serology - 83%, and specificity of all methods was 100%. These data corresponded to the other authors who reported 79-100% sensitivity for microbiological method, 77-100% for histology and 81-100% for serology and specificity - 100% /16, 18/.
Histological staining was the first method used to detect H.pylori and is still useful, rapid and demonstrative. This method has a disadvantage from several sites gastric mucosa must be examined /16, 19, 21, 24/. We used only 2 biopsy /one from the antrum and another from the corpus/. Maybe for that reason our diagnostic sensitivity was lower - 51 - 58%.
Culture technique had the disadvantage of being time-consuming, expensive and needs a quick transportation of the biopsy specimens to the laboratory. Our diagnostic sensitivity was lower - 39-30%.
The rapid disc urease test appeared to be both sensitive /93%/ and specific /100%/ when compared to culture and histology, and had the advantage of providing results rapidly. Serological tests also appeared to be highly accurate and ware non invasive, but ware not yet standardized and antibody titre cannot be read.
In conclusion, at the present time the best approach to use would probably be a histological stain plus a rapid urease test. Our results showed that the combination between histological and direct rapid disk urease test was with highest efficiency for detection of Helicobacter pylori infection - 98%.

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# TESTING THE BACTERICIDAL ACTIVITY OF PVA/TEOS/AG-NP HYBRID THIN FILMS ONTO CLINICAL STRAINS WITH PROVEN RESISTANCE TOWARD ONE OR MORE ANTIMICROBIAL AGENTS

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**SUMMARY:**
**The synthesized novel PVA/TEOS/Ag-Nps hybrid materials were tested for bactericidal activities onto 86 clinical isolates from patients with urinary tract infections, surface skin infections and wound infections. Disk diffusion method (DDM) was used in order to determine the resistance of the strains towards antimicrobial agents and their sensitivity to the disks impregnated with PVA/TEOS/Ag-Nps. The zones of inhibitions were determined using the main criteria of CLSI and for their interpretation and the EUCAST expert rules in antimicrobial susceptibility testing are taken into account. Strains of the three examined groups of bacteria – Staphylococcus aureus (gram-positive bacteria), Escherichia coli (gram-negative bacteria), Pseudomonas aeruginosa (non-fermentative gram-negative bacteria) showed resistance toward different groups of antimicrobial agents and some of them were also multiresistant. There was no strain to show resistance towards the disks impregnated with PVA/TEOS/Ag-Nps.**

*Key words: PVA, Antimicrobiologents*

## INTRODUCTION

Many studies on the antimicrobial resistance are focused on the development of new antimicrobial agents [1]. A large number of publications demonstrated the bactericidal properties of silver in all its variations in the terms of molecular composition [2-5]. With the growing development of the nanotechnology, many evidences for the bactericidal activity of silver nanoparticles included in variouse polymer materials are cited [6-11]. As a universal method for testing the bactericidal properties of liquid materials is the method using determination of Minimal Inhibitory Concentration (MIC) [12,13]. However, this method is not applicable when bactericidal properties of hybrid materials prepared as films or other composite materials must be tested [14, 15]. The experience gained in using of DDM (Disk diffusion method) indicates the possibility of its use for testing the bactericidal properties of this type of materials [16] on control bacterial strains representatives of different groups of bacteria. Its standardization by using of impregnated with such material disks allows obtaining of zones with correct diameter [17] and gives a possibility for a comparison of their inhibition zones with those obtained at testing of isolated from patients clinical strains with pronounced resistance toward antimicrobial agents from different groups.

Testing the bactericidal properties of materials with embedded silver nanoparticles on multi-resistant strains [18] confirms their position as active agents.

Subject of special observation in Worldwide is the contribution of several groups of bacteria, representatives of which are with established multi-resistance [19]. The challenge at multi-resistant microorganisms consists in the limited number of therapeutic possibilities (if there is any) at patients contaminated with them. Examples of widespread multi-resistant bacteria are: Methicillin–resistant Staphylococcus aureus (MRSA), Vancomycin resistant enterococci (VRE), Enterobacteriaceae, producing wide-spectrum beta lactamases (ESBL) (the most widespread are Escherichia coli and Klebsiella pneumoniae), multiresistant Pseudomonas aeruginosa and Clostridium difficile.

Representatives of three of these groups resistant bacteria are included for investigation in our study. The synthesized hybrid materials with embedded silver nanoparticles possess proven bactericidal properties established in our previous investigation against etalon strains of three different groups of bacteria – Staphylococcus aureus (gram-positive bacteria), Escherichia coli (gram-negative bacteria), Pseudomonas aeruginosa (non-ferment gram-negative bacteria) [17]. The aim of this study is to test the sensitivity of synthesized materials toward resistant to one or more antimicrobial agents, clinical strains with representatives from the three groups bacteria, isolated from patients with urinary tract infections, surface skin infections and wound infections.

## MATERIALS AND METHODS

2.1. Materials

Polyvinyl alcohol (PVA) (Sigma–Aldrich; 87–88% hydrolyzed, Mw = 13,000–23,000 mol-1); HNO3 (Riedel de Haln, standard solution 2 mol/L); silver nitrate (Acros Organics); tetraethyl orthosilicate (TEOS) (Fluka) were used as received without further purification. Nutrition media in disposable petri dishes are used. Antimicrobial disks, produced by Bul-Bio NCIPD and BBL, disks impregnated with PVA/AgNps/TEOS hybrid material with silver concentration 3.7 mg/mL were used [17]. Clinical bacterial strains (total number 86) were obtained from the National referent laboratories "Staphylococcus" and "Control and monitoring of antibiotic resistance" by NCIPD (total number 86) which are further subjected for testing using DDM [20] in order to establish their sensitivity toward antmicrobial substances and the presence of sensitivity toward the disks impregnated with PVA/AgNps/TEOS hybrid material. For testing were subjected strains from patients with urinary tract infections: 11 strains S. saprophyticus, 16 strains E.coli, 16 strains P.aeruginosa. Clinical isolates from patients with surface skin infections caused by S.aureus-10 strains and isolated from wound secretes 16 strains S.aureus, 10 strains P.aeruginosa and 7 strains E.coli are also tested in the present study. In vitro investigation for sensitivity of clinical strains toward antimicrobial agents is considered with the requirements of CLSI [21] and interpretative rules of EUCAST [22].

## RESULTS

E.coli strains were tested for sensitivity toward ampicillin (A), amoxicillin-clavulanic acid (AmC), aztreonam (Azt), imipenem (I), gentamicin (G), ciprofloxacin (Cp), cefuroxime (Cx), cefamandole (Cm), cefotaxime (Ct), ceftazidime (Cz), ceftriaxone (Cft), cefepime (Ce) and PVA/AgNps/TEOS. The zones were accounted according to the norms indicated in CLSI M100-S17.

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**Table 1**. Inhibition zone in mm at testing the sensitivity of clinical strains E. coli toward disks impregnated with antimicrobial materials and disks impregnated with PVA/AgNps/TEOS using DDM.

| E.coli strain № | A mm | AmC mm | Azt mm | I mm | G mm | Cp mm | Cx mm | Cm mm | Ct mm | Cz mm | Cft mm | Ce mm | PVA/ AgNps /TEOS mm |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 11 | 14 | 28 | 7 | 0 | 0 | 0 | 10 | 18 | 8 | 18 | 10 |
| 2 | 0 | 10 | 14 | 26 | 7 | 0 | 0 | 0 | 8 | 17 | 9 | 18 | 10 |
| 3 | 0 | 10 | 14 | 28 | 8 | 0 | 0 | 0 | 11 | 19 | 9 | 21 | 10 |
| 4 | 0 | 10 | 13 | 28 | 8 | 0 | 0 | 0 | 9 | 17 | 9 | 19 | 9,5 |
| 5 | 0 | 11 | 10 | 27 | 8 | 0 | 0 | 0 | 8 | 15 | 7 | 17 | 10 |
| 6 | 0 | 11 | 15 | 29 | 7 | 0 | 0 | 0 | 7 | 18 | 9 | 18 | 10 |
| 7 | 0 | 11 | 13 | 27 | 7 | 0 | 0 | 0 | 7 | 17 | 9 | 19 | 10 |
| 8 | 0 | 11 | 14 | 29 | 7 | 0 | 0 | 0 | 9 | 18 | 9 | 17 | 10 |
| 9 | 0 | 10 | 14 | 28 | 8 | 0 | 0 | 0 | 7 | 19 | 10 | 19 | 10 |
| 10 | 0 | 11 | 13 | 29 | 7 | 0 | 0 | 0 | 0 | 18 | 9 | 18 | 10 |
| 11 | 0 | 10 | 13 | 29 | 8 | 0 | 0 | 0 | 10 | 17 | 9 | 19 | 9,5 |
| 12 | 0 | 10 | 15 | 29 | 7 | 0 | 0 | 0 | 11 | 17 | 9 | 19 | 10 |
| 13 | 0 | 11 | 15 | 28 | 7 | 0 | 0 | 0 | 11 | 18 | 9 | 17 | 10 |
| 14 | 0 | 14 | 13 | 27 | 11 | 0 | 0 | 0 | 0 | 19 | 9 | 20 | 10 |
| 15 | 0 | 10 | 13 | 27 | 8 | 0 | 0 | 0 | 0 | 18 | 10 | 19 | 10 |
| 16 | 0 | 10 | 14 | 28 | 7 | 0 | 0 | 0 | 11 | 17 | 9 | 18 | 11 |
| 703 | 0 | 12 | 0 | 29 | 10 | 0 | 0 | 0 | 17 | 15 | 15 | 24 | 10 |
| 732 | 0 | 13 | 12 | 28 | 8 | 0 | 0 | 0 | 7 | 15 | 9 | 19 | 9 |
| 800 | 0 | 13 | 13 | 27 | 8 | 0 | 0 | 0 | 9 | 15 | 8 | 18 | 9,5 |
| 920 | 0 | 21 | 25 | 30 | 26 | 31 | 0 | 0 | 20 | 27 | 16 | 26 | 10 |
| 1616 | 0 | 8 | 0 | 27 | 10 | 0 | 0 | 0 | 0 | 11 | 9 | 16 | 9,5 |
| 1546 | 0 | 13 | 13 | 28 | 8 | 0 | 0 | 0 | 9 | 17 | 9 | 19 | 9,5 |
| 1769 | 0 | 21 | 25 | 28 | 25 | 32 | 0 | 0 | 20 | 27 | 17 | 26 | 10 |

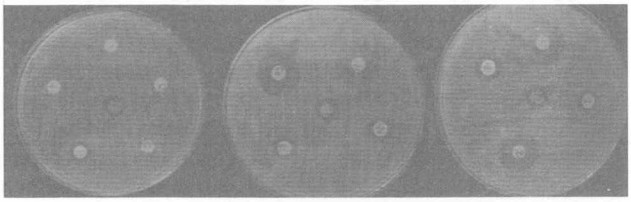


**Figure 1. DDM at testing the sensitivity of clinical strains E. coli toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/TEOS that is located in the middle of the petri dish using Muller Hintone Agar.**

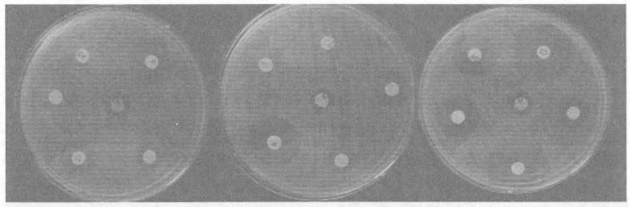


**Figure 2. DDM at testing the sensitivity of clinical strains P.aeruginosa toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/ TEOS that is located in the middle of the petri dish using Muller Hintone Agar.**

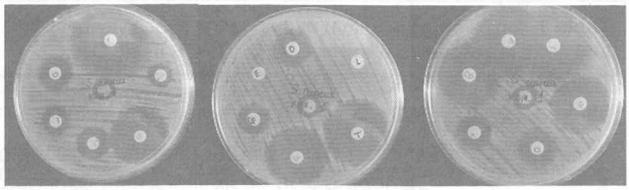
Additional tests for the presence of E.coli, producers of wide-spectrum betalaktamases (ESBL) were performed. Hodge test for detecting the metal betalactamases (MBL) were done. At P.aeruginosa, the sensitivity towards piperacillin (Pi), cefotaxime (Ct), ceftazidime (Cz), cefepime (Ce), imipenem (I), aztreonam (Azt), gentamicin (G), ciprofloxacin (Cp) and PVA/ AgNps/TEOS was examined. The zones were accounted according to the norms indicated in CLSI M100-S17.

