Indexed by
SCOPUS
from 2008

PROBLEMS
of Infectious and Parasitic Diseases

NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 44, NUMBER 2/2016

ISSN 0204-9155

1504 Sofia; 26, Yanko Sakazov Blvd.
Tel.: +359 2/ 846 83 07, Fax: +359 2/ 943 30 75
e-mail: infovita@ncipd.org

PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 44, NUMBER 2/2016

Editor-in-Chief
Prof. T. Kantardjiev, MD, DSc

Editorial Board
Acad. B. Petrunov, MD, DSc
Prof. I. Christova, MD, DSc
Prof. P.Teoharov, MD, DSc
Assoc. Prof. I. Rainova, MD, PhD

Preprinting and Printing by Maker Arts & Alliance print

CONTENTS

1. ETIOLOGICAL DIAGNOSIS OF MENINGOCOCCAL MENINGITIS AND SEROGROUP TYPING OF NEISSERIA MENINGITIDIS BY REAL-TIME PCR IN BULGARIA 5
V. Levterova, I. Simeonovski, A. Kurchatova, S. Panaiotov, I. Philipova, T. Kantardjiev

2. DISTRIBUTION OF CHLAMYDIA TRACHOMATIS SEROTYPES BY DNA SEQUENCING OF OMPA GENE IN SOFIA, BULGARIA 11
I. Philipova, I. Ivanov, A. Kouzmanov, Z. Ivanova, V. Levterova, I. Simeonovski, V. Kantardjiev

3. CHLAMYDIA TRACHOMATIS GENOTYPING BY HIGH-RESOLUTION MELTING ANALYSIS 15
I. Philipova, I. Ivanov, A. Kouzmanov, Z. Ivanova, V. Kantardjiev

4. DNA SEQUENCING OF ITS REGION FOR RAPID AND ACCURATE IDENTIFICATION OF MEDICALLY IMPORTANT FUNGI 19
A. Kouzmanov, I. Philipova, Z. Ivanova, I. Ivanov, V. Kantardjiev

5. HUMAN LEPTOSPIROSIS IN BULGARIA, 2010-2014 23
I. Christova, E. Tasseva

6. CHOLESTASIS SYNDROME IN HEPATITIS E – TWO-YEAR PERIOD ANALYSIS (2014-2015) 30
M. Pishmisheva, P. Teoharov, E. Naseva, N. Vatev,M. Stoycheva, M. Karamisheva, D. Velkova, I. Ivanov

7. COMPARATIVE STUDY OF METHODS FOR DETECTION OF CLOSTRIDIUM DIFFICILE DIRECTLYFROM STOOL 36
M.Pavlova, E.Dobreva, K.Ivanova, G.Asseva, I. N. Ivanov, P. Petrov, E.Savov, I.Todorova, A.Trifonova, T. Kantardjiev

8. CASE OF ICTEROHAEMORRHAGIC LEPTOSPIROSIS WITH PREMORBID LIVER DISORDER 41
G. Gancheva, T. Doichinova, I. Pakov

9. BACTERIOPHAGE THERAPY 46
A. Trifonova, I. Todorova, E. Savov

10. CELL SURFACE COMPONENTS DETERMINING AGGREGATION IN LACTOBACILLI (MINIREVIEW) 51
T. Yungareva

Instructions to Authors

Papers should not have been previously published or be currently under consideration for publication.

Manuscripts must be written in English, using British spelling. All manuscripts should be single-spaced, with wide margins and numbered pages. MS Word should be used for word processing, 12-point Times New Roman font.

Named authors must fit the following three criteria:

1. Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data;
2. Drafting the article or revising it critically for important intellectual content; and
3. Final approval of the version to be published.

All people who meet the three criteria should be named as authors. Those who participate in the study but do not meet the requirements of authorship should be acknowledged as contributors.

TITLE PAGE

The title page must contain: 1) title, name and surname of the authors; 2) names of the institution(s) where the research was carried out; 3) the name and full postal address, e-mail address and telephone numbers of the corresponding author; 4) three to five key words.

ABSTRACT

The abstract should contain about 250 words and must be structured as follows: background, material and methods, results, conclusions. Review Articles should have an informative, unstructured abstract of about 250 words. Brief reports should have a short abstract of no more than 150 words.

TEXT

The text should contain introduction, material and methods, results, discussion and references. No particular format is required for review articles.

ACKNOWLEDGEMENTS

Individuals who supplied facilities, strains or reagents, or gave advice may be acknowledged. Also, supporting grants may be mentioned.

REFERENCES

References should be numbered in order of appearance in the text, in parenthesis, not superscripts, as shown bellow:

Journal articles:

Vellinga A, Cormican M, Hanahoe B, Murphy AW. *Predictive value of antimicrobial susceptibility from previous urinary tract infection in the treatment of re-infection.* Br J Gen Pract. 2010

ETIOLOGICAL DIAGNOSIS OF MENINGOCOCCAL MENINGITIS AND SEROGROUP TYPING OF *NEISSERIA MENINGITIDIS* BY REAL-TIME PCR IN BULGARIA

V. Levterova, I. Simeonovski, A. Kurchatova, S. Panaiotov, I. Philipova, T. Kantardjiev

National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia – 1504, Bulgaria

ABSTRACT

Neisseria meningitidis is a leading cause of bacterial meningitis globally. The present study focused on diagnosing and serotyping of *N. meningitidis* in Bulgaria. We applied adapted protocols for DNA isolation with Chelex 100 resin from cerebospinal fluid (CSF) and diagnostic real-time PCR technique. One hundred and thirty CSF samples, collected from all over Bulgaria were tested for detection or confirmation the etiology of bacterial meningitis by *ctrA* and *sodC* marker genes. *N. meningitidis* accounted for 14.8% of the meningitis cases in Bulgaria. *N. meningitidis* was confirmed in 42% of patients over 20 years of age. We targeted *sacB, synD, synE, xcbB,* and *synF* marker genes for A, B, C, W135, X, and Y serogroup typing by real-time PCR. Serogroups B and C were predominant, accounting for 74% and 19% of the isolates, respectively. Our results confirm that in Bulgaria serogroups B and C are major causes of invasive meningococcal disease, in agreement with data from other European countries. The real-time PCR technique is useful for confirmation of isolates and detection of meningococci in clinical samples, regardless of the encapsulation status.

Keywords: *Neisseria meningitidis*, meningitis, real-time PCR diagnosis, genotyping, serotyping.

ADDRESS FOR CORRESPONDENCE:
Victoria Levterova
National Centre of Infectious and Parasitic Diseases, Sofia – 1504, Bulgaria
e-mail: vikis@abv.bg

INTRODUCTION

Meningitis is inflammation of the meninges. Meningitis infection is most commonly caused by bacteria, viruses or fungi. The etiology of bacterial meningitis is various, but most often associated with *Neisseria meningitidis, Haemophilus influenzae* or *Streptococcus pneumoniae*. The most serious and difficult to treat type of meningitis tends to be caused by *N. meningitidis*. The annual number of invasive meningitis cases worldwide is estimated to be at least 1.2 million, with 135 000 deaths related to *N. meningitidis* (1).

Invasive meningococcal disease (IMD) is rare in Europe with notification rate of 0.68 cases per 100 000 population in 2012 (2). Bulgaria reports low endemic rates of meningitis. The incidence of IMD in Bulgaria ranges between 0.2 – 1.1 cases per 100 000 of population during 2000-2015 (3, 4). The incidence rate does not vary significantly over time and is retained at a constant level in the last years. Data on IMD are obtained from annual analyses of infectious diseases prepared by the National Centre of Infectious and Parasitic Diseases (NCIPD) and published on the web page of the centre: (http://ncipd.org/index.php?option=com_docman&view=list&slug=analizi-na-zaraznite-zabolyavaniya&Itemid=1127&lang=bg).

Vaccination against meningitis in Bulgaria is not compulsory. Nimenrix (GlaxoSmithKline Biologicals S.A., Belgium) is the only registered vaccine in Bulgaria and it is not effective against *N. meningitidis* serogroup B strains.

The etiological agent *N. meningitidis* is a Gram-negative diplococcus. Meningococci colonise the mucous membrane of the nasopharynx. Frequently the colonisation is asymptomatic and occurs in approximately 10 % of the population (5). In winter season meningococcal meningitis is predominant (6). In 10% of the cases, meningitis can be potentially fatal. Meningitis is more often encountered among neonates and children. Symptoms in neonatal meningitis are associated with respiratory distress, hyper- or hypotonia, irritability, poor feeding, increased tonus. In children and adults the most common clinical characteristics of bacterial meningitis are associated with fever, vomiting, altered mental status, neck stiffness, seizures, focal neurological deficit, rash. The clinical manifestations could be accompanied by pareses of cranial nerves – depending on localisation of the inflammatory exudate. Approximately one third to one half of the people who survived after the infection, remain with permanent health consequences (7).

Manifestations of these sequelae might be hearing loss, chronic fatigue or insomnia, neurological problems, as well as loss of parts of skin and sometimes of extremities due to limited blood circulation to these tissues (7, 8). Often symptoms in children and adults are non-specific. Therefore absence of classical clinical symptoms should not exclude possible meningitis. In all children and adults suspected of bacterial meningitis, cerebrospinal fluid (CSF) examination is strongly recommended. The results from the CSF examination in suspected cases of meningitis should indicate higher CSF leukocyte count, lower CSF glucose concentration, lower CSF/ blood glucose ratio and higher total CSF protein content. The microbiological diagnosis includes blood and CSF cultures, analysis of CSF by Gram staining, latex agglutination to determine serogroups, immunochromatographic antigen testing, and PCR (9, 10). CSF culture is positive in 60-77% of the samples. Pre-treatment with antibiotics decreases positivity of the results with 10-20% (11). Latex agglutination testing has little incremental value in the diagnosis of bacterial meningitis. It has high risk (54%) of false-positive results (12). Nucleic acids (NA) amplification techniques are usefull for the identification of the pathogen in samples of negative CSF cultures or negative CSF Gram staining (11). NA amplification has several advantages, such as faster detection, detection of fastidious or non-growing microorganisms, detection of 'killed' bacteria when antibiotic therapy was initiated before lumbar puncture. Specific and multiplex PCR against common meningeal pathogens have sensitivity >95% (13). Whole genome sequencing (WGS) for diagnosis of meningitis is possible but it has been applied only for specific studies (14, 15).

Based on differences in the chemical composition of the polysaccharide antigens of the capsule, *N.meningitidis* was divided in twelve serogroups: *A, B, C, X, Y, Z, W135, 29E, H, I, K, L*. Most frequently six serogroups are associated with invasive meningococcal infections, namely - *A, B, C, X, Y, W135* (4). Serogroups A, B, and C are the most frequent causes of meningococcal mengitis (16). In Europe, serogroups B and C are major causes of invasive meningococcal disease (3, 17). Prevalence of meningitis in Europe varies between 0.2 and 14 cases per 100 000 cases. Most cases are due to *N. meningitidis* serogroup B strains, especially in countries where meningococcal conjugated vaccine for serogroup C is applied (17). Recently increase of meningococcal serogroups W and Y was reported (18, 19). Serogroup B is determined as leading for meningococcal disease in Thailand and Taiwan (16). *N. meningitidis* serogroups A, C, X, and Y have been identified in Asian countries (16, 20, 21).

Rapid microbiological diagnosis is required to identify the causative agent. At present the fastest and most accurate microbiological diagnosis of meningitis requires application of molecular methods such as real-time polimerase chain reaction (real-time PCR). The aim of our study is to introduce molecular diagnostic and typing methods for *N. meningitidis* for efficient management of patients with meningitis in Bulgaria.

MATERIAL AND METHODS

Written informed consent was obtained from all patients or legal guardian upon hospitalisation by the local hospitals. Additional ethics approval from the NCIPD Research Ethics Committee was not necessary because the received samples were part of the routine reference activities performed by the National Reference Laboratory of Molecular Microbiology.

The criteria for testing CSF samples required patients to comply with the definition of purulent meningitis: leukocytosis (>100 cells/mm^3) and/ or elevated protein content (>100 mg/dl) or reduced glucose (<40 mg/dl). The samples were split in two portions. The first was cultured and from the second one DNA was isolated in order to be tested by real-time PCR.

One hundred and thirty CSF samples from patients suspected of meningitis were tested by real-time PCR for detection and serotyping of *N. meningitidis* from January 2013 to January 2016. Samples were collected from all over Bulgaria.

CSF samples were examined with Gram staining. Culturing was performed on blood and chocolate agar plates. Latex agglutination test was performed with Pastorex Meningitis kit (Bio Rad, USA) (10).

Genomic DNA from CSF was isolated by 5 % Chelex 100 resin (Bio Rad, USA) and proteinase K (Sigma, USA) in concentration 20 mg/ml. 200 µl of the sample were placed in an Eppendorf tube and mixed with 150 µl of 5 % Chelex 100 and 6 µl proteinase K. The tube was incubated on dry thermal block at 56°C for 15 minuts and at 96°C for 15 minutes. Centrifugation for 5 minutes at 14,000 rpm followed. The DNA-containing supernatant was collected in a new tube. Isolated DNAs were stored at -20°C.

Target genes, primer sequences, and FAM fluorescently labeled probes applied for species

identification and serotyping of *N. meningitidis* were used as described (22) and listed in Table 1. The volume of the real-time PCR reaction was 25 µl, containing a mix of the following: 10x PCR Buffer (1X), $MgCl_2$ 4.5 mM, dNTP 0.2 mM, *Taq* DNA Polymerase 0.05 U/µl, (Invitrogen Ltd, USA), primers and probes have different optimised working concentrations as listed in Table 1 and 2. The PCR mix was set up to 20 µl with distilled sterile water and 5 µl of extracted DNA were added to a final volume of 25 µl.