Test, establishing the presence of VEB-1 code enzymes at the strains P.aeruginosa exhibiting a resistance toward third-generation cephalosporine and MHT/ Modified Hodge Test/ was performed. Antimicrobial sensitivity of strains S.saprophiticus – Table 3, Figure 3 and S.aures – Table 4 and 5, Figure 4 to oxacillin (O), linkomycin 15 μg (L), erythromycin (E), tetracycline (T), gentamicin (G), ciprofloxacin (Cp), chloramphenicol (C), rifampin (R), sulfamethoxazole/

**Table 2**. Inhibition zone in mm at testing the sensitivity of clinical strains P.aeruginosa toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/TEOS using DDM.

| P.aeruginosa strain № | Pi mm | Ct mm | Cz mm | Ce mm | I mm | Azt mm | G mm | Cp mm | PVA/ AgNps/ TEOS mm |
|---|---|---|---|---|---|---|---|---|---|
| | 21 | 0 | 0 | 15 | 28 | 0 | 15 | 11 | 10 |
| | 20 | 0 | 0 | 17 | 27 | 0 | 15 | 11 | 11 |
| | 21 | 0 | 0 | 17 | 28 | 0 | 15 | 10 | 11 |
| | 20 | 0 | 0 | 14 | 28 | 0 | 15 | 10 | 11 |
| | 21 | 0 | 0 | 16 | 27 | 0 | 15 | 10 | 10 |
| | 21 | 0 | 0 | 16 | 27 | 0 | 16 | 11 | 11 |
| | 20 | 0 | 0 | 16 | 27 | 0 | 15 | 10 | 11 |
| | 20 | 0 | 0 | 16 | 27 | 0 | 15 | 10 | 10 |
| | 21 | 0 | 0 | 16 | 28 | 0 | 15 | 10 | 10 |
| | 20 | 0 | 0 | 17 | 27 | 0 | 15 | 10 | 11 |
| | 21 | 0 | 0 | 16 | 28 | 0 | 15 | 12 | 10 |
| | 20 | 0 | 0 | 16 | 27 | 0 | 15 | 12 | 11 |
| | 21 | 0 | 0 | 14 | 27 | 0 | 16 | 12 | 12 |
| | 20 | 0 | 0 | 16 | 27 | 0 | 15 | 11 | 10 |
| | 21 | 0 | 0 | 16 | 27 | 0 | 15 | 11 | 10 |
| | 20 | 0 | 0 | 14 | 27 | 0 | 15 | 10 | 10 |
| 1515 | 23 | 21 | 20 | 25 | 23 | 0 | 24 | 36 | 11 |
| 1536 | 13 | 7 | 0 | 0 | 14 | 13 | 10 | 11 | 11 |
| 1537 | 30 | 21 | 29 | 28 | 27 | 28 | 7 | 42 | 11 |
| 1556 | 18 | 0 | 0 | 0 | 16 | 0 | 0 | 14 | 11 |
| 1570 | 14 | 0 | 0 | 8 | 16 | 14 | 8 | 10 | 10 |
| 1721 | 16 | 0 | 0 | 0 | 26 | 0 | 7 | 10 | 11 |
| 1758 | 25 | 21 | 22 | 20 | 14 | 12 | 24 | 29 | 11 |
| 1705 | 15 | 0 | 14 | 24 | 15 | 27 | 0 | 36 | 11 |
| 1773 | 15 | 0 | 0 | 0 | 12 | 0 | 0 | 7 | 8 |
| 1801 | 18 | 16 | 26 | 13 | 21 | 18 | 13 | 27 | 11 |

trimethoprim (S/T) and PVA/AgNps/TEOS was established. The zones were accounted according to the norms indicated in CLSI M100-S17 and for linkomycin 15 μg (L) according to the standard DIN [23]. For S. saprophiticus, differentiated disk novobiocin (Nb), which confirms the belonging to the species were performed. The sensitivity toward oxacilline was again tested with cefoxitin (Cn) (BBL). All staphylococcus strains, which displayed resistance toward erythromycin, were additionally tested for inducible $MLS_B$ resistance. In all cases, resistance was not proven, therefore there was no change in the interpretation of the results.



Because of the specific accounting of the results for the methicillin resistance, one more group of staphylococcus strains was tested. They were isolated from wound secretions where no resistance toward any antimicrobial from the used set has been proved.

**Table 3**: Inhibition zone in mm at testing the sensitivity of clinical strains S. saprophiticus toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/TEOS using DDM.

| № | S. saprophiticus strain № | O mm | Cn mm | L mm | E mm | T mm | G Mm | Cp mm | C mm | R mm | S/T mm | PVA/ AgNps /TEOS mm |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 8 744/04 | 13 | 27 | 39 | 32 | 30 | 34 | 29 | 28 | 35 | 37 | 11 |
| 2 | 8 256/04 | 13 | 25 | 36 | 32 | 31 | 32 | 28 | 27 | 34 | 33 | 11 |
| 3 | 8 44E/04 | 11 | 25 | 37 | 10 | 8 | 32 | 33 | 9 | 37 | 31 | 11 |
| 4 | 8214/05 | 16 | 27 | 33 | 32 | 31 | 35 | 31 | 30 | 35 | 33 | 11 |
| 5 | 319/05 | 13 | 27 | 37 | 33 | 9 | 35 | 31 | 28 | 35 | 34 | 11 |
| 6 | 3690/05 | 12 | 29 | 0 | 0 | 9 | 34 | 34 | 6 | 36 | 34 | 11 |
| 7 | 616/05 | 10 | 25 | 36 | 11 | 32 | 34 | 33 | 31 | 36 | 29 | 11 |
| 8 | 720/05 | 14 | 24 | 36 | 33 | 31 | 36 | 32 | 28 | 36 | 35 | 11 |
| 9 | 1311/05 | 9 | 28 | 37 | 33 | 32 | 30 | 32 | 29 | 38 | 34 | 11 |
| 10 | 8870/04 | 11 | 27 | 32 | 31 | 9 | 30 | 32 | 9 | 38 | 37 | 11 |
| 11 | 8100/04 | 11 | 27 | 35 | 12 | 32 | 33 | 33 | 30 | 36 | 36 | 11 |

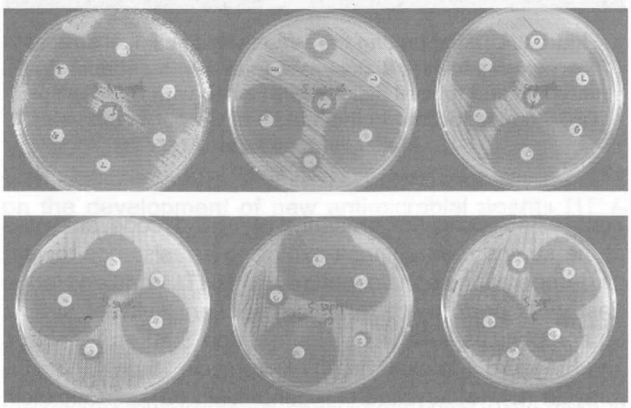


**Figure 3. DDM at testing the sensitivity of clinical strains S. saprophiticus toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/ TEOS that is located in the middle of the petri dish using Muller Hintone Agar.**

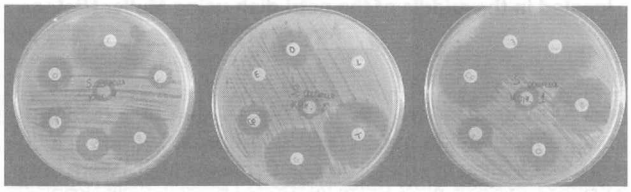


**Figure 4. DDM at testing the sensitivity of clinical strains S. aureus toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/TEOS that is located in the middle of the petri dish using Muller Hintone Agar.**

**Table 4**. Inhibition zones in mm at testing the sensitivity of clinical strains S. aureus, isolated from surface skin infections toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/TEOS.

| № | S.aureus skin infection Strain № | O mm | Cn mm | L mm | E mm | T mm | G mm | Cp mm | C mm | R mm | S/T mm | PVA/ AgNps/ TEOS mm |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 532/05 | 12 | 17 | 29 | 29 | 14 | 22 | 27 | 25 | 31 | 29 | 11 |
| | 245/05 | 0 | 16 | 29 | 14 | 27 | 26 | 30 | 25 | 30 | 30 | 11 |
| | 340/05 | 16 | 17 | 30 | 30 | 27 | 25 | 27 | 25 | 34 | 30 | 10 |
| | 394/05 | 0 | 16 | 29 | 13 | 10 | 26 | 16 | 25 | 30 | 30 | 11 |
| | 372/06 | 20 | 26 | 28 | 27 | 28 | 24 | 23 | 24 | 31 | 25 | 11 |
| | 92/06 | 0 | 16 | 0 | 0 | 26 | 26 | 13 | 24 | 30 | 30 | 11 |
| | 133/07 | 12 | 18 | 29 | 29 | 26 | 15 | 31 | 26 | 30 | 31 | 11 |
| | 227/07 | 19 | 25 | 29 | 30 | 27 | 29 | 32 | 25 | 32 | 31 | 11 |
| | 83/08 | 18 | 25 | 29 | 28 | 26 | 24 | 28 | 27 | 31 | 29 | 11 |
| | 144/08 | 20 | 24 | 29 | 29 | 11 | 24 | 28 | 25 | 28 | 28 | 11 |

**Table 5.** Inhibition zones in mm at testing the sensitivity of clinical strains S. aureus, isolated from the wound secrete toward disks impregnated with antimicrobial materials and toward disks impregnated with PVA/AgNps/TEOS using DDM.

| S.aureus-wound secrete Strain № | O mm | Cn mm | L mm | E mm | T mm | G mm | Cp mm | C mm | R mm | S/T mm | PVA/ Ag-Nps/TEOS mm |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 22 | 26 | 26 | 28 | 25 | 25 | 30 | 26 | 28 | 27 | 11 |
| 2 | 22 | 26 | 28 | 27 | 26 | 24 | 27 | 25 | 29 | 27 | 11 |
| 3 | 22 | 26 | 29 | 27 | 27 | 24 | 28 | 26 | 28 | 26 | 11 |
| 4 | 23 | 26 | 29 | 28 | 27 | 24 | 29 | 25 | 30 | 26 | 11 |
| 5 | 23 | 27 | 29 | 28 | 27 | 23 | 28 | 26 | 31 | 26 | 11 |
| 6 | 20 | 26 | 28 | 28 | 28 | 24 | 30 | 26 | 29 | 26 | 11 |
| 7 | 22 | 26 | 28 | 28 | 27 | 24 | 31 | 26 | 31 | 27 | 11 |
| 8 | 23 | 27 | 27 | 29 | 27 | 24 | 30 | 27 | 29 | 25 | 11 |
| 9 | 22 | 26 | 28 | 29 | 27 | 25 | 31 | 26 | 31 | 27 | 11 |
| 10 | 22 | 26 | 30 | 30 | 26 | 25 | 31 | 26 | 30 | 30 | 11 |
| 11 | 22 | 27 | 29 | 29 | 29 | 27 | 31 | 27 | 31 | 28 | 11 |
| 12 | 22 | 26 | 29 | 28 | 28 | 27 | 30 | 26 | 31 | 27 | 11 |
| 13 | 23 | 25 | 29 | 29 | 27 | 26 | 32 | 25 | 30 | 27 | 11 |
| 14 | 21 | 26 | 29 | 28 | 27 | 25 | 31 | 26 | 31 | 28 | 11 |
| 15 | 22 | 27 | 28 | 28 | 26 | 25 | 28 | 27 | 29 | 26 | 11 |
| 16 | 23 | 26 | 28 | 30 | 27 | 25 | 30 | 26 | 30 | 27 | 11 |

## DISCUSSION

Enterobacteriaceae are intrinsically resistant to penicillin G, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin,daptomycin and linezolid. Exceptional phenotypes of any Enterobacteriaceae are resistance to ertapenem, meropenem, imipenem (except Proteus spp.) [22]. Several mechanisms of resistance are known for E.coli.
Reduced permeability as a result of change in porines or overexpression of efflux systems.
Modification of the target.
Hydrolysis via β –lactamases.
The resistance of E.coli toward oxyimino-cephalosporines and monobactams could be a result of ESBL producers or non – ESBL producers. The second isolates are likely to be derepressed for Amp C, or to have plasmid-mediated Amp C [22].
According to CLSI E. coli that produce extended-spectrum betalactamase (ESBLs) may be clinically resistant to therapy with penicillins, cephalosporins, or aztreonam, despite apparently in vitro susceptibility to some of these agents. In all strains with ESBLs (Figure5), the zone diameters for one or more of the extended-spectrum cephalosporins or aztreonam should increase in the presence of clavulanic acid, as determined in phenotypic confirmatory testing (at Cz zone ≤ 22 mm, Cft zone ≤ 25 mm and ≥ 5-mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs. its zone when tested alone = ESBL). For all confirmed ESBL-producing strains, the test interpretation should be reported as resistant for all penicillins, cephalosporins, and aztreonam.

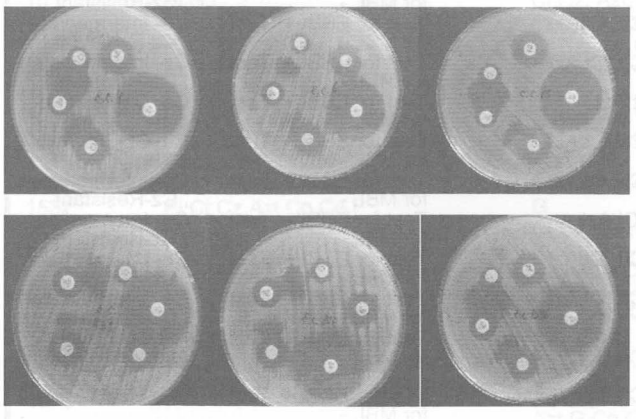


**Figure 5. Test for proving of ESBL producers at testing the clinical shtains E.coli.**

Resistance of E.coli to the carbapenem may results from:
Variable stability to Amp C hydrolysis, dependence on porins and susceptibility to the efflux pumps: "Test results regarding one carbapenem (imipenem, meropenem, ertapenem) can not be extrapolated to the other carbapenems" [22].
Metallo-β-lactamases, that can hydrolyse all β-lactams except monobactams. According to [22] if the production of metallo-β- lactamase is confirmed, the susceptible results should be reported as intermediate and intermediate results as resistant for any β-lactam. Aztreonam is an exception where the results should be reported as found.
KPC carbapenemase or combinations of ESBL or Amp C and impermeability. According to [22] if the susceptibility to carbapenems and oximinocefalosporines and aztreonam is reduced, resistance may reflect either KPC, IMP, GES β-lactamases or combination of AmpC or ESBL plus impermeability. Ertapenem can tend to be the most affected carbapenem. Synergy between carbapenems and clavulanate may arise with either KPC enzymes or with combinations of ESBL and impermeability.
Modified Hodge test (MHT) detects carbapenemase production in isolates of Enterobacteriaceae. Carbapenemase production is detected by the MHT when the test isolate produces the enzyme and allows growth of a carbapenem susceptible strain (E.coli ATCC 25922) towards a carbapenem disk. The positive result is a characteristic cloverleaf-like indentation (Figure 6) [24]. The results from the MHTs of the clinical isolates were negative (Figure7).

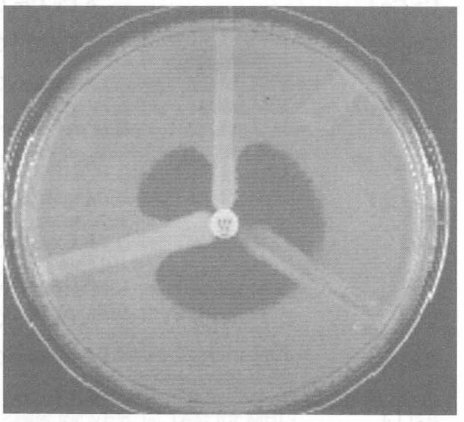


**Figure 6. The MHT performed on a 100 mm MHA plate. (1) K. pneumoniaeATCC BAA 1705, positive result (2) K. pneumoniaeATCC BAA 1706, negative result; and (3) a clinical isolate, positive result 312 [25]**

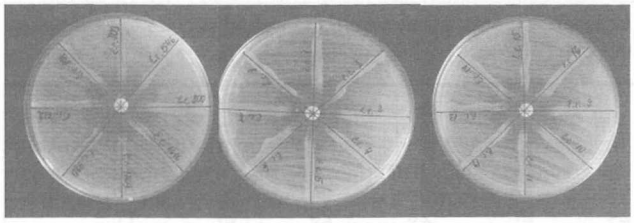


**Figure 7. The MHT performed on a three 100 mm MHA plates, each with 8 clinical isolates of E.coli– negative results.**

According to the recommendations of CLSI, all tested in this study clinical strains, which are producers of ESBL, should be reported as resistant to all penicillins, cephalosporins except cephamicin and aztreonam. For interpretation of the results obtained from testing of clinical strains of E.coli and P.aeruginosa were taking into account the rules of EUCAST [22]. Accroding to these rules if there is a resistance to any 3rd or 4th generation oxyimino-cephalosporin or aztreonam, a test for ESBL should be performed. If the results are positives, then any susceptible results for these cephalosporins (including fourth-generation agents) and for aztreonam should be reported as intermediate and any intermediate results should be reported as resistant. ESBL producers may appear susceptible to penicillin/β-lactamase inhibitor combinations. The use of these combinations against ESBL producers remains controversial, and should be approached with caution. If there is resistance to cefotaxime, ceftazidime and ceftriaxone, but the results are negative for ESBL and susceptible to cefepime and cefpirome, then the results should be reported as found. Because of that, the interpretation of some results was changed (Table 6).

Non-fermentative Gram-negative bacteria are also intrinsically resistant to penicillin G, cefazolin, cefoxitin, cefamandole, cefuroxime, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin, daptomycin and linezolid. At P.aeruginosa this resistance is to: ampicillin, amoxicillin-clavulanic acid, cefoxitin, cefotaxime, ceftriaxone, ertapenem, chloramphenicol, tetracycline, kanamycin and neomycin due to low level of APH(3')-IIb activity, to trimethoprim and moderately susceptible to sulphonamides (although it may appear susceptible in vitro to co-trimoxazole, it should be considered resistant) [22]. Exceptional phenotype of P.aeruginosa is resistance to colistin[22].

The mechanisms of resistance at P.aeruginosa as a result of mutation are:

Hyperexpression of efflux systems (Mex AB-OprM);

Hyperexpression of AmpC chromosomal betalactamase;

Lack of carbapenem-specific porin OprD

Acquiring of plasmid beta-lactamases (all penicillinase – class A enzymes, ESBL, MBL – class B enzymes and oxacilinase – class D enzymes).

In more cases the resistance is a result from the activity of different factors of resistance.

P. aeruginosa has an inducible, naturally occurring cephalosporinase that confers low-level resistance to aminopenicillins and narrow-spectrum cephalosporins such as cephalothin and cefoxitin [26]. Resistance to extended-spectrum cephalosporins may arise from overexpression of this cephalosporinase, acquired beta-lactamases, or both [26]. The acquired beta-lactamases may be either clavulanic-acid inhibited (mostly Ambler class A enzymes) or clavulanic-acid resistant (class B and class D enzymes)[27]. The class A

**Table 6.** Interpretation of the results obtained from testing the antimicrobial sensitivity of E.coli clinical strains.

| E.coli strain № | S (Susceptible) | I (Intermediate) | R (Resistant) | Note | Change in the interpretation |
|---|---|---|---|---|---|
| 1 | Cz,Ce,I | | A/S, A,Cx,Cm,G,Cp,AmC,Cft,Ct, Azt | Test for ESBL+, Test for MBL - | Cz,Ce- Intermediate |
| 2 | Ce,I | Cz | A/S, A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 3 | Cz,Ce,I | | A/S, A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Cz,Ce- Intermediate |
| 4 | Ce,I | Cz | A/S, A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 5 | I | Cz | A/S, A,Cx,Cm,G,Cp,AmC,Cft,Ce, Ct,Azt | Test for ESBL+, Test for MBL - | Cz-Resistant |
| 6 | Cz,Ce,I | | A/S, A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Cz,Ce- Intermediate |
| 7 | Ce,I | Cz | A/S, A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 8 | Cz,I | Ce | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Cz- Intermediate , Ce-Resistant |
| 9 | Cz,Ce,I | | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Cz,Ce- Intermediate |
| 10 | Cz,Ce,I | | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Cz,Ce- Intermediate |
| 11 | Ce,I | Cz | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 12 | Ce,I | Cz | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 13 | Cz,I | Ce | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Cz- Intermediate , Ce-Resistant |
| 14 | Cz,Ce,I | AmC | A,Cx,Cm,G,Cp, Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce, Cz- Intermediate |
| 15 | Cz,Ce,I | | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce, Cz- Intermediate |
| 16 | Ce,I | Cz | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 703 | Ce,I | Cz,Cft | A,Cx,Cm,G,Cp,AmC, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 732 | Ce,I | Cz | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 800 | Ce,I | Cz | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce- Intermediate , Cz-Resistant |
| 920 | G,Cp,AmC,Cz,Ce,I, Azt,Ct | Cft | A,Cx,Cm | Test for ESBL+, Test for MBL - | Azt,Ct,Cz,Ce-Intermediate, Cft – Resistant |
| 1616 | I | Ce | A,Cx,Cm,G,Cp,AmC,Cft,Cz, Ct,Azt | Test for ESBL+, Test for MBL - | Ce –Resistant |
| 1546 | Ce,I | Cz | A,Cx,Cm,G,Cp,AmC,Cft, Ct,Azt | Test for ESBL+, Test for MBL - | Ce-Intermediate, Cz - Resistant |
| 1769 | G,Cp,AmC,Cz,Ce,I, Azt,Ct | Cft | A,Cx,Cm | Test for ESBL +, Test for MBL - | Azt,Ct,Cz,Ce – Intermediate |

extended-spectrum beta-lactamases (ESBLs) may derive from narrow-spectrum beta-lactamases of TEM and SHV types, as extensively reported for Enterobacteriaceae and rarely for P. aeruginosa [28]. The strains with bla VEB-1-like sequences, located next to the 3'-CS end within class 1 integrons, showed decreased susceptibility to all beta-lactams except imipenem and piperacillin/tazobactam [28]. The veb1 gene cassette that encodes the extended spectrum beta-lactamase, VEB-1 is increasingly isolated from Gram-negative rods, where it is inserted into the variable region of class 1 integrons varying in size and composition [29]. Identification of ESBLs is of interest since they confer resistance to all extended-spectrum cephalosporin and aztreonam, whatever their MICs [28]. The test for the presence of VEB-1 gene is performed by examining the presence of synergism between disk imipenem and fourth generation cephalosporin (Figure 8).

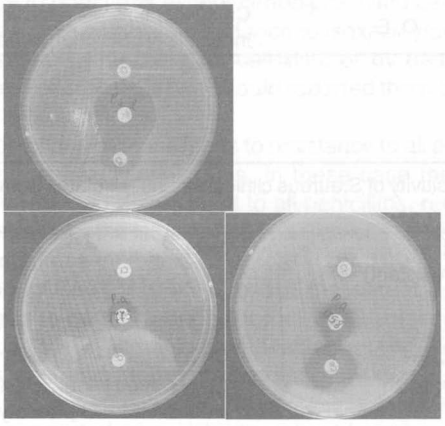


**Figure 8. Test for presence of VEB-1 gene in proven clinical strains P.aeruginosa.**
**The modified Hodge test for detection of metal beta-lactamase is performed (Figure 9). The obtained results were negative, therefore it wasn't necessity to change the interpretation of them.**

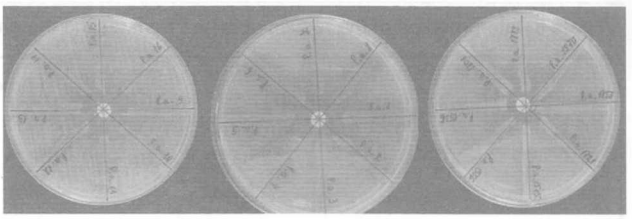


**Figure 9. The MHT performed on a 100 mm MHA plates, with clinical isolates – negative results.**

It is interesting to note that P.aeruginosa N1773, which shows resistance toward all tested antimicrobial agents gives an inhibition zone toward impregnated with PVA/AgNps/TOES hybrid material disks (Figure 10).