The PCR reaction conditions were as follows: 1 cycle at 95˚C for 10 minutes, 50 cycles: 95˚C for 15 seconds, 60˚C for 60 seconds. For typing of serogroups A, B, C, X, Y, and W135 we applied the same amplification conditions as for analysis of *sodC* and *ctrA* genes. When interpreting the results, if the cycle threshold value (Ct) was Ct ≤35 the sample was considered positive and if Ct >40, the sample was considered negative. If Ct was within the range of 36 to 40 the result was considered equivocal and the sample was retested after dilution of the matrix DNA 1:5 and 1:10 with water, aiming to reduce any inhibitors

Table 1. Primers and probes used for detection of the bacterial meningitis pathogen *N. meningitidis* (22).

Primer or probe name and target	**Real-time PCR primers and probes**	**Working stock (µM)**
ctrA F	5'-TGTGTTCCGCTATACGCCATT	3.75
ctrA R	5'-GCCATATTCACACGATATACC	11.25
ctrA Pr	5'FAM-AACCTTGAGCAA"T"CCATTTATCCTGACGTTCT	1.25
sodC F	5'-GCACACTTAGGTGATTTACCTGCAT	3.75
sodC R	5'-CCACCCGTGTGGATCATAATAGA	7.5
sodC Pr	5'FAM-CATGATGGCACAGCAACAAATCCTGTTT	1.25
RNazeP F	5'-CCA AGT GTG AGG GCT GAA AAG	4
RNazeP R	TGT TGT GGC TGA TGA ACT ATA AAA GG	4
RNazeP Pr	5'FAM- CCC CAG TCT CTG TCA GCA CTC CCT TC	1

Table 2. Primers and probes used for detection of *N. meningitidis* serogroups (22).

Target	**Primer or probe name**	**Real-time PCR primers and probes Nucleotide sequence (5' to 3')**	**Working Stock Conc (µM)**	**Final Conc (nM)**
Nm A	F2531	5'-AAAATTCAATGGGTATATCACGAAGA	3.75	300
sacB	R2624	5'-ATATGGTGCAAGCTGGTTTCAATAG	11.25	900
	Pb2591i	5' FAM CTAAAAG"T"AGGAAGGGCACTTTGTGGCATA AT	1.25	100
Nm B	F737	5'-GCTACCCCATTTCAGATGATTTGT	3.75	300
synD	R882	5'-ACCAGCCGAGGGTTTATTTCTAC	3.75	300
	Pb839i	5' FAM AAGAGATGGGYAACAAC"T"ATGTAATGTCTTT ATTT	1.25	100
Nm C	F478	5'-CCCTGAGTATGCGAAAAAAATT		11.25
synE	R551	5'-TGCTAATCCCGCCTGAATG		3.75
	Pb495i	5' FAM TTTCAATGC"T"AATGAATACCACCGTTTTTTT GC	1.25	100
Nm W135	F857	5'-TATTTATGGAAGGCATGGTGTATG	1.25	100
synG	R964	5'-TTGCCATTCCAGAAATATCACC	11.25	900
	Pb907i	5' FAM AAATATGGAGCGAATGATTACAGTAACTATAAT GAA	2.5	200
Nm X	F173	5'-TGTCCCCAACCGTTTATTGG	11.25	900
xcbB	R237	TGCTGCTATCATAGCCGCC	11.25	900
	Pb196	5' FAM 5'-TGTTTGCCCACATGAATGGCGG	1.25	100
Nm Y	F787	5'-TCCGAGCAGGAAATTTATGAGAATAC	11.25	900
synF	R929	5'-TTGCTAAAATCATTCGCTCCATAT	7.5	600
	Pb1099i	5' FAM TATGGTG"T"ACGATATCCCTATCCTTGCCTAT AAT	1.25	100

that might interfere with the reaction. After dilution of the matrix DNA, if Ct values declined to Ct ≤35 the sample was considered positive. Real-time PCR was perfromed with apparatus LightCycler® 480 Instrument II (Roche Diagnostics GmbH, Germany).

RESULTS AND DISCUSSION

From January 2013 to January 2016 we tested 130 CSF samples. Eleven cases were confirmed in 2013 and in 2014, as well as 9 cases in 2015. Thirty-one samples (23.8%) were positive for *N. meningitidis*. Of the tested samples, 20% (n=26) were with positive microscopy for meningococci, 15.3% (n=20) were culture-positive, 14.6% (n=19) were positive with latex agglutination, and 23.8% (n=31) were *N. meningitidis*-positive by real-time PCR. *N. meningitidis* accounted for 14.8% (n=31/130) of the meningitis cases in Bulgaria. The remaining 99 (75.2%) cases of meningitis were caused by other etiological agents.

The samples were from patients aged from 2 months to 83 years. *N. meningitidis* was confirmed in 42% of the patients over 20 years of age. Vaccination against meningococcal infection is not compulsory in Bulgaria and logically an increasing trend among children should be expected. Among the different age groups 16% (5/31), 13% (4/31), and 16% (5/31) were IMD cases among age groups 1-4, 5-9, and 10-19 years, respectively (Fig. 1). Contrary to our results, in the EU region infants younger than one year of age are at highest risk of meningococcal infection (2).

Figure 1. Number of identified IMD cases by year and age group.

The bacterial load in the CSF samples varies from $3x10^1$ to $4x10^9$ CFU/ml (23, 24). Real-time PCR has proven to detect the presence of meningococcal infection in a range of 8 to 50 meningococcal genomes per reaction (23) and results are obtained within 2.5 to 3 hours. In our study we applied real-time PCR tests by targeting two genes - *ctrA* and *sodC*. By applying 10X dilution series of *N. meningitidis* pure culture we compared the sensitivity of culture versus the DNA extraction protocol used and real-time PCR. Our results confirm that both real-time PCR methods for *ctrA* and *sodC* markers detect less than 20 genomes (data not shown). Unlike culture methods, the PCR technique does not require viable bacteria. Also, real-time PCR is more sensitive than culture.

The marker gene *ctrA* is highly conserved in *N. meningitidis* and acts as a transport protein in the capsule (22). This marker is located within the capsule locus. However, in approximately 16% of meningococci intact *ctrA* gene is missing, thus rendering this marker gene ineffective for identification of strains lacking ctrA marker (25). Bearing in mind the fact that *ctrA* is not present in all isolates (26, 27), it is appropriate to use this gene in combination with the target gene *sodC*. Recently there were reports on false-negative results with *ctrA*-based real-time PCR due to variations in the *ctrA* sequence (28). *sodC* encodes superoxide dismutase which is not genetically linked to the capsule locus and allows identification of *N. meningitidis* without capsule. It is assumed that *sodC* in *N. meningitidis* is obtained via horizontal transfer from *Haemophilus influenzae* (29). This marker gene is carried by all meningococci and there are no reports on meningococci with missing *sodC*. At the same time *sodC* is not found in other *Neisseria* spp. (30, 31). *sodC* analysis is intended for detection of *N.meningitidis* among samples which do not contain *ctrA*. In all invasive *N. meningitidis sodC* is present. Combined use of both markers *ctrA* and *sodC* significantly improves specificity and sensitivity (32).

Detection of *N. meningitidis* with culture is 100 % specific, but the method is limited by low sensitivity (33) and long incubation of 24 to

Table 3. Number of IMD cases and identified serogroups among studied age groups.

Across the EU region, the highest proportion of serogroup C cases among 25-44-year-olds was observed in 2012 (50). Our results demonstrate that in 2015 serogroups B and C had equal proportions among patients over 5 years of age. In that year, we confirmed strains of serogroups W135 and Y for the first time. Recent epidemiological surveillance data for the European region indicate an increase of serogroup Y in some EU countries, although it is less frequent compared to serogroups B and C (2). For a period of three years, trends in serogroup prevalences among different age groups are difficult to discern at national level.

CONCLUSIONS

Meningococcal meningitis requires fast and accurate diagnosis to ensure timely treatment with an appropriately chosen antibiotic agent. In our study we applied real-time PCR technique which proved to be rapid, sensitive, and specific tool. With this method results could be obtained whitin 3 hours after specimen collection. This is important for prompt and correct treatment of bacterial meningitis, especially in children.

N. meningitidis serogroups B and C are major serotypes of invasive meningococcal disease in Bulgaira. In 2015 we confirmed circulation of *N. meningitidis* strains of serogroups W135 and Y.

In conclusion, CSF specimens that do not yeld positive culture should be tested by sensitive molecular methods. Real-time PCR-based assay of *ctrA* and *sodC* marker genes is useful for confirmation of isolates and detection of meningococci in clinical samples, regardless of the encapsulation status.

REFERENCES

1. Rouphael N, Stephens D. *Neisseria meningitidis: biology, microbiology and epidemiology.* Methods Mol Biol. 2012; 799:1-20.
2. European Centre for Disease Prevention and Control. Annual epidemiological report 2014 – Vaccine-preventable diseases – invasive bacterial diseases. Stockholm: ECDC; 2015.
3. Jafri R, Ali A, Messonnier N, Tevi-Benissan C, Durrheim D, Eskola J. *Global epidemiology of invasive meningococcal disease.* Popul Health Metrics. 2013; 11:7.
4. http://ec.europa.eu/health/ph_information/dissemination/echi/docs/meningoccocal_en.pdf.
5. McIntyre Peter B, Katherine L O'Brien, Brian Greenwood, Diederik Van De Beek. *Effect of Vaccines on Bacterial Meningitis Worldwide*. Lancet. 2012; 380:1703-1711.
6. Paireau J, Chen A, Broutin H, Grenfell B, Basta N. *Serial dynamics of bacterial meningitis: a time-series analysis.* Lancet Glob Health. 2016; 4(6):e370-377.
7. Chaudhuri A. *Adjunctive Dexamethasone Treatment in Acute Bacterial Meningitis*. Lancet Neurol. 2004; 3(1):54-62.
8. "Neisseria meningitidis." Microbe Wiki. N.p., n.d. Web. 8 May 2013.
9. Janda WM, Gaydos CA. *Neisseria*. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of clinical microbiology. Washington, D.C.: ASM Press, 2007, 601–620.
10. Popovic T, Ajello G, Facklam R. *Laboratory manual for the diagnosis of meningitis caused by Neisseria meningitidis, Streptococcus pneumoniae and Haemophilus influenzae.* Geneva: World Health Organization. 1999, WHO/CDS/CSR/EDC/99.7
11. Tacon C, Flower O. *Diagnosis and management of bacterial meningitis in the paediatric population: A review*. Emerg Med Int. 2012; ID 320309.
12. Filho E, Horita S, Gilio A, Nigrovic L. *CSF lactate level as a diagnostic biomarker for bacterial meningitis in cildren.* Int J Emerg Med. 2014; 7(1):14.
13. Wang X, Theodore M, Mair R, Trujillo-Lopez E, du Plessis M, Wolter N, Baughman A. *Clinical validation of multiplex RT-PCR assays for detection of bacterial meningitis pathogens*. J Clin Microbiol. 2012; 50(3):702-708.
14. Jones C, Mohamend N, Rojas E, Andrew L, Hoyos J, Howkins J, et al. *Comparison of phenotipic and genotipic approaches to capsule typing of N. meningitidis by use of invasive and carriage isolate collections*. J Clin Microbiol. 2016; 54(1):25-34.
15. Mustapha M, Marsh J, Harrison L. *Global epidemiology of capsular group W meningococcal disease (1970-2015). Multifocal emergence of and persistent of hypervirulent sequence type (ST)-11 clonal complex.*Vaccine. 2016; 34(13):1515-1523.
16. "Pharmainfo.net." A Comprehensive Review on Meningococcal