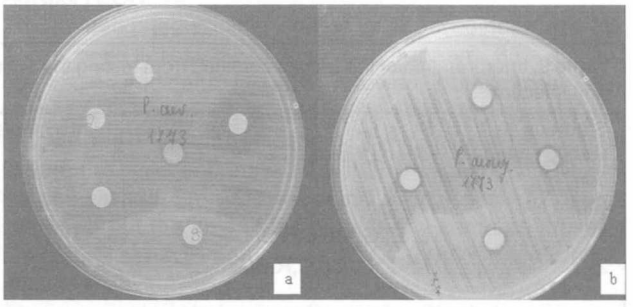



**Figure 10. Zone of inhibition of P.aeruginosa N1773 toward impregnated with PVA/AgNps/TOES hybrid material disks: a) in the middle, b) all tested PVA/AgNps/TEOS impregnated disks.**

**Table 7**. Interpretation of the results obtained from testing the antimicrobial sensitivity of P.aeruginosa clinical strains.

| P. aeruginosa strain № | S (susceptible) | I (intermediate) | R (resistant) | Note | Change in the interpretation |
|---|---|---|---|---|---|
| 1 | Pi,G,I | Ce | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 2 | Pi,G,I | Ce | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 3 | Pi,G,I | Ce | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 4 | Pi,G,I | | Ct,Cz, Azt,Cp,Ce | Test for VEB 1+, Test for MBL- | |
| 5 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 6 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 7 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 8 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 9 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 10 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 11 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 12 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 13 | Pi,G,I | | Ct,Cz, Azt,Cp,Ce | Test for VEB 1+, Test for MBL- | |
| 14 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 15 | Pi,G,I | | Ct,Cz, Azt,Cp | Test for VEB 1+, Test for MBL- | |
| 16 | Pi,G,I | | Ct,Cz, Azt,Cp,Ce | Test for VEB 1+, Test for MBL- | |
| 1515 | Pi, Ct,Cz,G,Cp,Ce,I | | Azt | Test for MBL- | |
| 1536 | Pi, | I | Ct,Cz, Azt,G,Cp,Ce | Test for VEB 1+, Test for MBL- | |
| 1537 | Pi,Ct,Cz,Azt,Cp,Ce,I | | G | Test for MBL- | |
| 1556 | Pi,I | | Ct,Cz, Azt,G,Cp,Ce | Test for VEB 1+, Test for MBL- | |
| 1570 | I | | Pi, Ct, Cz,Azt,G,Cp,Ce | Test for VEB 1+, MBL- | |
| 1721 | I | | Pi, Ct,Cz,Azt,G,Cp,Ce | Test for VEB 1+, Test for MBL- | |
| 1758 | Pi,Cz,G,Cp,Ct,Ce | I | Azt | Test for MBL- | |
| 1705 | Azt,Cp, Ce | I | Pi, Ct, Cz,G | Test for VEB 1-, Test for MBL- | |
| 1773 | | | Pi, Cz, Azt,G,Cp,Ct,Ce,I | Test for VEB 1-, Test for MBL- | |
| 1801 | Pi,Cz, Cp | Azt, G, Ct | Ce,I | Test for MBL- | |

**Table 8**. Interpretation of the results obtained from testing the antimicrobial sensitivity of S.saprophiticus clinical strains

| S. saprophiticus strain № | S (susceptible) | I (intermediate) | R (resistant) | Note |
|---|---|---|---|---|
| 1 | L,E,T, G,Cp,C,R,S/T,Cn | | O | Cn susceptible→ oxacillin "S" |
| 2 | L,E,T, G,Cp,C,R,S/T,Cn | | O | Cn susceptible → oxacillin "S" |
| 3 | L, G,Cp,R,S/T,Cn | | O, E,T,C | Cn susceptible → oxacillin "S" inducible MLSB phenotype - |
| 4 | L,E,T, G,Cp,C,R,S/T,Cn | | O | Cn susceptible → oxacillin "S" |
| 5 | L, E, G,Cp,C,R,S/T,Cn | | O, T | Cn susceptible → oxacillin "S" |
| 6 | G,Cp,R,S/T,Cn | | O, L, E, T, C | Cn susceptible → oxacillin "S" constitutive MLSB phenotype+ |
| 7 | L, T, G,Cp,C,R,S/T,Cn | | O, E | Cn susceptible → oxacillin "S" inducible MLSB phenotype - |
| 8 | L, E, T, G,Cp,C,R,S/T | | O, Cn | |
| 9 | L,E,T, G,Cp,C,R,S/T,Cn | | O | Cn susceptible → oxacillin "S" |
| 10 | L, E, G,Cp,R,S/T,Cn | | O, T, C | Cn susceptible → oxacillin "S" |
| 11 | L, T, G,Cp,R,S/T,Cn | | O, E | Cn susceptible → oxacillin "S" inducible MLSB phenotype - |

**Table 9.** Interpretation of the results obtained from testing the antimicrobial sensitivity of S.aureus clinical strains – isolates from surface skin infections.

| S.aureus кожskin infection Strain № | S (susceptible) | I (intermediate) | R (resistant) | Note |
|---|---|---|---|---|
| 1 | L, E, G, Cp,C,R,S/T | O | T,Cn | Cn resistant→ oxacillin "R" |
| 2 | L, T, G, Cp,C,R,S/T | | O, E,Cn | Cn resistant → oxacillin "R" inducible MLSB phenotype - |
| 3 | O, L, E, T, G, Cp,C,R,S/T | | Cn | Cn resistant → oxacillin "R" |
| 4 | L, G,C,R,S/T | | O, E, T, Cp,Cn | inducible MLSB phenotype - |
| 5 | O,L,E, T, G, Cp,C,R,S/T,Cn | | | |
| 6 | T, G,C,R,S/T | | O, L, E, Cp,Cn | Cn resistant → oxacillin "R" constitutive MLSB phenotype+ |
| 7 | L, E, T, G C,R,S/T | O | Cn | Cn resistant → oxacillin "R" |
| 8 | O,L,E, T, G, Cp,C,R,S/T,Cn | | | |
| 9 | O,L,E, T, G, Cp,C,R,S/T,Cn | | | |
| 10 | O, L, E, G, Cp,C,R,S/T,Cn | | T | |

**Table 10**. Interpretation of the results obtained from testing the antimicrobial sensitivity of S.aureus clinical strains- isolates from wound secretion.

| S.aureus- wound secrete Strain № | S (susceptible) | I (intermediate) | R (resistant) | Note |
|---|---|---|---|---|
| 1 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 2 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 3 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 4 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 5 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 6 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 7 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 8 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 9 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 10 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 11 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 12 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 13 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 14 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 15 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |
| 16 | O,Cn,L,E,T,G,Cp,C,R,S/T | | | |

The zone is smaller than any other tested clinical strains P.aeruginosa but matches in size with that obtained at testing with the control strain P.aeruginosa ATCC 27853 [17]. The reason for this observation will be established in next study with the aid of biochemical and molecular genetic methods. Gram-positive bacteria are also intrinsically resistant to aztreonam, temocillin, polymyxin B/colistin and nalidixic acid. Staphylococcus saprophyticus is intrinsically resistant to ceftazidime, fosfomycine, novobiocine, and the other coagulase-negative staphylococci and Staphylococcus aureus are intrinsically resistant to ceftazidime too [22]. Exceptional phenotypes of Staphylococcus aureus and coagulase-negative staphylococci are resistance to vancomycin, linezolid, quinupristin/dalfopristin, daptomycin, tigecycline [22].

Staphylococcus spp.resistant to β-lactams possesses the following resistance mechanisms:

Production of PBP2a (encoded by mecA) leads to cross resistance to β-lactams except ceftobiprole and ceftaroline. That's way if we establish resistance to isoxazolylpenicillins (as determined with oxacillin, cefoxitin, or by detection of mecA-gene or of PBP2a) we should reported them as resistant to all β-lactams.

Production of penicillinase leads to resistance to all penicillins except the isoxazolylanalogues. In these case the results should be reported as resistant to all penicillins, regardless of MIC, except for the isoxazolyl-penicillins and combinations with β-lactamase inhibitors.

According to CLSI M100-S17 oxacillin-resistant S. aureus and coagulase-negative staphylococci (MRS), other β-lactam agents, i.e., penicillins, β-lactam/β-lactamase inhibitor combinations, cephems, and carbapenems, may appear active in vitro, but are not effective clinically. Results for these drugs should be reported as resistant or should not be reported. By detection of oxacillin resistance strains, these isolates may be tested as susceptible to cefoxitin by disk diffusion. For S. aureus, the cefoxitin disk test is comparable to the oxacillin disk test for prediction of mecA-mediated resistance to oxacillin. But the cefoxitin disk test is easier to read and thus is the preferred method for testing coagulase-negative staphylococci. The cefoxitin disk test has higher specificity and equal sensitivity to the oxacillin disk test for coagulase-negative staphylococci and that is the reason to report the results based on the cefoxitin disk instead those from oxacillin as susceptible or resistant (Tables 8,9).

Staphylococci resistant to macrolides but susceptible to lincosamides (clindamycin and lincomycin) produce Erm ribosomal methylases conferring the inducible MLSB phenotype or express efflux pumps. In case of inducible MLSB resistance, constitutively resistant mutants can be selected by lincosamides. In case of resistance by efflux, the risk for selection of mutants resistant to lincosamides is not greater than that for erythromycin susceptible isolates. Both clinical failures and successes with clindamycin have been reported for staphylococci inducibly MLSB resistant. By a disk diffusion test, the inducible MLSB phenotype can be identified by the flattening of the clindamycin zone facing the erythromycin disk [30,31]. When were tested the clinical Staphylococus strains it was not established inducible MLSB phenotype, therefore there was not performed change in the interpretation of the results of lincomycine (Tables 8,9).

According to the interpretive rules for macrolides, lincosamides and streptogramins of EUCAST [22] if there is resistance to erythromycin but susceptible to clindamycin or lincomycin, a test for inducible MLSB resistance should be performed. If the result is negative, then the results should be reported as susceptible to clindamycin and lincomycin. If the result is positive, then the result should be reported as resistant to clindamycin and lincomycin. The resistance to clindamycin (associated with resistance to erythromycin) is a marker of the constitutive macrolide lincosamide streptograminB (MLSB) resistance phenotype [32,33] (Tables 8,9). The investigated 16 strains from S.aureus, isolated from wound secretion were susceptible toward all proofed antimicrobials (Table 10).

## CONCLUSION

The established antimicrobial resistance toward different groups antimicrobial materials is expressed in % to the total number of strains from given group bacteria taking into account the corrections made during the interpretation of the results obtained.

In total, 23% of strains of E. coli exhibit 100% resistance to ampicillin, cefuroxime и cefamandole, 96% exibit resistance to ceftriaxone, 91% is the resistance toward aztreonam, gentamicin, ciprofloxacin and cefotaxime, 87% toward amoxicillin-clavulanic acid, 48% toward ceftazidime, and only 17% toward cefepime. There is no established resistance at the tested E.coli clinical strains toward imipenem and PVA/AgNps/TEOS.

The investigated clinical strains of P.aeruginosa are 26. There is 88% resistance toward aztreonam, 85% toward cefotaxime and ceftazidime, 81% toward ciprofloxacin, 35% toward cefepime, 27% toward gentamicin, 15% toward piperacillin and 4% toward imipenem. The manifested resistance toward the disks impregnated with PVA/AgNps/ TEOS was not established. Smaller zone of inhibition at strain 1773 was observed but the size of the zone is the same as those of the control strain P. aeruginosa ATCC 27853, therefore it is accepted as sensitive.

The investigated strains from the genius Staphyloccocus are 37, as at 16 of them there was not established any resistance. When analyzing the rest 21 strains there was observed 33% resistance toward cefoxitin, erythromycin and tetracycline, 14%- toward chloramphenicol and 10% toward linkomycin and ciprofloxacin. There was not established resistance toward gentamicin, rifampin and PVA/AgNps/ TEOS.

At all tested 86 clinical bacterial strains, the inhibition zones of the impregnated with PVA/AgNps/ TEOS discs are with diameter from 8 to 12 mm.

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# NEURAMINIDASE FROM ENVIRONMENTAL AND CLINICAL ISOLATES OF AEROMONAS SPP. – BIOCHEMICAL STUDIES ON THE ENZYME PRODUCTION

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**SUMMARY:**
**A total of 40 Aeromonas spp. strains were screened for neuraminidase production and 72.5% of them were positive. No correlation was observed between the source of the strains and their ability to produce neuraminidase. Enzyme production depending on the growth phases, media and cultivation conditions was studied using the strain with the highest enzyme activity - Aeromonas sp.40/02. Neuraminidase production was found to be predominantly intracellular. It was most intensive at static aerobic cultivation – 14.7 U/ml for the exogenous and 40 U/ml for the endogenous form of the enzyme. No activity was detected at anaerobic conditions. Significant variations of the cultural liquid pH were observed during the cultivation at all aeration conditions. The possible role of neuraminidase in Aeromonas pathogenesis, nutrition and adaptation is discussed.**

*Key words: Aeromonas, neuraminidase, growth conditions*

## INTRODUCTION

The representatives of the genus Aeromonas are hemoorganotrophic, Gram-negative bacteria, ubiquitous for aquatic environments, including ground and surface waters, estuarine and marine waters, wastewater, as well as drinking water, fresh and packaged foods. The Aeromonas bacteria, in the water environment, could be a part of autochthonous microflora, or to be of another origin, including anthropogenic one. They usually are causative agents of diseases in invertebrate animals, fishes, amphibians, reptiles, farm animals, as well as in humans. This includes gastroenteritis and wound infections (5). Humans carry Aeromonas spp. in their gastrointestinal tract both in the presence and absence of disease (7).

The pathogenetic factors of these bacteria are not well known, and that is why the investigations of these factors (including neuraminidase) are of interest.

Neuraminidase (sialidase, E 3.2.1.18.) cleaves terminal sialic acids from oligosaccharides, glycoproteins, glycolipids, mucins, etc. The enzyme is a pathogenicity and virulence factor in many viruses and pathogenic bacteria that colonize mucous surfaces (6). Besides pathogenesis, neuraminidase takes part in the metabolism of the bacterial cell, providing it with free sialic acids as an alternative carbon and energy source (4, 15). Thus, the enzyme advances the survival of bacteria outside the macroorganism – in the natural substrates of environment (sediments, mud, etc.), containing sialic acids (9, 15). On the other hand, it provides an advantage of sialic acid utilizing bacteria before the other microorganisms in the intestine.