Meningitis. Web. 07 May 2013.
17. Harrison LH, Trotter CL, Ramsay ME. *Global epidemiology of meningococcal disease.* Vaccine 27(Suppl. 2). 2009; B51–B63.
18. Campbell H, Borrow R, Salisbury D, Miller E. *Meningococcal C conjugate vaccine: the experience in England and Wales*. Vaccine 27(Suppl. 2). 2009; B20–B29.
19. Ladhani SN, Flood JS, Ramsay ME, Campbell H, Gray SJ, Kaczmarski EB, et al. *Invasive meningococcal disease in England and Wales: implications for the introduction of new vaccines*. Vaccine. 2012; 30:3710–3716.
20. Stephens DS, Greenwood B, Brandtzaeg P. *Epidemic meningitis, meningococcaemia, and Neisseria meningitidis*. Lancet. 2007; 369:2196–2210.
21. Kim SA, Kim DW, Dong BQ, Kim JS, Anh DD, Kilgore PE. *An expanded age range for meningococcal meningitis: molecular diagnostic evidence from population-based surveillance in Asia*. BMC Infect Dis. 2012b; 12:310.
22. *Laboratory Methods for the Diagnosis of Men-ingitis caused by Neisseria meningitidis, Streptococcus pneumoniae and Haemophilus influenzae* WHO Manual, 2nd edition. / WHO/IVB.11.09.-2011, 311.
23. Bingen E, Lambert-Zechovsky N, Mariani-Kurkdjian P, Doit C, Aujard Y, et al. *Bacterial counts in cerebrospinal fluid of children with meningitis*. Eur J Clin Microbiol Infect Dis. 1990; 9:278–281.
24. La Scolea LJ Jr, Dryja D. *Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance*. J Clin Microbiol. 1984; 19:187–190.
25. Thomas J, Hatcher C, Satterfield D, Theodore MJ, Bach M, Linscott K, et al. *sodC-based RT-PCR for detection of N. meningitidis*. PlosOne. 2011; 6(5):e19361.
26. Claus H, Maiden MC, Maag R, Frosch M, Vogel U. *Many carried meningococci lack the genes required for capsule synthesis and transport*. Microbiology. 2002; 148:1813–1819.
27. Dolan-Livengood JM, Miller YK, Martin LE, Urwin R, Stephens DS. *Genetic basis for nongroupable Neisseria meningitidis*. J Infect Dis. 2003; 187:1616–1628.
28. Cavrini F, Liguori G, Andreoli A, Sambri V. *Multiple nucleotide substitutions in the Neisseria meningitidis serogroup C ctrA gene cause false-negative detection by real-time PCR*. J Clin Microbiol. 2010; 48:3016–3018.
29. Kroll JS, Wilks KE, Farrant JL, Langford PR. *Natural genetic exchange between Haemophilus and Neisseria: intergeneric transfer of chromosomal genes between major human pathogens.* Proc Natl Acad Sci. 1998; 95:12381–12385.
30. Wilks KE, Dunn KL, Farrant JL, Reddin KM, Gorringe AR, et al. *Periplasmic superoxide dismutase in meningococcal pathogenicity*. Infect Immun. 1998; 66:213–217.
31. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Res. 2009; 37:D26–31.
32. http://ncipd.org/index.php?option=com_docman&view=list&slug=analizi-na-zaraznite-zabolyavaniya&Itemid=1127&lang=bg.
33. Centers for Disease Control and Prevention. *Pediatric bacterial meningitis surveillance - African region, 2002–2008*. Morb Mortal Wkly Rep. 2009; 58:493–497.
34. Chiba N, Murayama SY, Morozumi M, Nakayama E, Okada T, et al. *Rapid detection of eight causative pathogens for the diagnosis of bacterial meningitis by real-time PCR*. J Infect Chemother. 2009; 15:92–98.
35. Zhu BQ, Xu L, Li MC, Ren HY, Tian GZ, et al. *Establishment and application of TaqMan real-time PCR in detection and serogrouping of Neisseria meningitidis*. Zhonghua Liu Xing Bing Xue Za Zhi. 2008; 29:360–364.

DISTRIBUTION OF CHLAMYDIA TRACHOMATIS SEROTYPES BY DNA SEQUENCING OF OMPA GENE IN SOFIA, BULGARIA

I. Philipova[1], I. Ivanov[1], A. Kouzmanov[1], Z. Ivanova[1], V. Levterova[1], I. Simeonovski[1], V. Kantardjiev[2]

[1]National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
2Military Medical Academy, Sofia, Bulgaria

ABSTRACT
In order to detect and characterise *Chlamydia trachomatis* serotypes in patients from Sofia, Bulgaria, 1124endocervicaland urethral swabs, and first-void urine samples were collected for cryptic plasmid PCR analysis.*C. trachomatis* serotypes were identifiedby performing DNA sequencing of the *ompA* gene. The phylogenetic tree was designed by alignment of the obtained sequences with chlamydial reference genotypes D-K sequences available in the GenBank database. The prevalence of *C. trachomatis* was 5.87% (66/1124). DNA sequencing results revealed the following distribution of serotypes: E (32%), G (20%), F (12%), D (11%), K (8%), Ia (7%), J, Ja, and H (4% each).
This is the first study providing information about the distribution of *C. trachomatis* genotypes among patients from Sofia, Bulgaria.
Keywords: ompA gene sequencing analysis Molecular epidemiology, Prevalence, Serotype distribution

INTRODUCTION

Chlamydia trachomatis continues to be the most prevalent bacterial infectious agent causing sexually tranThemitted infections
sution ueutrmaled tant thrpe were DNAJ

70), respectively. First-void urine samples (20 to 40 ml) were collected in sterile screw-cap plastic tubes. Urethral or endocervical specimens were obtained using sterile Dacron swabs. All collected specimens were directly transported to the laboratory and processed within one hour after collection. Diagnostic detection of *C. trachomatis* in the clinical samples was performed using AmpliSens® Chlamydia trachomatis-FRT PCR kit (Federal Budget Institute of Science "Central Research Institute for Epidemiology", Moscow, Russia) (8, 9).

DNA EXTRACTION

Genomic DNA from first-void urine, urethral and endocervical swabs was isolated with the Quick-DNA™ Universal Kit (Zymo Research, United States) according to the manufacturer's instructions. The urine was concentrated by centrifugation for 15 minutes at 2,000 × g prior to extraction. The DNA samples were stored at -79˚C untilanalysis.

OmpA PCR AND DNA SEQUENCING

Amplification of an approximately 1100-bp fragment of ompA was performed byOmpA PCR as previously described (10). The amplification product was visualised after electrophoresis through 1.5% agarose gel containing GelRed™. DNA Molecular Weight Marker was included in each electrophoresis. The obtained ompA fragment was purified as previously described (11). Sufficient amounts of purified DNA (15-30 ng/μl) were mixed with 1 μl of 10 μM concentration of each of the primers (CT1: 5′-TGA ACC GAG CCT TAT GAT CGA CGG A-3′; CT2: 5′-CGG AAT TGT GAA TTT ACG TGA G-3′; CT3: 5′-ACT TTG AAA TCG ACC GTG TTT TG-3′ and CT4: 5′-GAT TGA GCG TAT TGG TTT GAA GC-3′) in separate reaction mixtures and sequenced with the GenomeLab DTCS Quick Start Kit (Sciex, USA). To remove the dye terminators in the cycle sequencing reaction mixtures an improved ethanol purification method was used as described elsewhere (12). The reaction mixtures were loaded onto a GenomeLabGeXP™ Genetic Analysis System (SCIEX, USA). Each amplificated by OmpA PCR DNA fragment was sequenced twice in each direction and this gave an overlap of the whole *ompA*gene.

BLAST ANALYSIS AND ALIGNMENTS

The consensus sequences were compared with known *C. trachomatis* strains using the BLAST search tool at the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). The sequences were assembled into alignments by using the reference genotypes D-K and Chlamydia muridarumMoPn as an outgroup.

PHYLOGENETIC ANALYSIS

Residues corresponding to flanking primers were excluded from analysis. Sequences were manually aligned and adjusted to reference sequences (A/Sa1, B/IU-1226, B/TW-5, C/W3, D/B-120, D/IC-Cal8, E/Bour, F/IC-Cal3, G/UW57, H/UW4, I/UW-12, Ia/IU-4168, J/UW36, Ja-IU-A795, K/UW31, L1/440, L2/434 and L3/404; accession numbers DQ064279, AF063208, FM872308, CP016426, CP002054, X62920, CP015302, CP010569, KM369939, CP006678, DQ116398, HE601809, AF202457, JN795450, AF063204, DQ231368, DQ231369 and DQ231370) as described previously (13). The phylogenetic analysis was produced in Geneious® 7.1.9 and was performed by neighbour-joining method, Jukes-Cantor model, using 100 bootstraps.

RESULTS

In total, 66 out of 1124 samples (5.87%) were positive for *C. trachomatis* in the routine diagnostic PCR test during the three-year period. The gender- and age-dependent distribution of the chlamydial urogenital infection is shown in Fig. 1.

Approximately 1015 bp of the *ompA* gene were amplified and sequence analysis was achieved in 63 (95%) out of 66 *C. trachomatis*-positive cases in the studied population. The most prevalent genotype was serotype E (32%), followed by G (20%), F (12%), D (11%), K (8%), Ia (7%), J, Ja, and H (4% each) (Fig. 2 and 3).

DISCUSSION

In this study we used *ompA* gene-based sequencing of *C. trachomatis* to characterise all detected cases of chlamydia urogenital infections in Sofia, Bulgaria for a period of 3 years. When compared with previous studies in Bulgaria, the overall (both sexes, all age categories) *C.trachomatis* prevalence in our study was relatively lower (14-16). However, it is difficult to interpret whetherthis is a reflection of actual difference in chlamydia prevalence or rather of differences in chlamydia detection methods. The largest proportion of positive cases was among 15–24 age group, accounting for 37% of cases. The second largest group is the age group 25–29 years, accounting for 31% of cases. Therefore young adults aged 15–29 years account for more

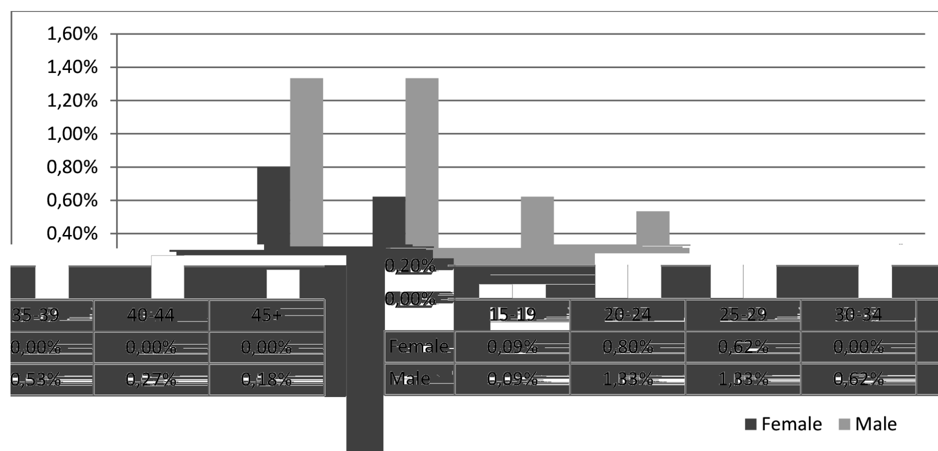

Figure 1. Age- and gender-specific rates of detected chlamydia infections (n=66 cases) in Sofia, Bulgaria, 2014-2016.

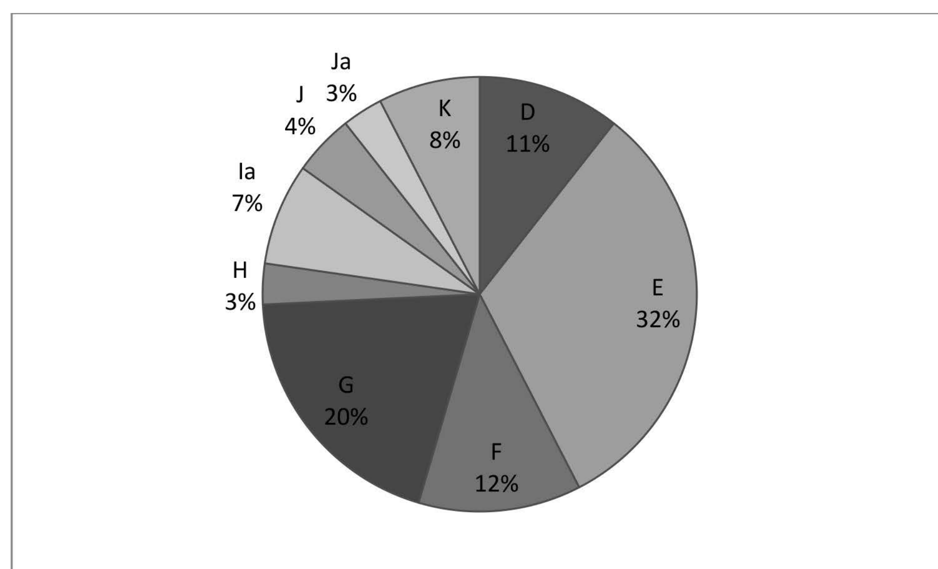

Figure 2. Distribution of *C. trachomatis* serotypes among detected chlamydia infections (n=66 cases) in Sofia, Bulgaria, 2014-2016.

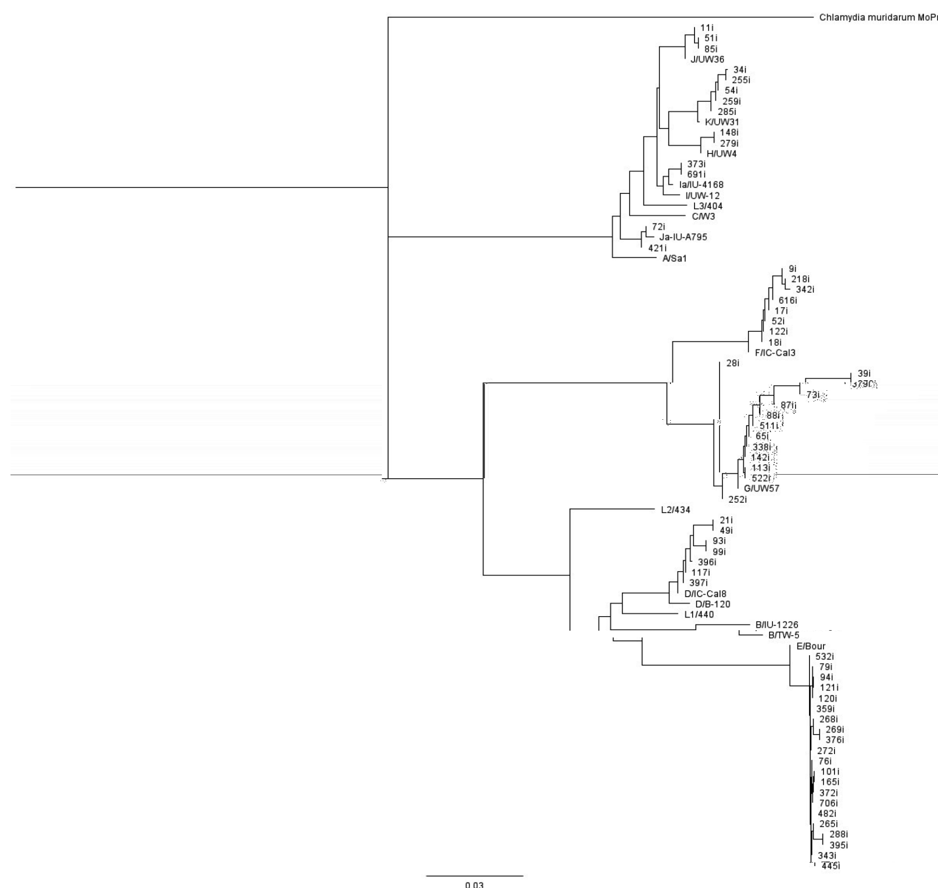

Figure 3. Phylogenetic tree displaying the *C. trachomatis* ompA sequences in relation to reference genotypes A-C, D-K and L1-L3. Chlamydia muridarum MoPn was used as outgroup (accession number M64171).

than two thirds of all cases (69%). The male-to-female ratio was 2.88 with 17 cases detected among women compared to 49 in men. In this study, we also present the serotype distribution of *C. trachomatis* in the studied patients. Sixty-three *C. trachomatis*-positive samples were classified by phylogenetic analysis, demonstrating the presence of nine different serotypes: E (32%), G (20%), F (12%), D (11%), K (8%), Ia (7%), J, Ja, and H (4% each). The serotype distribution is similar to that found in other investigations based on monoclonal antibodies, restriction fragment length polymorphism (RLFP), or gene sequencing (17-20). Similar levels of E and G prevalence were detected in European and American studies (21, 22). Asian population, to the contrary, is mainly F serotypic (23). This incoherence may be caused by different laboratory serotyping methods as well as different *C.trachomatis* serotype distribution in asymptomatic vs. symptomatic population. Three (5%) *C. trachomatis*-positive cases could not be amplified by the ompA PCR, which is comparable with failure rates in similar studies (21). In conclusion, this is the first study based on *ompA* gene sequence analysis showing the distribution of urogenital *C. trachomatis* serotypes in clinical specimens from Sofia, Bulgaria. Relatively low *C. trachomatis* prevalence, mainly restricted to E, G, F, and D serotypes, was highest in patients under 30 years of age. However, the true incidence of chlamydial infections in the Bulgarian population is very likely much higher due to the asymptomatic nature of this disease and incomplete screening coverage. These data also support the need of a more extensive screening for STIs caused by *C. trachomatis* promoting early detection, treatment and prevention.

REFERENCES

1. WHO, *Global incidence and prevalence of selected curable sexually transmitted infections – 2008*. 2012: Geneva, Switzerland.
2. Lagergard T, et al.*Distribution of Chlamydia trachomatis ompA genovars and the new variant of C. trachomatis in the Goteborg area, Sweden.*Eur J Clin Microbiol Infect Dis. 2010; 29(5): 609-611.
3. Ito S, et al.*Male non-gonococcal urethritis: From microbiological etiologies to demographic and clinical features.* Int J Urol. 2016; 23(4):325-331.
4. Price MJ, et al.*The natural history of Chlamydia trachomatis infection in women: a multi-parameter evidence synthesis.* 2016.
5. Gdoura R, et al.*Assessment of Chlamydia trachomatis, Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis, and Mycoplasma genitalium in semen and first void urine specimens of asymptomatic male partners of infertile couples.*J Androl. 2008; 29(2):198-206.
6. Anttila T, et al.*Serotypes of Chlamydia trachomatis and risk for development of cervical squamous cell carcinoma.*Jama. 2001; 285(1): 47-51.
7. Olsen AW, et al.*Protection Against Chlamydia trachomatis Infection and Upper Genital Tract Pathological Changes by Vaccine-Promoted Neutralizing Antibodies Directed to the VD4 of the Major Outer Membrane Protein.* J Infect Dis. 2015; 212(6):978-989.
8. Georgieva E, et al. *PCR detection of Chlamydia trachomatis in genitourinary specimens.* Probl Infect Paras Dis. 2003; 1(31): 12-13.
9. Tchoudomirova K and Kantardjiev T.*Manual for the laboratory diagnosis of infections caused by C. trachomatis (urogenital chlamydial infection)*, in *Etiological diagnosis of sexually transmitted infections - chlamydial and mycoplasma infections, gonorrhoea and syphilis.* National Center of Infectious and Parasitic Diseases: Sofia. 2012, 9-35.
10. Zheng H, et al. *Genotyping of Chlamydia trachomatis by sequence analysis of VS1-VS2 region of omp1 gene.* Chin J Dermatol. 2007; 40:277-280.
11. Rosenthal A, Coutelle O, Craxton M. *Large-scale production of DNA sequencing templates by microtitre format PCR.* Nucleic Acids Res. 1993; 21(1):173-174.
12. Fregel R, Gonzalez A, Cabrera VM.*Improved ethanol precipitation of DNA.*Electrophoresis. 2010; 31(8): 1350-1352.
13. Yuan Y, et al.*Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 Chlamydia trachomatis serovars.* InfectImmun. 1989; 57(4):1040-1049.
14. Tchoudomirova K, Nuhov P, Tchapanova A.*Prevalence, epidemiological and clinical correlates of genital Chlamydia trachomatis infection.*J EurAcadDermatolVenereol. 1998; 11(3):214-220.
15. Ouzounova V.*Prevalence of Chlamydia trachomatis infection in symptomatic patients in Bulgaria.*Journal of IMAB–Annual Proceeding (Scientific Papers). 2004; 10(1):11-14.
16. Kučinskienė V, et al.*Prevalence and risk factors of genital Chlamydia trachomatis infection.* Medicina (Kaunas). 2006; 42(10): 885-894.
17. Lysen M, et al.*Characterization of ompA genotypes by sequence analysis of DNA from all detected cases of Chlamydia trachomatis infections during 1 year of contact tracing in a Swedish County.* J Clin Microbiol. 2004; 42(4):1641-1647.
18. Gao X, et al. *Distribution study of Chlamydia trachomatis serovars among high-risk women in China performed using PCR-restriction fragment length polymorphism genotyping.* J Clin Microbiol. 2007; 45(4):1185-1189.
19. Jurstrand M, et al.*Characterization of Chlamydia trachomatis omp1 genotypes among sexually transmitted disease patients in Sweden.* J Clin Microbiol. 2001; 39(11):3915-3919.
20. Ossewaarde J, et al.*Comparison of two panels of monoclonal antibodies for determination of Chlamydia trachomatis serovars.*J ClinMicrobiol. 1994; 32(12):2968-2974.
21. Cabral, T., A.M. Jolly, and J.L. Wylie, *Chlamydia trachomatis omp1 genotypic diversity and concordance with sexual network data.* J Infect Dis, 2003. 187(2): p. 279-86.
22. Geisler, W.M., et al., *The relationship of serovar to clinical manifestations of urogenital Chlamydia trachomatis infection.* Sex Transm Dis, 2003. 30(2): p. 160-5.
23. Ikehata, M., K. Numazaki, and S. Chiba, *Analysis of Chlamydia trachomatis serovars in endocervical specimens derived from pregnant Japanese women*. FEMS Immunol Med Microbiol, 2000. 27(1): p. 35-41.
24. Bosnjak Z, et al. *Distribution of Chlamydia trachomatis serotypes in clinical urogenital samples from north-eastern Croatia.* Curr Microbiol. 2012; 64(6):552-560.

CHLAMYDIA TRACHOMATIS GENOTYPING BY HIGH-RESOLUTION MELTING ANALYSIS

I. Philipova[1], I. Ivanov[1], A. Kouzmanov[1], Z. Ivanova[1], V. Kantardjiev[2]

1National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
2Military Medical Academy, Sofia, Bulgaria

ABSTRACT
High-resolution melting analysis (HRMA) is an inexpensive, single-step, closed-tube methodusing real-time PCR technology to investigate DNA sequence variation. Recent studies suggest that HRMA could be applied as a cost-effective alternativeto*ompA*DNAsequencing for *C. trachomatis* genotyping. Here we provide the first data on the successful implementation of HRMA for *C. trachomatis* genotypingin Bulgaria directly from clinical specimen.
Keywords: High-resolution melting analysis, *Chlamydia trachomatis* genotyping

INTRODUCTION

High-resolution melting analysis (HRMA) is a relatively new technique, introduced by Wittwer et al. in 2003 (1), which characterises thermal denaturation of a double-stranded DNA (dsDNA) in much more detail and with much higher information yield than the classical melting curve analysis. DNA samples can be discriminated according to their sequence, length, GC content, and strand complementarity in an extent that even a single base change, such as SNP(single nucleotide polymorphism),is readily identified. This technique has been widely used in mutation detection (gene scanning), SNP genotyping, and DNA methylation analysis (2, 3). Over the last five years the number of HRMA microbiological applications has significantly expanded and it has been successfully used in the following areas: species identification of microorganisms (4-6), genotyping within microbial species (7-9), detection of genotypic variants leading to antimicrobial resistance (10-12), and detection of human genetic variants associated with susceptibility to infectious diseases or treatment response(13). High-resolution typing methods for *Chlamydia trachomatis,* such as *ompA* gene sequencing, multilocus sequence typing (MLST) and whole genome sequencing (WGS), are expensive, laborious and time-consuming (14). Here, we have implemented HRMA for the first time in a Bulgarian microbiological laboratory for genotyping of *C. trachomatis* and applied it specifically to the sexually transmitted infection (STI)-related genotypes.

MATERIAL AND METHODS

Strains and clinical *C. trachomatis* samples

To determine the melting temperatures (Tm) and GC content of the STI-related *C. trachomatis* genotypes, eight different DNA samples with previously established genotypes were subjected to HRMA. The DNA samples were selected from *C. trachomatis*-positive cases at the National Reference Laboratory for STIs, Sofia, Bulgaria, and represent eight of the most widespread urogenital genotypes (including genotypes D-K). Another eight clinical urogenital specimens with an unknown genotype from Sofia, Bulgaria, 2016, previously identified by AmpliSens® Chlamydia trachomatis-FRT PCR kit (Federal Budget Institute of Science “Central Research Institute for Epidemiology”, Moscow, Russia), were used as an assay validation panel. For confirmation of the HRMA specificity, a DNA panel of microorganisms commonly isolated from urogenital tract samples was selected as follows: *Gardnerellavaginalis, Lactobacillus* spp*., Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, Candida albicans, Mycoplasma hominis, Ureaplasmaurealyticum, Mycoplasma genitalium, Neisseria flava, Neisseria sicca, Neisseria gonorrhoeae, Trichomonasvaginalis, Toxoplasma gondii*, HSV type 1 and 2, CMV, and HPV. To determine

the Quick-DNA™ Universal Kit (Zymo Research, United States) according to the manufacturer's instructions. To normalise the amount of template DNA for HRMA, all DNA samples were eluted in the same buffer, spectrophotometrically quantified and adjusted to the same concentration with elution buffer.

Design of high-resolution melting analysis experiment

In order to obtain reliable results, HRMA was performed with the use of **a)** a special DNA-binding dye that works at high, saturating concentrations with minimal PCR inhibition and homogeneous staining of dsDNA (EvaGreen®, Biotium); **b)** an instrument with suitable excitation/emission wavelengths, high data acquisition rates, and outstanding temperature homogeneity (LightCycler® 480 Instrument II, Roche, Switzerland); and **c)** a software algorithm that analyses the shape of the melting curves and groups those that are similar (LightCycler 480 Software release 1.5.1.62 SP2). HRMA assay involved an optimised nested PCR followed by a melting curve collected using a fluorescent dye. The outer primers for the nested PCR were selected to amplify two of the variable domains, VS1 and VS2, of the *ompA* gene as described elsewhere (15). The nested primers extended the *ompA* conserved regions flanking the VS2 and produced a 168-bp amplicon(16). The first round of the nested PCR was performed as previously described by the authors (datanot published). The nested real-time PCR for amplification of VS2 and HRMA was performed on the LightCycler® 480 II instrument (Roche, Basel, Switzerland). A half microliter of primary DNA product was added to ٢٥ µL of a reaction mixture containing homemade reaction buffer (with 2,5 mmol/L $MgCl_2$), 0,3 mmol/L of each deoxynucleoside triphosphate, 1,5 U of DNA polymerase, 1,5 X EVA Green, and 750 nmol/L of each primer. Twenty five amplification cycles were performed, each consisting of 95°C for 30 s, 47°C for 40 s, and 72°C for 35 s with a transition rate of 4,4°C/s. A high resolution melting curve was acquired by melting the amplified fragment from to 72°C to 95°C with a ramp rate of 0.02°C/s and 25 acquisitions/°C. The experiment data were analysed by using the LightCycler 480 Software release 1.5.1.62 SP2. The amplified DNA was confirmed by capillary electrophoresis (Qiaxcel, Qiagen). To confirm the sensitivity and specificity relevant analyses were performed.

Genotype validation by ompA DNA sequencing

To confirm the genotype identity of the validation panel determined by HRMA, the *ompA* gene of *C. trachomatis* was sequenced as described elsewhere (15). The amplification products of the eight positive specimens were purified and bidirectionally sequenced with a GenomeLab DTCS Quick Start Kit and GenomeLabGeXP™ Genetic Analysis System (SCIEX) according to the manufacturer's instructions. Genotypes of the *ompA* gene were analysed using Geneious® 7.1.9.