There are no fundamental investigations on Aeromonas spp. neuraminidase up to now. Vertiev et al.(1978) found for the first time neuraminidase activity in 4 of 22 Aeromonas strains investigated (18). There was another report in 1984 that established neuraminidase activity in 2 strains of Aeromonas hydrophila out of 8 (9). It is found that Aeromonas strains, isolated from meet, water and patients in Bulgaria show neuraminidase activity (2).

We started a detailed research on dynamics and optimal conditions for biosynthesis of neuraminidase during the different growth phases of the Aeromonas 40/02 strain. That is the purpose of the present investigation.

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## MATERIALS AND METHODS

### Strains, media and cultivation conditions

A total of 40 strains Aeromonas spp., from the collection of the National Center of Infectious and Parasitic Diseases (NCIPD) in Sofia were studied. The strains were isolated from various water environments in Bulgaria. Their taxonomic affiliation was identified by an appliance for automated identification VITEK 2. The screening procedure was carried out with 24 and 48 hour cultures of the isolates grown in Hottinger broth, pH 8.0, at 30°C.

The strain showing highest enzyme activity – Aeromonas spp.40/02, was cultivated in the following media: Nutrient broth (NB) (Difco, Detroit, MI, USA), Tryptic soy broth (TSB), Hottinger broth (Bulbio, NCIPD), or in Brain heart infusion broth (BHI) (Becton Dickinson and Co., MD, USA), pH 8.0, at 30°C.

For the neuraminidase production were used 18-36 hour cultures of Aeromonas spp., grown in 100 ml BHI broth. They were cultivated in Erlenmeyer flasks at the following different aeration regimes: aerobic cultivation on shaker (40 rpm), static aerobic cultivation, microaerophilic cultivation, and anaerobic cultivation by addition of sterile paraffin layer. A sample of 1.5 ml cultural liquid was taken from the statically grown culture every 30 minutes and its pH, OD and neuraminidase activity was estimated to observe the correlation between growth phases and enzyme production. The growth was estimated turbidimetrically and expressed as optical density at 660 nm (OD660) with a spectrophotometer (UV/VIS Spectrophotometer ΗΕΛΙΟS β, UNICOM).

### Neuraminidase and protein assays

The neuraminidase activity of the supernatants was measured quantitatively by the thiobarbituric acid method of Uchida (17). One unit of neuraminidase activity is defined as the amount that releases 1 μg of N-acetyl-neuraminic acid for 1 min under standard conditions using glucomacropeptide (3) as a substrate. The extracellular and the intracellular neuraminidase activities and were assayed as the supernatants or the cell pellets were used. The cultures were centrifuged at 3000 rpm for 20 min, and the neuraminidase activity of the cell-free supernatants was determined. The cell pellets were suspended in phosphate buffer at pH 7.2 and homogenized. The supernatants of disrupted cells were collected after centrifuging at 5 000 rpm for 5 min. Protein concentrations were measured by the Lowry procedure (12) using bovine serum albumin as standard.

## RESULTS

### Screening of isolates for neuraminidase activity

We found that the enzyme activity of these bacteria is low, between 0.3 and 5.6 U/ml. No relation between presence of neuraminidase and the origin of the strains was observed. Enzyme activity was found both in strains isolated from healthy persons as well as from patients. The strain showing highest activity (Aeromonas 40/02), was in future experiments.

### Neuraminidase production in various media and at different aeration conditions

The cultivation of Aeromonas sp. 40/02 in different nutrient

**Table 1**. Neuraminidase activity of the screened strains

| Source | Number of Aeromonas spp. strains | Neuraminidase activity (U/ml) | |
|---|---|---|---|
| | | 18 h | 24 h |
| human isolates | 2 | - | 0.6-0.8 |
| human isolates | 7 | 0.9-2 | 0.9-3 |
| human isolates | 2 | 2-3 | 2-3.8 |
| human isolates | 2 | 0.3-0.6 | 2.5-3.8 |
| patients with enteritis | 7 | - | - |
| patient | 1 | - | 2.85 |
| patients | 5 | 0.6-2 | 1.9-3.5 |
| river water | 4 | 1.5-2.7 | 2.5-2.8 |
| river water | Aeromonas sp. 40/02 | 3.1 | 5.6 |
| drinking water | 1 | - | - |
| drinking water | 2 | 0.6-1.3 | 1.8-2.8 |
| meat | 1 | 3 | 2.6 |

media at identical other conditions demonstrated that the highest enzyme production was observed at growth in BHI – 11.6 U/ml, and the lowest – in NB, 1.6 U/ml. The enzyme production at cultivation in TSB was 8 U/ml, and in Hottinger broth - 5.9 U/ml.

The cultivation of Aeromonas sp. 40/02 at different aeration conditions in BHI revealed that the highest enzyme activity is observed at static aerobic cultivation – 14.7 U/ml and no activity was observed at anaerobic conditions. The enzyme activities at microaerophilic conditions and cultivation on shaker were approximately equal - about 4.4 U/ml (fig. 1). Significant variations of the cultural liquid pH were observed during the cultivation at all aeration conditions (table 2).

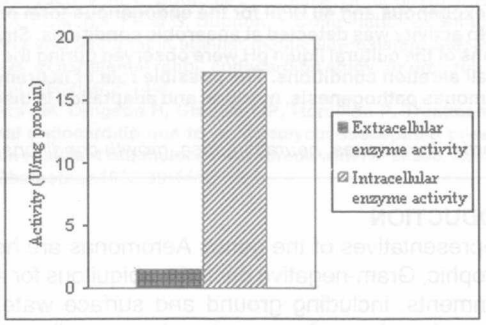


**Fig.3. Specific enzyme activities of the extra- and intracellular neuraminidase from Aeromonas sp. 40/02**

**Table 2.** Extracellular neuraminidase production and pH variations at different aeration regimes.

| Aeration regime | OD660 | Neuraminidase activity (U/ml) | Initial pH | Final pH |
|---|---|---|---|---|
| anaerobic | 0.48 | - | 8.0 | 5.5 |
| microaerophilic | 0.98 | 4.1 | 8.0 | 5.5 |
| static aerobic | 1.7 | 14.7 | 8.0 | 9.5 |
| aerobic on shaker | 1.8 | 4.4 | 8.0 | 9.5 |

**Dynamics of enzyme accumulation depending on the growth phases**

Using the favorable conditions, estimated for neuraminidase production from Aeromonas sp. 40/02 – BHI broth and static aerobic cultivation, the enzyme accumulation dynamics depending on growth phases was observed. According to our investigation the exponential phase begins at about the third hour and continues till the eight hour of cultivation (Fig. 2). Neuraminidase production becomes detectable at about the third hour. Maximal enzyme activity was found between the 12th and the 14th hour of cultivation, during the stationary phase.

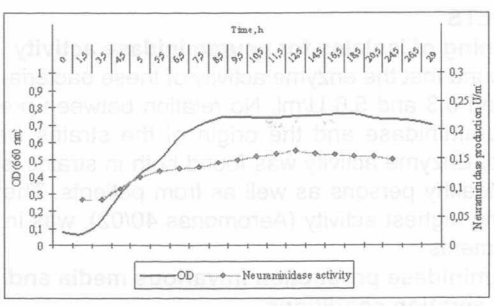


**Fig.2. Growth and neuraminidase activity of Aeromonas sp. 40/02 in BHI**

The intracellular neuraminidase activity of Aeromonas sp. 40/02 at the 14th hour of cultivation was 17.2 U/mg protein and the extracellular – 2 U/mg protein. The ratio of extracellular to intracellular enzyme activity at this hour is presented on fig 3.

## DISCUSSION

The term neuraminidase is used for a group of different enzymes with the common ability to hydrolyse the α-O-ketosidic bond between sialic acids and carbohydrates. They differ in many properties and may have different functions (15). The ability of sialic acid conversion is not frequently observed among bacteria and is associated mainly with pathogenic species (14). It is not evenly distributed among bacterial taxa too. Very close species or even strains of certain species differ in their ability to produce the enzyme.

The role of neuraminidase as a factor of pathogenicity in Aeromonas is still in process of investigation and is mainly hypothetic. In many pathogenic bacteria such as Vibrio cholerae, Clostridium spp. Erysipelothrix rhusiopatiae, Streptococcus spp., Bacillus spp Bacteroides fragilis etc., (6, 15) there is clear connection between neuraminidase production and virulence. The ability of some pathogenic bacteria to use the sialic acids as carbon, nitrogen and energy source reflects one of the mechanisms of surviving and reproduction in the cells of the macroorganism (11). Most pathogenic

bacteria, once infected the host, usually enter the respiratory and intestinal tract, which is covered with mucin substances, susceptible to neuraminidase action. In this way, the microbe neuraminidase might be of importance for arising of infection. It is supposed, that all bacteria showing high neuraminidase production, use the enzyme as part of their adaptation to the conditions of respiratory and intestinal tract (8).

There is a lack of data in the literature, concerning the relationship between neuraminidase production and the pathogenesis of Aeromonas infections. Our data do not establish any relation between enzyme activity and strains isolated. The enzyme is present both in strains isolated from patients as well as from healthy people and the strain with highest enzyme activity is isolated from river water (Table1). The lack of strong connection between pathogenicity, origin of the strains and level of enzyme synthesis is determined, also, in other investigations. For example, higher neuraminidase activity was observed in strains isolated from drinking water and meat, compared with isolates from patients (2). Many authors, however, regarding the enteropathogenicity of some Aeromonas bacteria as function of certain set of extracellular virulence factors (enterotoxins, hemolysins, some enzymes). It is quite possible that the Aeromonas neuraminidase participates in desialization of mucin substances in the microorganism, facilitating the penetration of bacteria. On the other hand, it is supposed that the presence of mucin-containing substances in environmental water and soil induce synthesis of neuraminidase in vibrios and clostridia. Obviously, this facilitates adaptation to environmental conditions in these bacteria (15). It was found that Pomorie lake mud, which is rich of bounded and free sialic acids, acts as inducer for secretion of neuraminidase in Aeromonas strains (1). The presence of enhanced enzyme activity in strain Aeromonas 40/02, isolated from environment could be a result of such an adaptive mechanism.

Compared with similar research of neuraminidase production in taxonomically close Vibrio cholerae, the data of present investigation showed some interesting features (unpublished data). Thus, the extracellular neuraminidase activity in Aeromonas strains investigated is lower, compared with the same in Vibrio cholerae non-O1 strains. The Aeromonas enzyme activity varied from 0.3 to 5.6 (U/ml), while in Vibrio they were in the limits of 19-36 (U/ml). In both microbe models (Vibrio and Aeromonas), the maximal enzyme activity was observed during stationary phase of growth. In Aeromonas, the highest neuraminidase activity was determined when aerobic, static cultivation was used, whereas in Vibrio, the most intensive enzyme synthesis is accomplished under microaerophilic conditions (unpublished data). These probably reflect the role of vibrionic neuraminidase as pathogenic factor in intestinal tract conditions, whereas in Aeromonas, this role is still unclear. In Aeromonas 40/02, the intracellular neuraminidase activity is higher than in V. cholerae. It is known that in some bacteria (Pseudomonas aeroginosa), there is endogenous neuraminidase, but they are not strong producers of extracellular neuraminidase (10). According to Muller (1974), the function of such neuraminidases may be seen not alone in hydrolysis of sialoconjugates, but also in the cell-own synthesis of such compounds (15). Our data concerning the intracellular enzyme activity in Aeromonas may reflect such a biological characteristic.

The role of Pseudomonas aeruginosa and Aeromonas caviae as causative agents of opportunistic infections is well known (13). Although with lower activity, the P. aeroginosa extracellular neuraminidase takes part in formation of biofilm (16). It is possible that the Aeromonas 40/02 enzyme has not only trophic function, but also has some relation to the pathogenicity. More research on this problem is in progress.