RESULTS

HRMA genotyping

Eight different *C. trachomatis* genotypes (D-K) were amplified by the nested real-time PCR on LightCycler® 480. Amplification plots with a crossing point (Cp) values less than 25 cycles were produced and the amplified DNA was confirmed by agarose gel electrophoresis. Eight melting curves were obtained by melt curve genotyping analysis. Genotypes D, E, F, and G had clearly distinguishable melting peaks and could be differentiated through direct visualisation; H, I, J, and K had somehow similar melting peaks, but correct interpretation could be achievedwith reliable software (Table 1).

Table 1. Sequence analysis, GC content, and melting temperature (Tm) of the eight STI-related *C. trachomatis*genotypes.

Sample No.	Source	*ompA*gene DNA sequencing	GC content (%)	Tm (°C)
396i	Urethra	Genotype D	41.21	82.01
269i	Cervix	Genotype E	41.46	82.25
218i	Urine	Genotype F	45.12	84.47
338i	Urine	Genotype G	46.95	85.34
148i	Vagina	Genotype H	42.51	82.97
373i	Urine	Genotype I	43.71	82.55
072i	Urine	Genotype J	43.11	84.03
255i	Urine	Genotype K	43.71	83.55

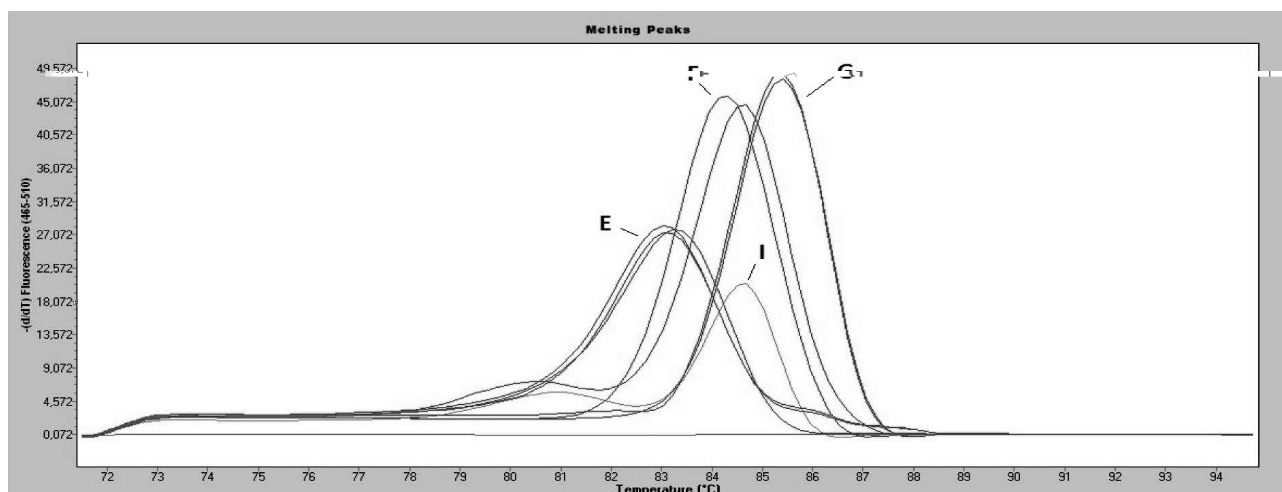

Figure 1. Eight melting curves were obtained by HRMA from *C. trachomatis*-positive cases from Sofia, Bulgaria, 2016. Four unique melting patterns were defined that corresponded to genotypes E, F, G, and I.

All eight *C. trachomatis*-positive samples from the validation panel, that were genotyped by the nested real-time PCR followed by HRMA, were discriminated correctly (Fig. 2) in accordance with the *ompA*sequencing genotyping. The sequencing and HRMA results are presented in Table 2.

Sensitivity and specificity analysis

The analytical specificity of nested real-time PCR was established by amplifying all eight STI-related genotypes of *C. trachomatis* without any cross-reaction with human DNA as well as DNA panel of the most common microorganisms isolated from urogenital tract samples. The performed sensitivity analysis with serially diluted *C. trachomatis* reference strain serotype D showed a detection limit of 500 GE/ml. The dilutions run using the routine diagnostic method also showed a detection limit of 500 GE/ml (data not shown).

Table 2. Clinical specimens genotyped by *ompA*sequencing and HRMA from *C. trachomatis*-positive cases in Sofia, Bulgaria, 2016.

Method					
	F	G	E	I	Total
Whole *ompA*gene DNA sequencing	2	2	3	1	8
HRMA	2	2	3	1	8

DISCUSSION

A number of molecular methods for genotyping *C. trachomatis* have been developed based on the use of restriction fragment length polymorphism (RLFP), *ompA*gene sequencing, multiple-locus variable number of tandem repeats (VNTR) analysis (MLVA), multilocus sequence typing (MLST), and whole genome sequencing (17-21). However, all these methods are expensive and require post-PCR analysis which is laborious, time-consuming, and prone to contamination. With advances in DNA saturating fluorescent dyes, precise instruments and software, HRMA has shown to be a robust, cost-effective, sensitive, and simple closed-tube approach for DNA variation analysis. We have confirmed that at least eight urogenital *C. trachomatis*genotypes can be readily differentiated by HRMA. In the validation panel HRMA successfully genotyped *C. trachomatis*-positive samples detected by AmpliSens® Chlamydia trachomatis-FRT PCR kit and the results were in accordance with the *ompA* sequencing data.

Sensitivity is crucial to any genotyping method in molecular epidemiologic studies especially those applied directly to clinical samples (22-25). The sensitivity of HRMA has been improved by implementing a nested real-time PCR approach as *ompA* is presented in a single genome copy. The detection limit of HRMA method was 500 GE/ml, which is equivalent to the detection limit of AmpliSens® Chlamydia trachomatis-FRT PCR kit.

Probably the main limitation of *C. trachomatis* genotyping by HRMA is that some genotypes have only minor nucleotide variations and could produce very similar melting profiles, which require DNA sequencing for confirmation. Nevertheless, the higher information yield for minimal effort with HRMA could significantly reduce the burden of expensive molecular typing methods such as DNA sequencing.

Unfortunately, genotypes A, B, C, and L1-L3 were unavailable for our study. Although

genotypes A-C (responsible for trachoma and preventable blindness) are rarely isolated from urogenital samples, genotypes L1-L3 are the cause of lymphogranulomavenereum (LGV), a disease with increasing clinical significance (26). Following in-silico analysis of the L1-L3 DNA sequences available in GenBank, it was established that HRMA also has the capacity to discriminate these genotypes. Therefore, further studies are needed to determine whether HRMA can differentiate LGV *C. trachomatis* genotypes.

REFERENCES

1. Wittwer CT, et al.*High-resolution genotyping by amplicon melting analysis using LCGreen.*Clin Chem. 2003; 49(6 Pt 1):853-860.
2. Chambliss AB, et al.*Rapid screening for targeted genetic variants via high-resolution melting curve analysis.*Clinical Chemistry and Laboratory Medicine (CCLM), 2016.
3. Simko I.*High-resolution DNA melting analysis in plant research.* Trends Plant Sci. 2016; 21(6):528-537.
4. Odell ID, et al. *Rapid species identification within the Mycobacterium chelonae-abscessus group by high-resolution melting analysis of hsp65 PCR products.*Am J ClinPathol. 2005; 123(1):96-101.
5. Ozbak H, et al.*Combined molecular gram typing and high-resolution melting analysis for rapid identification of a syndromic panel of bacteria responsible for sepsis-associated bloodstream infection.*J MolDiagn. 2012; 14(2):176-184.
6. Yang S, et al.*Rapid identification of biothreat and other clinically relevant bacterial species by use of universal PCR coupled with high-resolution melting analysis.*J ClinMicrobiol. 2009. 47(7):2252-2255.
7. Merchant-Patel S, et al.*Campylobacter jejuni and Campylobacter coli genotyping by high-resolution melting analysis of a flaA fragment.*Appl Environ Microbiol. 2010; 76(2):493-499.
8.bStephens AJ, et al.*High-Resolution Melting Analysis of the spaRepeat Region of Staphylococcus aureus.*Clin Chem. 2008; 54(2):432-436.
9. Cousins MM, et al.*Use of a high resolution melting (HRM) assay to compare gag, pol, and env diversity in adults with different stages of HIV infection.*PLoS One. 2011; 6(11):e27211.
10. Chen X, et al.*Rapid Detection of Isoniazid, Rifampin, and Ofloxacin Resistance in Mycobacterium tuberculosis Clinical Isolates Using High-Resolution Melting Analysis.*J ClinMicrobiol.2011; 49(10):3450-3457.
11. Tong SYC, et al.*Rapid Detection of the H275Y Oseltamivir Resistance Mutation in Influenza A/H1N1 2009 by Single Base Pair RT-PCR and High-Resolution Melting.*PLOS ONE. 2011; 6(6):e21446.
12. Daniels R, et al.*Rapid, Field-Deployable Method for Genotyping and Discovery of Single-Nucleotide Polymorphisms Associated with Drug Resistance in Plasmodium falciparum.*Antimicrob Agents Chemother. 2012; 56(6):2976-2986.
13. Kovanda A, PoljakM.*Real-time polymerase chain reaction assay based on high-resolution melting analysis for the determination of the rs12979860 polymorphism involved in hepatitis C treatment response.*JVirol Methods. 2011; 175(1):125-128.
14. de Vries HJC, Schim van der LoeffMF, BruistenSM.*High-resolution typing of Chlamydia trachomatis: epidemiological and clinical uses.*CurrOpinInfect Dis. 2015; 28(1):61-71.
15. Zheng H, et al. *Genotyping of Chlamydia trachomatis by sequence analysis of VS1-VS2 region of omp1 gene.* Chin J Dermatol. 2007; 40:277-280.
16. Li J-H, et al. *A high-resolution melting analysis for genotyping urogenital Chlamydia trachomatis.*DiagnMicrobiol Infect Dis. 2010; 68(4):366-374.
17. Frost EH, DeslandesS, Bourgaux-RamoisyD.*Chlamydia trachomatis Serovars in 435 Urogenital Specimens Typed by Restriction Endonuclease Analysis of Amplified DNA.*J Infect Dis. 1993; 168(2):497-501.
18. Bandea CI, et al.*Typing of Chlamydia trachomatis strains from urine samples by amplification and sequencing the major outer membrane protein gene (omp1).* Sex Transm Infect. 2001; 77(6):419-422.
19. Pedersen LN, PodenphantL, MollerJK.*Highly discriminative genotyping of Chlamydia trachomatis using omp1 and a set of variable number tandem repeats.*ClinMicrobiol Infect. 2008; 14(7):644-652.
20. Klint M, et al.*High-resolution genotyping of Chlamydia trachomatis strains by multilocus sequence analysis.*J ClinMicrobiol. 2007; 45(5):1410-1414.
21. Harris SR, et al.*Whole-genome analysis of diverse Chlamydia trachomatis strains identifies phylogenetic relationships masked by current clinical typing.* Nat Genet. 2012; 44(4):413-419.
22. Tchoudomirova K,KantardjievT.*Manual for the laboratory diagnosis of infections caused by C. trachomatis (urogenital chlamydial infection)*, in *Etiological diagnosis of sexually transmitted infections - chlamydial and mycoplasma infections, gonorrhoea and syphilis*. 2012, National Center of Infectious and Parasitic Diseases: Sofia. 9-35.
23. Brankova N, et al.*Development of a modified multi-locus variable number of Tandem repeats analysis for typing of B. pertussis applicable to clinical samples.*Probl Infect Parasit Dis. 2012; 40(2):5-11.
24. Todorova B, et al.*First clinical cases of NDM-1-producing Klebsiellapneumoniae from two hospitals in Bulgaria.*J InfectChemother. 2016; 22(12):837-840.
25. Kardjeva V, et al.*Forty-eight-hour diagnosis of onychomycosis with subtyping of Trichophytonrubrum strains.*J ClinMicrobiol. 2006; 44(4):1419-1427.
26. de Vrieze NH, de VriesHJ.*Lymphogranulomavenereum among men who have sex with men. An epidemiological and clinical review.* Expert Rev Anti Infect Ther. 2014; 12(6):697-704.

DNA SEQUENCING OF ITS REGION FOR RAPID AND ACCURATE IDENTIFICATION OF MEDICALLY IMPORTANT FUNGI

A. Kouzmanov[1], I. Philipova[1], Z. Ivanova[1], I. Ivanov[1], V. Kantardjiev[2]

[1]National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
[2]Military Medical Academy, Sofia, Bulgaria

ABSTRACT

Infections caused by fungi have increased during the last decades mainly due to the increasing population of immunocompromised patients. Therefore, the number of medically important yeast species, not covered by conventional identification kits, and human infections associated with moulds that are difficult to identify with classical methods, also increased. These issues created the necessity for rapid and robust molecular identification techniques. We have recently implemented successfully ITS sequencing at the National Reference Mycology Laboratory in Sofia, Bulgaria as a rapid and accurate method to facilitate conventional identification of medically important fungi.

Keywords: Fungal identification, DNA sequencing, ITS region

INTRODUCTION

In recent decades, advances in medicine have led to increase in the number of infections by pathogenic fungi (1, 2). This is mainly due to expansion of life expectancy of selected patient populations, particularly those with a compromised immune system – transplant recipients, cancer patients, individuals receiving immunosuppressive therapy, etc. (3). Delays in diagnosis are associated with high mortality and severe organ dysfunction, such as respiratory failure (chronic pulmonary aspergillosis), neurologic deficits (cryptococcosis), blindness and visual impairment (fungal keratitis) (4, 5). There is a growing need for a rapid and accurate identification of pathogens for better understanding, control, and treatment of these diseases. Morphological and biochemical identification methods are time-consuming, require well-trained experts and lack sufficient specificity (6). Alternatively, molecular methods such as DNA sequencing of suitable molecular targets offer a powerful and easy tool for rapid identification.