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# DIAGNOSTIC CAPACITY OF CFA AND ELISA METHODS FOR DETECTION OF ANTIBODIES AGAINST CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS IN PATIENTS SERUM

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**SUMMARY:**
**Complement Fixation Assay (CFA) and Enzyme-linked Immunosorbent Assay (ELISA) were used to test 16 serum samples from patients divided in three basic groups: 1st group included nine patients suspected to be infected with Crimean-Congo haemorrhagic fever virus (CCHFV) according to their clinical history and status; 2nd group - six patients immunized with a vaccine against CCHFV; 3rd group - one patient who has recovered from CCHF. The results obtained by the two methods for detection of specific antibodies against CCHFV were compared. Specific IgG antibodies against CCHFV were found in 4 patients. IgM specific antibodies were found in only one patient. The coincidence in diagnostic capacity of CFA and ELISA was achieved in small percentage of the examined samples.**

*Key words: Crimean-Congo haemorrhagic fever (CCHF); immunodiagnostics of CCHF; serology.*

Crimean-Congo haemorrhagic fever (CCHF) is particularly dangerous endemic viral infection. The main natural reservoirs in nature are ticks, mainly Hyalomma marginatum. Further, relatively short-term reservoirs of the virus are different species of mammals, mainly wild and farm animals in the stage of viremia after infection by the tick. The disease is caused by viruses of the genus Nairovirus, belonging to the family Bunyaviridae. The illness progresses with toxic, craniopharingeal and hemorrhagic syndromes (2). Human is also a source of infection during the acute period of disease (3).

CCHF is spread worldwide. The disease is endemic in large areas of Asia, Southern Russia, Africa, Middle East and Southeast Europe. Cases and outbreaks in recent years occurred in Kosovo, Albania, Iran and Pakistan, South Africa, Turkey and Greece (8). In Bulgaria, CCHF is described in various natural foci: Shumen region, Bourgas region, Sredna Gora Mountain, Eastern Rhodopes and Veliko Tarnovo region (5).

Laboratory methods for diagnosis of CCHF are based on direct detection of the etiological agent or on serological methods, detecting highly specific "antigen-antibody" complex and identifying both virus and antibodies. Efficiency of examination of clinical materials from the patient should comply with certain infectious conditions: the high pathogenicity of the Crimean-Congo haemorrhagic fever virus (CCHFV) to humans; lack of viral stability in the external environment; about 10 days of viremia duration; appearances of IgM antibodies 5-7 days after initial symptoms; appearance of IgG antibodies after 7-10 days followed by the increase in titre 15-20 days after the onset of the disease (5, 7). Two serum samples have to be tested by the serological methods – one at the onset of clinical manifestations and the second, two weeks later. To confirm the diagnosis, the antibodies titre in the second serum sample should be at least 4 times higher than in the first one (4).

In this paper, we present the results from the diagnostic capacity of immunodiagnostic methods: Complement Fixation Assay (CFA) and Enzyme-linked Immunosorbent Assay (ELISA) for detection of IgM and IgG antibodies against CCHFV in human sera.

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## MATERIALS AND METHODS

### Clinical materials

Sixteen serum samples from patients divided into three groups were collected and analyzed: 1st group included nine patients suspected to be infected with CCHFV according to their clinical history and status; 2nd group - six patients immunized with a vaccine against CCHFV; 3rd group - one patient who has recovered from CCHF. All specimens were taken intentionally by infectious diseases specialists after previous examination or during therapeutic manipulations. The studied clinical samples were brought together from the following infectious disease clinics: Regional Hospital - Pazardjik, Regional Hospital "Dr. Atanas Dafovski", Kardjali, Regional Hospital "St. Panteley", Yambol and Infectious Disease Hospital "Prof. Ivan Kirov", Sofia. Storage and transportation to the laboratory were done accordingly (4). The distribution of clinical materials for the study is shown in Table 1.

### Complement Fixation Assay (CFA)

CFA for detection of specific antibodies against CCHFV was held by the method of Casals (7). The reaction was carried out by heating the serums diluted 1:4 with isotonic buffer pH 7,4 for 30 minutes at 580C in a water bath. The supplies during the titration were as follows: specific CCHF antigen (obtained from laboratory infected newborn mice with known CCHFV strain); normal antigen (obtained from healthy newborn mice); the patient serum -subject of the study; and haemolytic complement system. All specific reagents used for the elaboration of CFA were produced by Bulbio® (Bulgaria).

### Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs for detection of specific IgM and IgG antibodies were held by standard kits Vectocrimean-CHF-IgM and IgG (Vector BEST, Russia). Serum samples involved in the study were carried out in accordance with the manufacturers' instructions. Applied specific antigen in the immunosorbent test was inactivated and concentrated. The "antigen-antibody" complex was visualized using horseradish peroxides. Values of optical density below 0,9 in the samples were reported as negative, values within 0,9 to 1,1 were reported as borderline, and those above 1,1 accepted as positive.

## RESULTS AND DISCUSSION

A total of 16 serum samples were tested with CFA for detection of antibodies in patients suspected for CCHF infection, immunized by CCHF vaccine or recovered from CCHF. The obtained results showed 2 (12.5%) positive and 14 negative (87.5%) reactions. The positive ones belonged to the first and the second group of patients: Vs 252/09 is a patient without

a history of tick bite, but presented an unexplained febrile syndrome. Vs 257/09 is a patient vaccinated against CCHF in 1992 and revaccinated for the last time in 1997. (Table 1) In parallel, all serum samples were also tested by ELISA for detection of IgG antibodies against CCHFV. After performing the test, a total of 4 (25%) positive and 12 (75%) negative reactions were proven. The positive results belonged to: one patient in the first group (Vs 247/09) suspected for CCHF after a history of contact with ticks and presented with unspecific febrile syndrome; two patients from the second group: Vs 253/09 - a patient vaccinated in 1999 with complications developed after the vaccination, expressed by increasing of temperature and epistaxis, and revaccinated in 2004; Vs 257/09 is the second positive patient from the second group vaccinated in 1992 and lastly revaccinated in 1997. The third group patient - Vs 258/09 was virologically proven (by infection of newborn mice with blood from the patient during the acute phase of the disease) in 2005. This patient recovered from CCHF. (Table 1)

Comparison of all 16 serum samples examined for antibodies against CCHFV held at the same time by CFA and ELISA showed positive results that matched only in one patient (25%) being part of the second group (Vs 257/09) with a full course of vaccination in 1992 and revaccination in 1997 (Table 1). We assumed that the discrepancy between the two methods is in the difference in the sensitivity due to the broader spectrum of antibodies that could be detected by ELISA.

When testing serums from the first group, a patient (Vs 247/09) who had a history of a tick bite and who removed the tick by hanhs, presented by unspecific febrile sndrome and manifested malaise. He was serologically positive only by ELISA. This patient was initially treated with antibiotics, and subsequently confirmed serologically as Mediterranean Spotted fever. However, several months later within this study, a test for CCHF was performed. Vs 252/09 was a patient without a history of a tick bite, with unexplained febrile syndrome, bleeding in the places of infusions and antibiotic treatment (Table 1). Although the result was negative by ELISA, a low titre of antibodies (1:4) was found by CFA in the first serum sample taken during hospitalization. In this case, we assumed cross reaction.

Patients belonging to the second group were with history of vaccination and revaccination against CCHF for 10 and more years. One of the patients (Vs 256/09) has been vaccinated, and in addition, was in contact with blood from an infected CCHF patient during the haemorrhagic stage in the past. However, in this case there were no antibodies detected by the two methods. The other patient (Vs 253/09) who had a history of complications after the vaccination was positive only by ELISA. This is an evidence of higher sensitivity of the immunosorbent test in comparison with CFA. Vs 257/09 is the only patient positive using the two test together (Table 1). In the rest of the samples from this group, there were no specific antibodies detected neither by CFA nor by ELISA. Most likely this was due to the titer reductions in the absence of revaccination as it's recommended - every five years.

The third group was formed only by one patient suffered by CCHF in May 2005. According to the history, he picked out and smashesed a tick by bare hands. Three days later, his temperature rose to 390C, and vomiting, weakness and pains on all over the body were presented. The diagnosis was confirmed by experimental infection of newborn white mice injected with his blood taken during the acute stage of infection. After transfusion and application of 36 ml of CCHF-bulin®, the patient recovered. Testing by ELISA, confirmed the history of the disease and demonstrated the presence of specific IgG antibodies against CCHF virus years after. When conducting the CFA in the same sample (Vs 258/09) (Table 1), antibodies were not found, confirming the limited sensitivity of this method.

All patients were also tested for IgM specific antibodies against CCHFV by ELISA and CFA. Only in one (Vs 250/09) serum IgM antibodies were found by the immunosorbent test. This patient belonged to the first group and his history revealed contacts with ticks and a moderate haemorrhage in his eye. No second serum samples were available. We assumed that this serum was collected during the very early stage of infection.

**Table 1**. General characteristics of sixteen tested serum samples examined for specific antibodies against CCHFV by two serological methods (CFA and ELISA) from patients divided into three main groups.

| Lab № | Gender | Status | Stage | CFA | ELISA IgM | ELISA IgG |
|---|---|---|---|---|---|---|
| 1st group | | | | | | |
| Vs 246/09 | F | Suspected | Reconvalescent | neg. (-) | neg.(-) | neg.(-) |
| Vs 247/09 | F | Suspected | Reconvalescent | neg. (-) | neg.(-) | pos. (+) |
| Vs 248/09 | M | Suspected | Reconvalescent | neg. (-) | neg.(-) | neg.(-) |
| Vs 249/09 | M | Suspected | Reconvalescent | neg. (-) | neg.(-) | neg.(-) |
| Vs 250/09 | M | Suspected | Haemorrhagic | neg. (-) | pos.(+) | neg.(-) |
| Vs 251/09 | M | Suspected | Reconvalescent | neg. (-) | neg.(-) | neg.(-) |
| Vs 254/09 | M | Suspected | Reconvalescent | neg. (-) | neg.(-) | neg.(-) |
| Vs 255/09 | F | Suspected | Reconvalescent | neg. (-) | neg.(-) | neg.(-) |
| Vs 252/09 | M | Suspected | Haemorrhagic | pos. (+) | neg.(-) | neg.(-) |
| 2nd group | | | | | | |
| Vs 256/09 | F | Vaccin./Susp. | Reconvalescent | neg. (-) | neg. (-) | neg. (-) |
| Vs 253/09 | F | Vaccinated | - | neg. (-) | neg. (-) | pos. (+) |
| Vs 257/09 | F | Vaccinated | - | pos. (+) | neg. (-) | pos. (+) |
| Vs 259/09 | F | Vaccinated | - | neg. (-) | neg. (-) | neg. (-) |
| Vs 260/09 | F | Vaccinated | - | neg. (-) | neg. (-) | neg. (-) |
| Vs 261/09 | M | Vaccinated | - | neg. (-) | neg. (-) | neg. (-) |
| 3rd group | | | | | | |
| Vs 258/09 | M | Recover | Reconvalescent | neg. (-) | neg. (-) | pos. (+) |

Balkan Peninsula is endemic for CCHF and sporadic cases or outbreaks are often described. Over the past two decades, in Bulgaria and the rest of the world the cases of this and other tick-borne infections have been increasing (1, 6, 8). It should be noted that the serum samples examined in this study were collected from patients with history and status of CCHF, residing in different endemic regions of the country (Pazardjik, Kardzhali and Yambol).

CFA and ELISA for detection of specific antibodies against CCHF are among the most widely used immunological diagnostic tests. In our country, CFA is used routinely as a diagnostic method. The reaction is based on the ability of serum antibodies to bind with specific viral antigens in the presence of complement and thus preventing haemolysis in the haemolytic system. Still, two questions stay opened: are antigens enough specific and how cross reactivity of sera could be escaped?

The ELISA test is not performed routinely for laboratory diagnosis of CCHF in Bulgaria. The method, however, is suitable for routine examinations of clinical samples and could be carried out not only qualitatively, but also as a quantitative method. The final result might be defined by comparing with standards of known titer as positive controls.

The results of this study confirmed that the diagnostic capabilities of the two methods (CFA and ELISA) match in a small percentage of the examined samples. Introducing the immunosorbent test for routine diagnostic of CCHF would

contribute to exact and accurate final results. Nevertheless, this study draws attention on the fact that diagnosis of this viral haemorrhagic fever needs more than one serological and/or virological method.