The aim of this study was the evaluation of morphological, biochemical, and molecular methods for the identification of medically important fungi.

MATERIAL AND METHODS

For the purpose of this study a total of twenty-five yeast and mould isolates were selected as a validation panel: seven reference strains from international collections (ATCC – American Type Culture Collection, Manassas, VA, USA; CNM-CL: Yeast collection of the Spanish National Centre of Microbiology; CNM-CM: Spanish National Centre for Microbiology, Filamentous Fungi Culture Collection) and eighteen control strains from INSTAND External Quality Assessment Program 2015-2016.

From January to September 2016, fourteen mould isolates received for confirmation at the National Reference Mycology Laboratory in Sofia, Bulgaria failed to identify to species level with conventional identification methods and consequently they were subjected to ITS sequence analysis. Criteria for referral included clinical relevance which was defined as detection of medically important fungi from primarily sterile specimen sites and from nonsterile specimen sites with positive direct microscopy and/or clinical evidence of infection.

The identification of medically important fungi using conventional identification methods included determination of key characteristics such as growth rate, colony and microscopic morphology (7-9). Biochemical identification of the yeasts was performed by commercially available miniaturised identification systems (API 20C Aux (bioMerieux) and Auxacolor yeast identification system (Bio-Rad Laboratories)) and by automated identification system (VITEK ID-YST card by the VITEK 2 system) (9).

ADDRESS FOR CORRESPONDENCE:
A. Kouzmanov, MD
National Reference Laboratory Mycology and Sexually Transmitted Infections (STIs), NCIPD
26 Yanko Sakazov Blvd
Sofia, Bulgaria
E-mail: akouzmanov@gmail.com
Tel: +359 2 944 6999/319

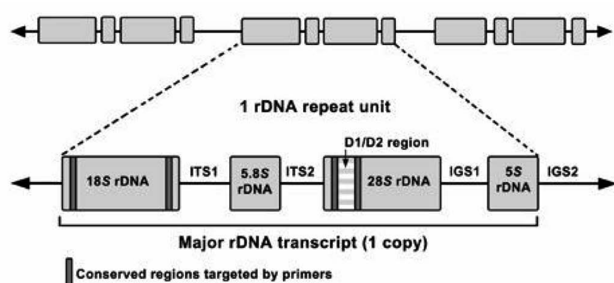

Figure 1. Schematic diagram of the fungal rDNA genes. The 18S, 5.8S and 28S rDNA genes are separated by the two internal transcribed spacers ITS1 and ITS2. The 28S and 5S rDNA genes are separated by the intergenic spacer 1 (IGS1). The intergenic spacer 2 (IGS2) separates the rDNA repeat units from each other (reprinted with permission from http://www.mycology.adelaide.edu.au/).

Molecular identification methods involved isolating and purifying genomic DNA by commercially available yeast DNA purification kit (Epicentre® - an Illumina company), amplification and sequencing of the Internal Transcribed Spacer regions (ITS1-5.8S-ITS2) of the fungal rDNA genes (Fig.1). The sizes of complete ITS sequences varied in different species and were between 285 and 791 bp (10). Data analysis was performed by comparison of the obtained sequences with quality-controlled ISHAM-ITS reference database. The correct ID was assigned at query rate more than 98.8% identity.

RESULTS

Morphological methods for identification allowed the yeasts to be identified to yeast level, the moulds could be identified to genus level, and in case of dermatophytes identification could be carried out to species level. Commercial biochemical systems identified the yeasts to species level but they were inapplicable to moulds and dermatophytes.

DNA sequencing of the ITS region could identify all the pathogenic fungi to species level (Supplement 1).

Eleven (78.5%) of the 14 isolates that have failed species identification using conventional methods, were successfully identified by ITS sequence analysis. Most of these isolates (64.2%) were assigned to species level. Genus level assignment was achieved for 14.3%; inability to differentiate at species level for these isolates was mostly due to high interspecies homology of the genera involved. Two (14.2%) could not be identified by sequencing because matching ITS reference sequences for these were lacking in the database and 1 (7.1%) failed to yield an ITS amplicon (Table 1.)

Table 1. Identification of fungal isolates referred for confirmation to the National Reference Laboratory from January to September 2016.

No. isolate	Specimen	Conventional identification	ITS		
			Identification	ISHAM-ITS ID	Similarity %
1,2,3	Sputum	*Aspergillus sp*	*Aspergillus fumigatus*	MITS3048	100%
4	BAL	*Aspergillus sp*	*Aspergillus niger*	MITS284	99,6%
5	BAL	*Aspergillus sp*	*Aspergillus nidulans*	MITS262	100%
6	Nasal swab	*Aspergillus sp*	*Aspergillus nomius*	MITS289	100%
			Aspergillus flavus	MITS2805	98,8%
7	BAL	*Penicillium sp*	*Penicillium decumbens*	MITS2117	100%
8	Feces	*Penicillium sp*	No taxonomic assignment		
9	Sputum	*Penicillium sp*	*Penicillium spinulosum*	MITS3232	99,1%
10	BAL	*Penicillium sp*	*Penicillium decumbens*	MITS2115	99,1%
11	Skin lesion	*Unidentified*	Unsuccessful amplification		
12	BAL	*Aspergillus sp*	*Aspergillus nomius*	MITS3050	100%
			Aspergillus bombycis	MITS112	98,9%
13	BAL	*Aspergillus sp*	*Aspergillus fumigatus*	MITS179	100%
14	Nasal swab	*Aspergillus sp*	No taxonomic assignment		

Supplement 1. Identification level of the reference (ATCC, CNM-CL and CNM-CM) and control (INSTAND EQA, 2015-2016) fungal strains by morphological, biochemical, and molecular methods.

Fungal culture	Strain	Source	Morphology	API 20C Aux	Auxacolor	VITEK 2	Molecular method
Candida krusei	ATCC 6258		**Yeast level**	Species level	Species level	Species level	Species level
Candida parapsilosis	ATCC 22019		**Yeast level**	Species level	Species level	Species level	Species level
Candida albicans	CNM-CL F8555		**Yeast level**	Species level	Species level	Species level	Species level
Candida krusei	CNM-CL-3403		**Yeast level**	Species level	Species level	Species level	Species level
Aspergillus fumigatus	ATCC 204305		**Genus level**	-	-	-	Species level
Aspergillus flavus	ATCC 204304		**Genus level**	-	-	-	Species level
Aspergillus flavus	CNM-CM-1813		**Genus level**	-	-	-	Species level
Candida kefyr		INSTAND EQA, 2015	**Yeast level**	Species level	Species level	Species level	Species level
Trichosporon inkin		INSTAND EQA, 2015	**Genus level**	Species level	Species level	Species level	Species level
Exophiala dermatitidis		INSTAND EQA, 2015	**Genus level**	-	-	-	Species level
Sarocladium kiliense		INSTAND EQA, 2015	**Genus level**	-	-	-	Species level
Madurella mycetomatis		INSTAND EQA, 2015	**Genus level**	-	-	-	Species level
Arthroderma benhamiae		INSTAND EQA, 2015	Species level	-	-	-	Species level
Microsporum canis		INSTAND EQA, 2015	Species level	-	-	-	Species level
Trichophyton tonsurans		INSTAND EQA, 2015	Species level	-	-	-	Species level
Microsporum gypseum		INSTAND EQA, 2015	Species level	-	-	-	Species level
Aspergillus tamarii		INSTAND EQA, 2016	**Genus level**	-	-	-	Species level
Candida orthopsilosis		INSTAND EQA, 2016	**Yeast level**	**Genus level**	**Genus level**	**Genus level**	Species level
Curvularia lunata		INSTAND EQA, 2016	**Genus level**	-	-	-	Species level
Candida pararugosa		INSTAND EQA, 2016	**Yeast level**	**Genus level**	**Genus level**	**Genus level**	Species level
Candida sinolaborantium		INSTAND EQA, 2016	**Yeast level**	**Genus level**	**Genus level**	**Genus level**	Species level
Trichophyton violaceum		INSTAND EQA, 2016	**Genus level**	-	-	-	Species level
Microsporum gypseum		INSTAND EQA, 2016	Species level	-	-	-	Species level
Scopulariopsis brevicaulis		INSTAND EQA, 2016	**Genus level**	-	-	-	Species level
Candida albicans		INSTAND EQA, 2016	**Yeast level**	Species level	Species level	Species level	Species level

DISCUSSION

ITS sequence analysis proved to be a reliable identification tool. Within the study period, all of the reference and external quality control fungal strains and most of the difficult to identify isolates were successfully determined to species level with ITS region sequencing. Therefore, the identification rate at the Reference Laboratory was clearly enhanced and this represents an excellent complement to the longtime effort to improve the etiological diagnosis and treatment of mycoses in Bulgaria (11-16). Internal Transcribed Spacer regions (ITS1-5.8S-ITS2) were selected as a molecular target because are easily amplified with universal primers that are compatible with most fungal species. ITS have been adopted as the official standard barcoding region for fungi as they have shown largely sufficient genetic variability for identification at interspecies level (17). However, the use of molecular identification of medically important fungi is still limited by incomplete quality-controlled reference databases (10) and the evolving recognition and definition of new fungal species/ complexes (18). Yet, because the ITS regions of some fungal genera (such as *Aspergillus*) are not polymorphic enough, a secondary identification marker is needed to identify a given species with confidence (19). Therefore, we recommend a polyphasic identification approach to pathogenic fungi with the use of combined morphological, biochemical, and molecular methods.

REFERENCES

1. Brown GD, et al. *Hidden Killers: Human Fungal Infections.* Sci Transl Med. 2012; 4(165):165rv13-165rv13.
2. Fisher MC, et al. *Emerging fungal threats to animal, plant and ecosystem health.* Nature. 2012; 484(7393):186-194.
3. Goldman L, et al. *Advancing the Health of Communities and Populations.* Vital Directions for Health and Health Care, 2016.
4. Chen SC-A, et al. *Clinical Manifestations of Cryptococcus gattii Infection: Determinants of Neurological Sequelae and Death.* Clin Infect Dis. 2012; 55(6):789-798.
5. Keay LJ, et al. *Clinical and Microbiological Characteristics of Fungal Keratitis in the United States, 2001–2007: A Multicenter Study.* Ophthalmology. 2011; 118(5):920-926.
6. Kantardjiev T. *Laboratory methods in clinical mycology*, in *Etiologic diagnosis and therapy of mycosis*. NCIPD: Sofia 2012, 70-103.
7. Procop GW. *Medically Important Fungi: A Guide to Identification–5th Edition.* Laboratory Medicine. 2014; 45(2):e68-e69.
8. Boyanova L, Kouzmanov A. *Laboratory diagnosis of invasive fungal diseases.* Probl Infect Parasit Dis. 2012; 40(2):17-19.
9. Boyanova L, Kouzmanov A, Ivanova Z. *Comparative study of laboratory methods for identification of medically important yeasts.* Probl Infect Parasit Dis. 2006; 34(1):25-27.
10. Irinyi L, et al. *International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database—the quality controlled standard tool for routine identification of human and animal pathogenic fungi.* Med Mycol. 2015; 53(4):313-337.
11. Kantardjiev T. *Etiologic diagnosis and therapy of mycosis*. Sofia: NCIPD, 2012.
12. Stavrakieva V, et al. *PCR as a method for diagnosis of Scopulariopsis brevicaulis.* Probl Infect Parasit Dis. 2003; 31(1):14-16.
13. Kardjeva V, et al. *Forty-eight-hour diagnosis of onychomycosis with subtyping of Trichophyton rubrum strains.* J Clin Microbiol. 2006; 44(4):1419-1427.
14. Angelov P, Nashev D, Kantardjiev T. *Whole-genome amplification significantly improves the diagnostics of systemic candidosis.* Probl Infect Parasit Dis. 2009; 37(2):34-36.
15. Viviani MA, et al. *Molecular analysis of 311 Cryptococcus neoformans isolates from a 30-month ECMM survey of cryptococcosis in Europe.* FEMS Yeast Res. 2006; 6(4):614-619.
16. Kantardjiev T, et al. *Molecular taxonomy of Cryptococcus neoformans varieties displaying phenotypic similarities.* Biotechnology and Biotechnological Equipment. 2006; 20(2):101-103.
17. Schoch CL, et al. *Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi.* Proc Natl Acad Sci. 2012; 109(16):6241-6246.
18. Huffnagle GB, Noverr MC. *The emerging world of the fungal microbiome.* Trends Microbiol. 2013; 21(7):334-341.
19. Gautier M, Normand AC, Ranque S. *Previously unknown species of Aspergillus.* Clin Microbiol Infect. 22(8):662-669.

HUMAN LEPTOSPIROSIS IN BULGARIA, 2010-2014

I. Christova, E. Tasseva*

* National Reference Laboratory for Leptospirosis, Department of Microbiology, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

ABSTRACT

The study revealed current trends in incidence, epidemiology, clinical symptoms, circulating Leptospira serogroups, mode of transmission, and source of infection in laboratory confirmed human leptospirosis cases in Bulgaria between 2010 and 2014. Serogroup Icterohaemorrhagiae had the leading role in aetiological structure in 2010-2013 and serogroup Sejroe was the most common in 2014. Following heavy rainfall and floods in 2014, 5-time increase in leptospirosis cases was observed in comparison with 2013. Water was found as the main source of infection. Leptospirosis in Bulgaria, located in temperate climate region of southeastern Europe, is endemic with low incidence but significant case fatality rate. General understanding of specific relations between aetiology, epidemiology and clinical manifestations of leptospirosis would help in prevention and management of the disease.