## ACKNOWLEDGMENTS

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# FEATURES OF CRIMEAN-CONGO HAEMORRHAGIC FEVER IN PATIENTS WITH FEBRILE SYNDROME IN BULGARIA

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**SUMMARY:**
**Crimean-Congo haemorrhagic fever (CCHF) is an acute tick-borne viral disease, affecting only humans and newborn mice, with haemorrhagic manifestations and considerable mortality in humans. A series of investigations have been conducted regarding circulation of CCHF virus (CCHFV) in Bulgaria. General epidemiological and ecological investigations have been completed in different geographic regions of Bulgaria. The aim of this study was to reveal to what extent the infection with Crimean Congo hemorrhagic fever virus (CCHFV) could be the cause for an acute febrile syndrome in Bulgaria. A total of 302 paired serum samples (acute and reconvalescent) from 149 patients from Sofia, Bourgas, Sliven and Plovdiv area were tested by ELISA for antibodies against CCHFV. Serological data were obtained for 9 (6,04%) patients from districts of Bourgas, Sliven and Sofia. Most of the CCHF cases were reported in summer with a peak in July - 3/9 (33,33%). People aged 10-19 years were more affected. An analysis of the frequency of the clinical signs was made. Only one patient had bleeding (11,11%). Four patients (44,44%) had a rash. Seven of the studied patients (77,78%) recovered completely. Continued surveillance is required to monitor suspected CCHF cases, not only within known foci, but also outside endemic areas as reported in this study.**

*Key words: Crimean-Congo haemorrhagic fever, antibodies, seroconversion, vectors*

## INTRODUCTION AND PURPOSE

Crimean-Congo haemorrhagic fever (CCHF) is an acute, tick-borne viral disease, affecting only humans and newborn mice, with haemorrhagic manifestations and considerable mortality in humans. CCHFV circulates in nature in an enzootic tick-vertebrate-tick cycle: migrating birds and livestock can transfer large numbers of infected ticks from endemic to non-endemic areas thus spreading CCHFV into novel areas (22). The formation of natural foci is typical as for most tick-borne diseases as for CCHV. Interspecies relationships between the agents of the diseases, animal donors and their vectors are developed in these foci. A lot of factors promote that circulation in the foci (21). A lot of tick genera could be infected with CCHFV, but the most efficient and common vectors for CCHFV are members of the genus Hyalomma (8). The infection emerged or re-emerged in Bulgaria, Albania, Kosovo (5), and Turkey (14) from 2000 till 2008 year. It has also recently emerged in Greece (12), where the first human case has been recognized.

A series of investigations have been conducted regarding circulation of CCHFV in Bulgaria. General epidemiological and ecological investigations have been completed in different geographic regions in our country (18). The disease is widely distributed in Bulgaria. Endemic regions are districts of: Shoumen, Razgrad, Veliko Tarnovo, Plovdiv, Pazardzhik, Haskovo and Bourgas (16). The endemic focuses are associated with the main vector Hyalomma marginatum ticks (18). The tick population was increased due to mild winters and limited agricultural activity. Tick-infested migratory birds contribute spreading of CCHFV. Most cases are reported from central and eastern parts of the country (16,17). A cluster of cases, observed in southwestern Bulgaria was reported in 2008 year. This area was previously considered at low risk for CCHF (4).

Diagnosis of patients with acute febrile syndrome of unknown origin is often difficult. Patients often do not recall a tick bite. Specific serologic diagnosis is needed in such cases.

The aim of this study was to reveal to what extend infection with CCHFV could be the cause for acute febrile syndrome of unknown origin in Bulgaria.

## MATERIAL AND METHODS

A total of 302 paired serum samples (acute and reconvalescent) from 149 patients (63 patients from the district of Plovdiv, 33 patients from the district of Bourgas, 25 patients from district of Sofia, 21 patients from district of Pazardzhik and 7 from district of Sliven) were tested by ELISA for antibodies against CCHFV. This study was completed during the period June 2008 – February 2009 in the National Reference Laboratory of vector-borne infections, leptospirosis and listeriosis. Paired serum samples from these patients were tested for detection of IgG and IgM antibodies by commercial kits (Vector Best, Russia). Capture ELISA was used for detection of IgM antibodies. The reaction was read by spectrophotometer at 450 nm. The presence of IgM antibodies in the serum of patients was indicative for early stages of the disease. Either the presence of IgM or a four-fold rise in the titer of IgG antibody in serum samples between the acute and convalescence phases was diagnostic.

Patients with positive results for CCHF were tested further for the presence of antibodies against the agents of Q fever, Hemorrhagic fever with renal syndrome and Tick-borne encephalitis in order to exclude other uncommon causes of the febrile syndrome.

## RESULTS AND DISCUSSION

Serological data for CCHFV infection were found for patients from the districts of Bourgas, Sliven and Sofia, but not from the district of Plovdiv, despite a lot of investigations in the district of Plovdiv regarding etiology, epidemiology, clinical manifestations and pathology of CCHF (15,16). Pre-selection criteria for patients from the district of Plovdiv, based on investigations of patients mainly with Mediteranean spotted fever and much less on patients with unclear febrile syndrome, may explain why no patients with CCHF from the district of Plovdiv were found.

According to the official data in 2008, 13 patients with CCHF (incidence 0,17% 000) are recorded from Ministry of Health and one patient died (mortality 0,01% 000) (10). For comparison, patients with CCHF in 2009 are less – 6 patients (incidence 0,08 % 000) and one died (mortality 0,01% 000).

Our serological study showed presence of antibodies against CCHFV in three patients 3/9 (33,33%) from Bourgas district. IgM antibodies were found in one patient . Both IgM and IgG antibodies were found simultaneously in 2 patients. Seroconversion was detected in all of the three patients.

Serological study showed presence of antibodies against CCHFV in three patients 3/9 (33,33%) from Sofia area (Godech and two villages) and in other three (33,33%) from district of Sliven. Both IgM and IgG antibodies were found

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simultaneously in only one patient and IgM antibodies solely were detected in the rest five patients. Seroconversion was detected in three patients. Serological findings correlated with known abundance of the ticks Hyalomma marginatum in Bourgas (33,3%) and Sofia areas (24,4%) (18). From the literature, it is known that IgM remains detectable for up to four months, and IgG levels decline but remain detectable for up to five years (13).
Seasonal distribution of seropositive patients is shown in figure 1.

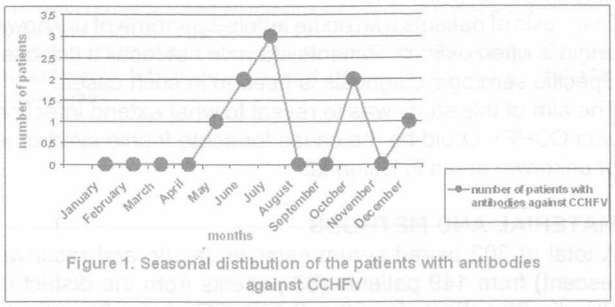


Figure 1. Seasonal distibution of the patients with antibodies against CCHFV

Most of the cases were reported in summer with a peak in July. The lowest incidence was recorded in May and December 1/9 (11,11%). There were no cases of CCHF in January, February, March and April. This is in accordance with the seasonal activity of tick H. marginatum (18). Morbidity is as greater as activity of the ticks is longer. There were cases of the disease from April to December in the district of Bourgas, whereas this activity is shorter in the district of Sofia. Changes in climatic conditions have been suggested to be one of the factors that have facilitated reproduction of the tick population, and consequently the increased incidence of tick-borne infectious diseases (6, 7).
Distribution of seropositive patients according to age is shown in figure 2. People aged between 10-19 years were more affected - 3/9 (33,33%), followed by the two age groups 60-69 years and 70-79 years - 2/9 (22,22%). Men were more affected - 7/9 (77,78%) than women - 2/9 (22,22%).
Analysis of patients with antibodies against CCHFV showed that more cases were detected in the cities - Bourgas 2/9 (22,22%), Aitos 1/9 (11,11%), Sliven 3/9 (33,33%), Godech 1/9(11,11%), while only two cases 2/9 (22,22%) were detected in two villages near Sofia (fig. 3).
Identification of the symptoms of CCHF is very important for accurate diagnosis. Incubation period ranged from 2 to 9 days (18). Time to onset of disease continues 3,2 days after the tick bite or 5,6 days after human blood exposure (20). There is a variety of potential clinical manifestations following

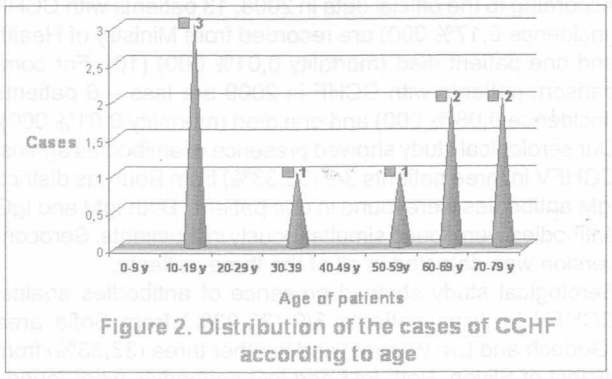


Figure 2. Distribution of the cases of CCHF according to age

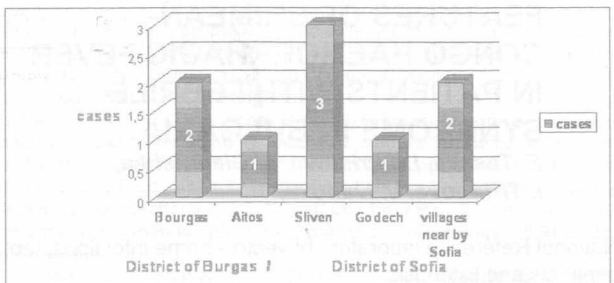


Figure 3. Comparative results of distribution of patients from districts of Bourgas, Sofia and Sliven.

infection with CCHFV. Some patients do not develop the classic form of CCHF syndrome. Patients exhibit a nonspecific prodrome initially, which typically lasts less than one week (8). Six patients had fever with duration from 4 to 8 days 6/9 (66,67%), in one patient this period was shorter (1-3 days) and two patients did not develop fever 2/9 (22,22%) (fig. 4).

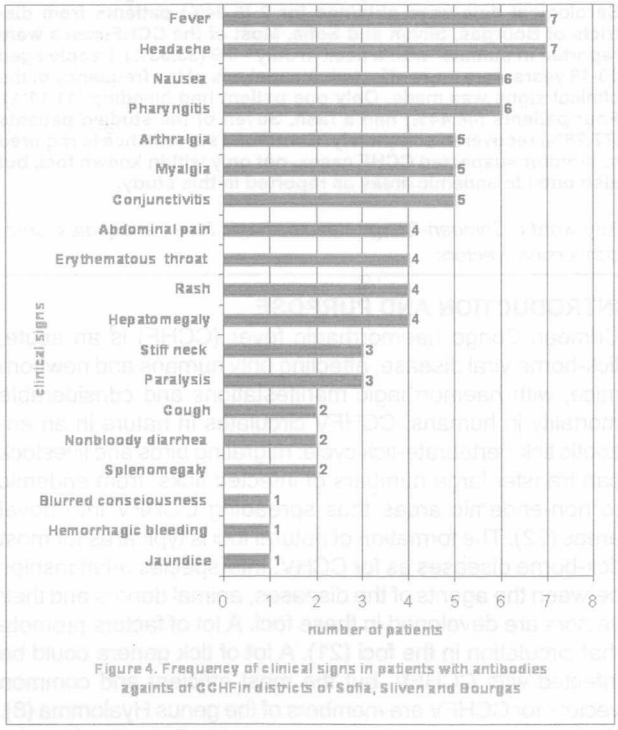


Figure 4. Frequency of clinical signs in patients with antibodies against of CCHFin districts of Sofia, Sliven and Bourgas

Clinical manifestations could include non-specific or typical symptoms. Typical symptoms include high fever, headache, malaise, arthralgia, myalgia, nausea, abdominal pain, and nonbloody diarrhea.
Seven patients had a headache 7/9 (77,78%). Arthralgia and myalgia were detected in five patients 5/9 (55,56%). Abdominal pain was developed in four patients 4/9 (44,44%) (fig. 4). Nausea was found in six patients 6/9 (66,67%) and only two had a nonbloody diarrhea 2/9 (22,22%). Early signs typically include: erythematous throat 4/9 (44,44%), cough in two patients 2/9 (22,22%), conjunctivitis in 5/9 (55,56%), and pharyngitis - in 6/9 (66,67%). Three patients had stiff neck (fig. 4). There are some reports showing that most cases in Europe and Asia are associated with skin redness or rash (8). Rash was detected only in 4/9 patients (44,44%). Central nervous system disfunction was manifested by paralysis in three patients 3/9 (33,33%). Blurred consciousness was detected in one patient (fig.4).
Only one patient had bleeding (11,11%). Haemorrhagic

period is usually short (2 - 3 days). It develops rapidly, and usually begins between third and fifth day of the disease (8). The bleeding is companioned with abdominal pain. This is very important because haemorrhage within the abdominal muscles can simulate acute appendicitis in CCHF patients (2).

Hepatomegaly and splenomegaly have been reported to occur in one-third of patients according to the literature (8). Hepatomegaly solely was detected in two of our cases 2/9 (22,22%) and simultaneous hepatomegaly and splenomegaly were detected in the other two cases in this study. Jaundice was observed only in one of the cases (fig. 4).

The most effective vectors for CCHF are ticks of species H. marginatum. CCHFV generally circulates in nature in an enzootic tick-vertebrate-tick cycle. Essential for spread of the disease is increased abundance of the tick population, as well as the migration or transportation of tick-infested birds or animals (22). Four 4/9 (44,44%) of the examined patients reported a tick bite. Two other patients had contacts with parrots. Domestic ruminant animals, such as cattle, sheep, and goats develop viremia for one week after becoming infected (1). They play a role in maintaining the epizootic process, acting as reservoirs of infection. The potential functions of migratory birds and the movement of livestock carrying ticks in the spread of the virus over distant geographic areas have been studied (8,10). Many birds are resistant to the infection, but some, such as ostriches, crows, parrots and partridges are susceptible to it. They can contribute to the prevalence of the disease in endemic areas. Only one patient announced contact with rodents in this study. It is proven that the role of rodents in the epidemic process is minimal.

The convalescence period of the disease is prolonged; the intoxication and the haemorrhagic manifestations gradually disappeared, but patients have neurasthenic complaints for a long time. Seven of the studied patients 7/9 (77,78%) recovered completely, but muscle, joint complaints and fatigue remained in two of them (22,22%).

Clinical symptoms of our patients, addressed to pre-diagnosis, were compatible with: viral meningitis, sepsis, Lyme borreliosis, Mediterranean spotted fever, other rickettsiosis and unspecific febrile syndrome. (Table1).

riketsiosis, Lyme borreliosis; in haemorrhagic cases - leptospirosis, sepsis, viral haemorrhagic fevers and hemorrhagic fevers Ebolla and Lassa.

Data analysis revealed one case of possible coinfection of CCHF with tick borne encephalitis - 1/9 (11,11%) from the district of Bourgas.

Presence of co-infection is most likely due to common reservoirs and vectors of these infections. Rodents and their ticks form typical epidemiological unit where viruses and bacteria circulate. Rapid and accurate diagnosis could be obtained by laboratory tests, especially in cases associated with co-infection.

## CONCLUSION

We did not detected patients with antibodies against the virus of CCHF in the district of Plovdiv. This does not diminish the fact that it is better to know the biology and species composition of vectors in this region and to resort to emergency prevention measures. It is necessary to assume tick-transmitted infections in monitoring of patients with acute febrile syndrome. There is evidence that genetically similar strains of CCHFV are circulating in Bulgaria, Greece and Iran (3,17). Therefore it is important to conduct monitoring. Furthermore, as for everyone emerging infection, as well as for the tick-borne transmitted infections it is necessary to develop effective early warning systems in case of new outbreaks and acts of bioterrorism (9). Continued surveillance of suspected CCHF cases, not only within known foci, but also outside endemic areas as reported herein, is required. Accurate etiological diagnosis is crucial to prevent the spread of infection.

**Table 1** Patients with febrile syndrome of unknown origin who had IgM and/or IgG antibodies against CCHFV

| Classes of antibodies | № of sample | Description (sex, age) | Leading clinical signs |
|---|---|---|---|
| IgM | Bul-Bug 018 | man/75 years | toxic infectious syndrome, hemorrhagic bleeding, abdominal pain |
| | Bul-Sof 009 | man/69 years | toxic infectious syndrome, erythematous throat, nausea |
| | Bul-Sof 055 | man/18 years | toxic infectious syndrome , erythematous throat, cough, nausea |
| | Bul-Sof 061 | man/32 years | rash |
| | Bul-Sof 188 | man/19 years | toxic infectious syndrome, stiff neck, cough , abdominal pain, nausea, nonbloody diarrhea, rash |
| | Bul-Sof 220 | woman 71 years | toxic infectious syndrome, abdominal pain, nausea, rash |
| IgG and IgM simultaneously | Bul-Bug 002 | man/13 years | toxic infectious syndrome, nausea, nonbloody diarrhea |
| | Bul-Bug 012 | woman 13 years | toxic infectious syndrome, stiff neck, erythematous throat, abdominal pain, nausea, rash |
| | Bul-Sof 057 | man/34 years | arthralgia, myalgia, paralysis |

Clinical manifestation of CCHF is not specific, particularly in early phase. Therefore, early clinical diagnosis is difficult. The symptoms are difficult to distinguish from those of many other acute viral and bacterial infections such as influenza, rickettsiosis, plague, leptospirosis and others (16). Differential diagnosis in the pre-haemorrhagic stages includes

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# COMPARISON OF A COMPLEMENT FIXATION ASSAY, ELISA AND IMMUNOBLOT FOR SEROLOGIC DIAGNOSIS OF HANTAVIRUS INFECTIONS IN BULGARIA

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**SUMMARY:**
**We introduced species-specific ELISAs and confirmatory immunoblot (lineblot) to determine hantavirus types that cause infections in Bulgaria and to compare their abilities for serologic diagnosis with those of routinely used complement fixation assay (CFA). A total of 44 serum samples from patients suspected to have hantavirus infection were tested in paralel by ELISAs and CFA and 26 of them were tested by immunoblot. Species-specific ELISAs revealed Dobrava reactivity. We found coincidence of the results between ELISA and CFA in 77,27%. Positive predictive value of ELISA and CFA was 90% and 69,6% when negative predictive value was 100% and 33,3% resp. Immunoblot confirmed all but two ELISA-positive and 9 CFA-positive results. ELISA-positive result revealed uniform antibody reactivity against Dobrava viruses but no reactivity to Puumala hantavirus. We concluded that with a very high reliability, ELISA could be used as confirmatory assay to reduce false-positive and false-negative results by CFA.**

**Key words:** *Hantavirus, ELISA, immunoblot*

Introduction

Hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia - Old world hantaviruses, while in Americas New World hantaviruses cause hantavirus pulmonary syndrome (HPS). HFRS is characterized by fever, hemorrhages and acute renal insufficiency. HPS is manifested by fever, pulmonary edema and respiratory failure. Case fatality rate is 3-10% for HFRS and up to 40% for HPS. Moreover, pulmonary manifestations can be found in HFRS and renal disorders – in HPS.

More than 30 hantavirus species are currently recognized. Pathogenic Old World hantaviruses are Hantaan, Dobrava, Puumala, Seoul, Saaremaa and Amur. Each of them is carried predominantly by its own rodent species. Apodemus agrarius (stripped field mouse) is associated with Hantaan and Saaremaa hantaviruses, Apodemus flavicollis (yellow necked field mouse) – with Dobrava, Myodes glareoulus (bank vole) – with Puumala, Rattus norvegicus and Rattus rattus (Norway rat and black rat) – with Seoul, and Apodemus peninsulae (Korean field mouse) is the host of Amur hantavirus. Besides rodents, some insectivores also can host hantaviruses (1).

Hantaan virus is distributed in China, Korea and Russia, Amur – in Far East and Russia, Dobrava is distributed in the Balkans, Puumala and Saaremaa - throughout Europe, and Seoul – worldwide.

Two viruses coexist in the Balkans - Dobrava and Puumala. Dobrava virus causes more severe HFRS that is spread not only in the Balkans but also in Russia. Puumala virus causes a milder disease, known as nephropathia epidemica, in Scandinavia, Western Europe and western Russia (2-5). In Bulgaria, serologic diagnosis of hantavirus infections is based routinely on complement fixation assay. New diagnostic techniques need to be introduced. In addition, hantaviruses affecting humans have never been classified. As a first step to elucidate status, we introduced polyvalent and species-specific ELISAs and immunoblot for confirmation of the positive results, that were able to distinguish between different hantaviruses. Then, we compared their abilities for serologic diagnosis with those of routinely used complement fixation assay.

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## MATERIALS AND METHODS

### Serum samples

A total of 44 serum samples were tested. The samples originated from patients suspected to be infected with hantaviruses and were stored in Bulgarian Reference Laboratory of tick-borne infections.

### Complement fixation assay

Reaction was carried out by heating the serums diluted 1:4 with isotonic buffer pH 7,4 for 30 minutes at 580C in a water bath. The supplies during the titration were as follows: specific antigen; normal antigen; tested serum sample; and haemolytic complement system – sheep red blood cells (sRBC), anti-sRBC and complement. If specific antibody is present in the patient's serum, then antigen-antibody complexes are yielded after addition of the specific antigen, the complement is completely utilized and sRBC added subsequently will not undergo hemolysis. However, if the antibody is not present in the patient's serum, then the complement binds anti-sRBC antibody and lysis of the sRBC ensues.

### ELISA

Two different commercially available ELISA tests were used to detect specific IgM antibodies against hantaviruses.

Puumala & Hantaan ELISA (ProGen, Germany) – serum samples were tested by species-specific ELISA detecting antibodies against Puumala and Hantaan (known to cross-react with Dobrava). Measured optical density of the samples at the end of the reaction was divided by optical density of the cut-off and was interpreted as positive if was above 2.

Dobrava ELISA (IBL, Germany) – in this case, wells of the microtitre plate were coated by nucleocapsid antigen of Dobrava hantavirus. Index values above 2 were interpreted as positive as suggested by the manufacturer.

### Immunoblot

To increase specificity of the investigations, Lineblot (Euroline anti-hanta profile) (Euroimmun, Germany) was applied. Each strip was coated by nucleocapsid antigens to Puumala, Hantaan and Dobrava viruses. This allowed simultaneous detection of and discrimination between antibodies against the tree hantavirus species on one strip.

## RESULTS

A total of 44 serum samples from patients suspected to have hantavirus infection were tested first by ELISA, complement fixation assay (CFA) and immunoblot.

Species-specific ELISAs revealed Dobrava reactivity.

When sera reactivity by ELISA was compared with reactivity by complement fixation assay, 77,27% (34/44) coincidence of the results from the two methods was found. The discrepancy was due to either false-positive or false-negative results by

CFA (Table 1). CFA-positive results were confirmed by ELISA in 16/23 (69,57%) of the cases. CFA-negative results were confirmed in 17/21 (80,95%) of the cases.

**Table 1.** ELISA versus complement fixation assay (CFA) for detection of antibodies against hantaviruses

| Results | CFA positive | CFA negative | Total |
|---|---|---|---|
| ELISA positive | 16 | 4 | 20 |
| ELISA negative | 7 | 17 | 24 |
| TOTAL | 23 | 21 | 44 |

Next step was to investigate serum reactivity by immunoblot (lineblot) test. In all but two out of 26 (7,7%) serum samples immunoblot showed the same reactivity as ELISA, when 9 of 26 (34,6%) serum samples showed different reactivity when tested by CFA and immunoblot (Tables 2 and 3).
All lineblot tests revealed uniform antibody reactivity against both Hantaan and Dobrava viruses but no reactivity to Puumala hantavirus.
Positive predictive value of ELISA and CFA was 90% and 69,6% when negative predictive value was 100% and 33,3% resp. (Tables 2 and 3)

**Table 2.** Comparison of results from ELISA and immunoblot

| | Immunoblot positive | Immunoblot negative | TOTAL | Predictive Value |
|---|---|---|---|---|
| ELISA-positive | 18 | 2 | 20 | Positive 18/20 |
| ELISA-negative | 0 | 6 | 6 | Negative 6/6 |
| TOTAL | 18 | 8 | 26 | |

**Table 3.** Comparison of results from CFA and immunoblot

| | Immunoblot positive | Immunoblot negative | TOTAL | Predictive Value |
|---|---|---|---|---|
| CFA-positive | 16 | 7 | 23 | Positive 16/23 |
| CFA-negative | 2 | 1 | 3 | Negative 1/3 |
| TOTAL | 18 | 8 | 26 | |

## DISCUSSION

In Bulgaria, hemorrhagic fever with renal syndrome (HFRS) is a notifiable disease for more than 50 years. A few cases are reported per year - usually 2-5 cases with fluctuations every 5-6 years. For example, 0 cases were reported in 1994, only 1 case in 2000 and 2001, 0 in 2006 and only 2 in 2010. Mostly Southern regions of the country are affected.
HFRS in Bulgaria is a rather severe disease. Renal failure is often seen and recovery period is prolonged. Even by clinical manifestation, one can predict Dobrava virus prevalence. Serologic investigations also led us to Dobrava prevalence. All lineblot reactions showed Hantaan and Dobrava reactivity, the two viruses known with their serologic cross-reactivity. Investigations by RT-PCR of frozen serum samples are ongoing to elucidate distribution of hantaviruses affecting people in Bulgaria.
The main goal of this study was to compare complement fixation assay (CFA), ELISA and lineblot for detection of antibodies against hantaviruses. We found coincidence of the results between ELISA and CFA in 77,27% and almost complete coincidence between ELISA and immunoblot.
CFA was the routine diagnostic test in the past in our country. Recently, polyvalent and species-specific ELISAs were introduced. Currently, ELISA is the main method for serologic diagnosis of patients with hantavirus infections (6-10). ELISA is routinely used also to detect antibodies against hantaviruses in rodents for epidemiological and ecological studies (11). The proportion of confirmed by lineblot ELISA-positive results was very high (90%). On the other hand, CFA omitted about 1/5 of the positive results and gave false-positive results in about 1/10 of the cases. It means that with a very high reliability, ELISA could be used as confirmatory assay to reduce false-positive and false-negative results by CFA.
HFRS is a problem also for other countries on Balkan Peninsula (6, 8-10). Adequate diagnostic tools are necessary to obtain reliable diagnosis. The disease is severe and proper diagnosis can avoid disease complications by adequate treatment.

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