Key words: Leptospira serogroup, leptospirosis, epidemiology, clinical manifestation.

INTRODUCTION

Leptospirosis is a worldwide distributed zoonotic disease that affects humans. Human leptospirosis is identified as an emerging infectious disease in many countries (1). Infection is endemic and occurs more frequently in warm-climate countries than in temperate regions because of longer survival of *Leptospira* spirochetes in warm humid environment.

Various species of wild and domestic animals serve as maintenance hosts. Humans are accidental hosts of the infection. While in maintenance hosts infection is spread by direct contact, humans can be infected by direct contact with urine or tissues of infected animal or indirectly with contaminated water (2). Clinical symptoms vary from mild to severe and two clinical forms can be distinguished: icteric (Weil's disease) and milder anicteric form. Icteric leptospirosis is a severe febrile illness often associated with multiple organ failure and fatality rate is up to 30%; anicteric leptospirosis usually presents as unspecific febrile disease with myalgia and abdominal pain (2).

Human cases of leptospirosis in Bulgaria have been reported since 1952 (3). Analysis of all confirmed cases for 13-year period, 1989-2001, revealed overall incidence of 0.42/100.000 population and overall case fatality rate of 6.6% (3). Mode of transmission was more often recreational exposure (mostly fishing) than occupational exposure (mostly livestock farming). In the late 1990s, serogroup *Pomona* lost its leading role in aetiology of human leptospirosis in Bulgaria, most probably due to veterinary control measures, while serogroup Icterohaemorrhagiae increased its proportion, causing 56% of the reported cases (3).

The present study was intended to describe current trends in incidence, epidemiology, clinical symptoms, circulating *Leptospira* serogroups, mode of transmission, and source of infection in laboratory confirmed human leptospirosis cases in Bulgaria between 2010 and 2014. In addition, association of flooding with emergence of leptospirosis and with infecting serogroups was analyzed.

METHODS

Epidemiological Data

This study was based on all reported laboratory confirmed cases of human leptospirosis in Bulgaria for 5 years, from 2010 to 2014. Confirmed case of leptospirosis was a patient with clinical evidence of leptospirosis and laboratory confirmation of infection. All cases were laboratory confirmed in the National reference laboratory for leptospirosis, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.

Patients with confirmed leptospirosis were analyzed using questionnaires that included data such as age, sex, location, month of onset, data from epidemiological investigation concerning possible source of infection and mode of transmission as well as symptoms of clinical manifestation of the disease evaluated by infectious diseases specialists.

Microscopic agglutination test

Microscopic agglutination test (MAT) was used for laboratory confirmation of the disease and for identification of the infecting *Leptospira* serovar

ADDRESS FOR CORRESPONDENCE:
Dr. Iva Christova,
National Center of Infectious and Parasitic Diseases,
26 Yanko Sakazov blvd.,
Sofia 1504,
Bulgaria; tel. +3592 8465042,
fax: +3592 9433075,
e-mail: iva_christova@yahoo.com

as a reference method for serological diagnosis. Suspensions of live serovars from 9 different *Leptospira* serogroups known to circulate in Bulgaria were used as antigens: serogroup Australis (serovar Bratislava Jez Bratislava); Autumnalis (serovar Nikolaevo); Ballum (serovar Ballum); Bataviae (serovar Bataviae van Tienen); Canicola (serovar Canicola); Icterohaemorrhegiae (serovar Copenhageni); Pomona (serovar Pomona); Sejroe (serovar Sejroe); Tarassovi (serovar Tarassovi Perepelicin) and Semaranga (serovar Patoc). Serum samples were tested first at 1:100 dilution and positive sera were further examined for reactivity to the detected *Leptospira* serovar at 2-fold dilutions starting with 1:100. The end point titer was the highest dilution of serum at which 50% agglutination occurred. In the current Bulgarian case definition, a titer ³ 400 is used for laboratory confirmation. All patients had titers of agglutinins above 400, in some cases as higher as above 12,800.

RESULTS

Occurrence of leptospirosis in Bulgaria

A total of 68 laboratory confirmed leptospirosis cases were registered in Bulgaria from 2010 to 2014 (12 cases in 2010, 10 in 2011, 10 in 2012, 6 in 2013 and 30 in 2014). Average annual incidence of the disease was 0.19/100,000 population (range 0.08-0.42/100,000).

Analysis of case distribution according to sex showed high predominance of male patients (83%) over female (17%). The most affected age groups were 40-49 years (24.6%), 50-59 years (23.1%) and 60-69 years (24.6%), and then 30-39 years (13.8%).

An overall case fatality rate was 8.8 % (6 patients). Aetiological agent in all fatal cases was serogroup Icterohaemorrhagiae and acute renal failure was proposed as the cause of death.

Leptospira serogroups in aetiology

Serovars belonging to 7 different serogroups caused infection during the 5-year study period (Table.1). Serogroup Icterohaemorrhagiae had the leading role in aetiological structure in 2010-2013. However, in 2014 Sejroe was the most commonly detected serogroup.

Overall proportion of serogroup Icterohaemorrhagiae was 33.8% (23/68 cases) and overall proportion of serogroups Sejroe and Pomona was 25% (17/68 cases caused by each serogroup) for the whole study period (Table 2). Serogroups Tarassovi and Australis were less common, causing a total of 5 and 3 cases resp. (incidence 7.4% and 4.4% resp.). In fact, all cases caused by serogroup Tarassovi were detected in 2014. Serogroups Autumnalis and Bataviae were extremely rare (2/68 and 1/68), giving proportions of 2.9% and 1.5% resp.

Monthly distribution of leptospirosis cases showed that most of the cases appeared in summer and fall with a peak incidence in August-September-October (Table 2). However, leptospirosis patients were detected throughout the year. Serogroup Icterohaemorrhagiae was found from May to December, serogroup Pomona – from January to October and serogroup Sejroe – throughout the year. Serogroup Tarassovi was recorded mostly in August (4 of 5 cases) and only in 2014 after the heavy floods in Central Bulgaria.

Analysis of the rainfall and number of leptospirosis cases showed correlation in 4 flooded districts (Table 3). In the district of Vratsa, only one leptospirosis case was registered in 2010 and none in 2011-2013. However, following excessive rainfall in September 2014, two cases appeared in September and October 2014. In the district of Gabrovo, there were no leptospirosis cases in 2010-2013, but in 2014 six cases were registered. Excessive flooding in September led to two cases – one in September and one in October. The rainfall caused repeatedly flooding in 2014 and leptospirosis cases appeared for the first time in the district. In the district of Lovech, excessive rainfalls in July and September 2014 flooded large areas and led to manifestation

Table 1. Proportions of Leptospira serogroups in aetiological structure of human leptospirosis in Bulgaria, 2010-2014

Year	Leptospira serogroups number of cases/total cases per year (%)						
	Icterohaemorrhagiae	**Pomona**	**Sejroe**	**Tarassovi**	**Autumnalis**	**Australis**	**Bataviae**
2010	**5/12 (42%)**		**5/12 (42%)**			**2/12 (17%)**	
2011	**4/10 (40%)**	**3/10 (30%)**	**1/10 (10%)**		**1/10 (10%)**		**1/10 (10%)**
2012	**6/10 (60%)**	**3/10 (30%)**	**1/10 (10%)**				
2013	**3/6 (50%)**	**3/6 (50%)**					
2014	**4/30 (13%)**	**8/30 (27%)**	**11/30 (37%)**	**5/30 (17%)**	**1/30 (3%)**	**1/30 (3%)**	

Table 2. Distribution of Leptospira serogroups causing human infections in Bulgaria by months, 2010-2014

Leptospira serogroups (number of cases)	January	February	March	April	May	June	July	August	September	October	November	December	Total
Sejroe	1	1				2	4	2	3	3		1	**17**
Icterohaemorrhagiae					2	2	2	6	5	4	1	1	**23**
Pomona	1			1			2	2	5	6			**17**
Tarassovi								4		1			**5**
Autumnalis										1			**1**
Bataviae					1					1			**2**
Australis						1					1	1	**3**
Total	**2**	**1**		**1**	**3**	**5**	**8**	**14**	**13**	**16**	**2**	**3**	**68**

of four leptospirosis cases in August-October. Finally, higher rainfall in June 2014 in the district of Dobrich was followed by clinical manifestation of leptospirosis in two patients, while none cases appeared in the previous years (Table 3).

Epidemiological data

Information on possible mode of transmission and source of infection was available for 56 (82.4%) of the patients with leptospirosis.

Analysis of the data about possible mode of transmission showed that infection was acquired 1) mainly through occupational exposure: 24 (42.9%) cases, mostly livestock farming – 15 (26.8%), slaughtering, agriculture, and working in forests – a total of 6 (10.7%); 2) less frequently by recreational exposure: 17 (30.4%) patients, mostly fishing – 9 (16.1%) and bathing – 5 (8.9%); and 3) accidentally, related to flooding - 10 (17.9%) (Table 4).

Analysis of possible source of infection revealed that water was the main risk factor to contract leptospirosis during 2010-2014 – 20/56 (35.7%) cases (Table 4). Thirteen (23.2%) patients were infected through direct or indirect contact with rodents. Overall, flood mud was the third common source of infection – for 9 (16.1%) cases, all of them in 2014 (9/30 – 30% of the cases in 2014). Contact with domestic animals (cattle and pigs) caused infection in 10 (17.9%) patients.

Twenty (35.7%) of the patients were exposed to several risk factors – livestock farming, presence of rodents in houses and/or working places, consumption of food contaminated with rodent excreta, etc.

Clinical data

Clinical manifestation data were available for all 56 patients with analyzed epidemiological data. The most commonly observed symptoms were: fever (n=50, 89.3% patients), diffuse myalgia (n=42, 75.0%), oliguria (n=39, 69.6%), jaundice (n=38, 67.9%), nausea (n=37, 66.1%), hepatomegaly (n=35, 62.5%), and headache (n=34, 60.7%). The disease was often presented by abdominal pain, arthralgia, vomiting, conjunctival suffusion, diarrhea, and splenomegaly. Rarely, the disease manifested as meningitis, rash, and pneumonia (Fig. 1). Anicteric cases (n=18, 32.1%) had milder flu-like course, while all icteric cases manifested as severe diseases, meaning that clinical jaundice is an important predictive marker. Acute renal failure as complication of the disease appeared in 6 (10.7%) patients, all of them with jaundice, and was the main cause of death in lethal cases.

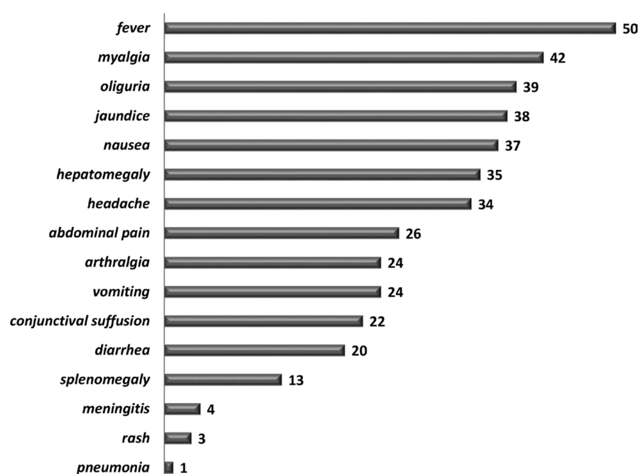

Fig. 1. Clinical manifestations of leptospirosis in Bulgaria, 2010-2014

DISCUSSION

Human leptospirosis is officially notified infection in Bulgaria. In this study, all clinically and laboratory confirmed leptospirosis cases in Bulgaria for 5 years were analyzed.

Between 2010 and 2014, an average of 13

Table 3. Rainfall (L/m²) and number of leptospirosis cases in the 4 flooded districts in 2014

District		Month					Total
	Rainfall / Leptospira cases	June	July	August	September	October	
	Rainfall 2010						
	2011	87	60	32	24	105	308
	2012	62	129	16	4	53	264
	2013	40	12	33	14	29	128
		113	43	13	20	61	250
	Rainfall 2014						
Vratsa		74	114	99	189	53	**529**
	No. cases						
	2010	-	-	1	-	-	1
	2011	-	-	-	-	-	0
	2012	-	-	-	-	-	0
	2013	-	-	-	-	-	0
	No. cases 2014	1	-	-	1	1	**3**
	Rainfall 2010						
	2011	132	121	26	37	77	393
	2012	59	77	99	3	129	367
	2013	67	16	39	28	51	201
		142	83	20	18	81	344
	Rainfall 2014						
Gabrovo		119	123	47	191	148	**628**
	No. cases						
	2010	-	-	-	-	-	0
	2011	-	-	-	-	-	0
	2012	-	-	-	-	-	0
	2013	-	-	-	-	-	0
	No. cases 2014	1	2	1	1	1	**6**
	Rainfall 2010						
	2011	136	128	22	42	96	424
	2012	79	104	80	5	94	362
	2013	39	23	42	27	46	177
		193	91	22	22	80	408
Lovech	Rainfall 2014	79	151	59	215	94	**598**
	No. cases						
	2010	-	-	-	-	-	0
	2011	-	-	-	-	-	0
	2012	-	-	-	-	-	0
	2013	-	-	1	-	-	1
	No. cases 2014	-	-	1	2	1	**4**
	Rainfall 2010						
	2011	78	142	3	32	57	312
	2012	29	55	30	9	158	281
	2013	21	20	11	13	40	105
Dobrich	Rainfall 2014	213	57	35	57	88	**450**
	No. cases						
	2010	-	-	-	-	-	0
	2011	-	-	-	-	-	0
	2012	-	-	-	-	-	0
	2013	-	-	-	-	-	0
	No. cases 2014	-	2	-	-	-	**2**

Table 4. Probable mode of transmission and source of infection in human leptospirosis cases in Bulgaria, 2010-2014.

Mode of transmission			**Source of infection**		
Activities	**Frequency**	**%**	**Contact with**	**Frequency**	**%**
Occupational exposure	24	42.9%			
			Water	20	35.7%
Livestock farming	15	26.8%	Rodents	13	23.2%
Agriculture	2	3.6%	Flood mud	9	16.1%
Slaughtering	2	3.6%	Livestock	6	10.7%
Forest worker	2	3.6%	Pigs	4	7.1%
Rice cultivation	1	1.8%	Unknown	4	7.1%
Mine worker					
RodEents in workplacesm=	Rode	e			

confirmed leptospirosis cases were reported per year and an overall incidence was 0.19/100,000 population. Comparing with the previous years (3), incidence gradually declined (53 reported cases in 1999 and only 6 cases in 2013) from the maximum level of 0.64/100,000 in 1999 to the minimum level of 0.08/100,000 in 2013. In 2014 however, 5-times increase in number of leptospirosis cases was observed (total 30 cases, incidence 0.42/100,000) in comparison with 2013. An overall case fatality rate for the whole 5-year period was 8.8 %. Marked increase in leptospirosis infections in 2014 was reported from Netherlands (4) and was attributed to the warmer weather. Germany also reported increased incidence of leptospirosis cases in 2014 and attributed it to a warm and humid climate (5). In Bulgaria, we also detected similar peak in human leptospirosis cases in 2014, which in our case correlated well with heavy rainfall and floods in central Bulgaria.

All 68 registered confirmed leptospirosis cases analyzed in the study contracted disease in Bulgaria with two exceptions of imported leptospirosis from Greece - a rice worker and a cowherd. A recent publication describes situation with leptospirosis in Greece (6). In accordance with previous investigations in Bulgaria as well as with numerous studies from Europe and Asia (7-11), our study showed that the disease affected mainly men of working age. Despite described more severe course of leptospirosis in men (12), this could hardly be the only reason for the much higher incidence of the disease observed in men than in women. More factors are likely included, such as more activities of Bulgarian men in fishing, farming, etc., which are at risk for contracting the disease. A report from Mexico, however, showed no statistical difference between male and female cases but higher mortality in male cases (13).

Most leptospirosis cases in Bulgaria for the last 5 years occurred in late summer and early autumn as has been reported also from Europe, Japan and Korea (9,11). Obviously, appropriate combination of optimal temperature and rainfall at this time supports efficient survival of leptospirae in the environment. However, in Bulagria leptospirosis cases could occur throughout year. Our analysis showed that in the years before (3) and after 2010, the main source of infection continued to be water. Fishing and bathing in lakes with stale water accounted for nearly one third of the infections. Before 1990, the disease was relatively common in rice workers and miners (3). In the following years, along with radical change of economic sector in Bulgaria, these professions faded and now occupationally most exposed people are livestock farmers, followed by slaughter workers, forest workers and those engaged with agriculture. Notably, occupational risk was higher in breeding cattle farmers than in breeding pig farmers.

Overall, the most common mode of transmission now is by occupational exposure like in countries on various continents such as Denmark, the Netherlands, Mexico, Japan, Korea (4,10,11,13). Now through recreational activities (fishing and

bathing) is less likely to contract leptospirosis in Bulgaria than is the occupational risk. Although the mode of transmission of leptospirosis for fishermen in Bulgaria and fish farmers in Denmark (10) is different, the source of infection is similar.

Contact with rodents, mainly rats, was another common source of infection as concluded from the results of our study. Patients reported having rodents in their farms and country houses. Generally, leptospirosis was contracted more frequently through indirect contact with animal excretions (via contaminated water, soil, or food) and less commonly through direct contact with animal urine or tissues.

2014 was a year with severe floods in Bulgaria, mainly in central Bulgaria (districts of Vratsa, Gabrovo, Lovech and Dobrich). Wading in flood mud was the only risk factor for 9 of the 15 cases from these districts in 2014. For comparison, a total of only 2 cases were detected in these districts for the previous 4 years.

Furthermore, analysis of the clinical signs and symptoms of registered leptospirosis cases in Bulgaria 2010-2014 showed prevalence of the severe Weil's disease over flu-like forms of the disease. The most likely explanation is that the majority of milder forms were not clinically recognized. The severe form of leptospirosis was usually accompanied by jaundice and oliguria. In about one third of the patients acute renal failure was established. As in the previous years, acute renal failure was the main cause of death in fatal cases (3). Even rare, some manifestations of leptospirosis are important and should not be understated - meningitis, pneumonia, endocarditis.

Remarkable changes in aetiological structure of leptospirosis in Bulgaria before and after 1989 were established. Before 1989, serogroup Pomona was the main cause of leptospirosis, causing more than half of all leptospirosis cases and serogroup Icterohaemorrhagiae caused nearly one fifth of the cases. In the years preceding our study, serogroup Pomona caused about one fifth of the cases, while serogroup Icterohaemorrhagiae caused more than half of the infections (3). This tendency continued in the coming years until 2013. In 2014, the most prevalent serogroup in the aetiological structure was Sejroe. For the whole 5-year study period, serogroups Icterohaemorrhagiae and Sejroe together caused nearly 60% of the infections. Full explanation of these changes is difficult. Pig and cattle vaccination is a plausible explanation of the decreasing role of serogroup Pomona. However, increased incidence of serogroup Icterohaemorrhagiae revealed inadequate measures for rodent control. Infections caused by serogroup Sejroe, extremely rare in the years preceding this study, became in the last 5 years the second most common leptospirosis after infections caused by serogroup Icterohaemorrhagiae. Furthermore, most of the cases caused by serogroup Sejroe and all infections caused by serogroup Tarassovi appeared in 2014, the year with unusual heavy floods. Icterohaemorrhagiae is the dominant serogroup across Europe - in France, Denmark, Ireland, the Netherlands (7,10,14,15).

Leptospirosis in Bulgaria, located in temperate climate region of southeastern Europe, is endemic with low incidence but significant fatality rate. Higher rainfall and flooding most probably increased the risk of infection with *Leptospira* serogroups Sejroe and Tarassovi. Since water appeared as the main source of infection throughout the study and not only after flooding, recommendations to avoid stale water and use personal protective clothing are of great importance. In addition, more efficient measures for rodent control are needed since contact with rodents was also shown to be an important risk factor. General understanding of specific relations between aetiology, epidemiology and clinical manifestations of leptospirosis would help in prevention and management of the disease.

REFERENCES

1. Pappas G, Papadimitriou P, Siozopoulou V, Christou L, Akritidis N. The globalization of leptospirosis: worldwide incidence trends. Int J Infect Dis. 2008;12:351-7. 10.1016/j.ijid.2007.09.011
2. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001;14:296-326. PMID: 11292640
3. Christova I, Tasseva E, Manev H. Human leptospirosis in Bulgaria, 1989-2001: epidemiological, clinical, and serological features. Scand J Infect Dis. 2003;35:869-72. PMID: 14723364
4. Pijnacker R, Goris MG, Te Wierik MJ, Broens EM, van der Giessen JW, de Rosa M, et al. Marked increase in leptospirosis infections in humans and dogs in the Netherlands, 2014. Euro Surveill. 2016 Apr 28;21(17). doi: 10.2807/1560-7917.ES.2016.21.17.30211.
5. Bundes Institut für Risikobewertung (BfR). Robert Koch-Institut. Leptospirose - eine seltene, aber immer häufiger auftretende Erkrankung [Leptospirosis - a rare but increasingly prevalent disease]. Berlin: BfR; 2014. German. Available from: http://www.bfr.bund.de/cm/343/leptospirose-eine-seltene-aber-immer-haeufiger-auftretende-erkrankung.pdf.
6. Papa A, Kotrotsiou T. Leptospirosis in Greece. Acta Trop. 2015;149:135-7. doi: 10.1016/j.actatropica.2015.05.025
7. Goris MG, Boer KR, Duarte TA, Kliffen SJ, Hartskeerl RA. Human leptospirosis trends, the Netherlands, 1925-2008. Emerg Infect Dis. 2013;19:371-8. Available on: http://dx.doi.org/10.3201/eid1903.111260
8. Vieira ML, Gama-Simoes MJ, Collares-Pereira M. Human leptospirosis in Portugal: A retrospective study of eighteen years. Int J Infect Dis. 2006;10: 378-86. DOI: 10.1016/j.ijid.2005.07.006
9. Ciceroni L, Stepan E, Pinto A, et al. Epidemiological trend of human leptospirosis in Italy between 1994 and 1996. Eur J Epidemiol. 2000;16:79-86.

10. Van Alphen LB, Lemcke Kunoe A, Ceper T, Kahler J, Kjelso C, Ethelberg S, et al. Trends in human leptospirosis in Denmark, 1980 to 2012. Euro Surveill. 2015;20(4):21019. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=21019.
11. Lee MJ, Miki S, Kitagawa M, Lee WC. Comparative study on the epidemiology of human leptospirosis in Korea and Japan, 2006-2012. Jpn J Infect Dis. 2016;69:259-61. Available online: http://doi.org/10.7883/yoken.JJID.2014.589.
12. Jansen A, Stark K, Schneider T, Shöneberg I. Sex differences in clinical leptospirosis in Germany: 1997-2005. Clin Infect Dis. 2007;44:e69-72. Available on: http://dx.doi.org/10.1086/513431.
13. Sánchez-Montes S, Espinosa-Martínez DV, Ríos-Muñoz CA, Berzunza-Cruz M, Becker I. Leptospirosis in Mexico: epidemiology and potential distribution of human cases. PLoS One. 2015; 10(7):e0133720. doi: 10.1371/journal.pone.0133720.
14. Baranton G, Postic D. Trends in leptospirosis epidemiology in France. Sixty-six years of passive serological surveillance from 1920 to 2003. Int J Infect Dis. 2006;10:162-70. Available on: http://dx.doi.org/10.1016/j.ijid.2005.02.010
15. Garvey P, Connell J, O'Flanagan D, McKeown P. Leptospirosis in Ireland: annual incidence and exposures associated with infection. Epidemiol Infect. 2014;142:847-55. http://dx.doi.org/10.1017/S0950268813001775

CHOLESTASIS SYNDROME IN HEPATITIS E – TWO-YEAR PERIOD ANALYSIS (2014-2015)

M. Pishmisheva[1], P. Teoharov[2], E. Naseva[3], N. Vatev[4],M. Stoycheva[5], M. Karamisheva[1], D. Velkova[1], I. Ivanov[1]

[1]Regional Hospital – Pazardzhik, Department of Infectious Diseases
[2]National Centre of Infectious and Parasitic Diseases - National Reference Laboratory for viral hepatitis, Sofia
[3]Medical University – Sofia, Department of Economy of Public Health, Faculty of Public Health
[4]Medical University – Plovdiv, Department ofEpidemiology of Infectious Diseases, Faculty of Public Health
[5]Medical University – Plovdiv, Department of Infectious Diseases, Parasitology and Tropical Medicine

ABSTRACT:
Introduction: Hepatitis E is a viraldiseasewit htheclinicalandmorphologicalcharacteristics of acutehepatitis.In endemic areas the disease is a major health problem. The increase in the number of patients with indigenous hepatitis in developed countries also becomes an issue. Hepatitis E clinically manifests most often with ictericform, and subjective complaints do not differ from those of hepatitis caused by other hepatotropic viruses. Caroli et al. report for the first time cholestatic form of acute viral hepatitis in 1942. Cholestasis syndrome is observed in all types of viral hepatitis. According to Caroli, 80% of cases of intrahepatic cholestasis are associated with viral hepatitis B and C, and only 20% - withotheretiologicalfactors.HBV, HCV, CMV, EBV can cause serious cholestasis in immunocompromised patients, as well as transplant patients. They can develop severe infection known as fibrosing cholestatic hepatitis, which can progress to liver failure if not treated.Unlike the above mentioned viruses, enterally transmitted hepatotropic viruses HAV and HEV can cause significant cholestasis in immunocompetent individuals.

ADDRESS FOR CORRESPONDENCE:
Dr. M. Pishmisheva,
Multi-profile hospital for active treatment
Department of Infectious Diseases,
15 “Bolnichna” str.
Pazardjik 4400
Bulgaria; tel. +3593 4408600, fax: +3593 4408766
e-mail: pishmishevampeleva@abv.bg

The **aim** of thispaperis to analyse the clinical manifestations in patients with hepatitis E and cholestasis. **Material and methods:**Ninety patients diagnosed with acute hepatitis E were treated at the Infectious Diseases ward of the Regional Hospital - Pazardzhik during the period 1 January 2007 – 30 June 2016, with the majority (83) being hospitalised between 1 January 2014 – 30 June 2016. Patients were subjected to clinical monitoring and observing the objective condition,combined with paraclinical and instrumental methods of examination. The diagnosis was confirmed at the NRL for hepatitisviruses, National Inspectorate - Sofia, withdetection of anti-HEV IgM andanti-HEV IgG antibodies with ELISA methodology. **Results:** Out of 90 patients with hepatitis E, 25 (27.77%) were with signs of cholestasis. Patients with cholestasis were aged between 40-76 years (average 58.04). Gender distribution was 20 (80%) male patients and 5 (20%) female patients. The average age of men was 58.75 years, ranging from 40 to 76, and for women - 55.2, with a range from 47 to 60 years. Of all patients with cholestasis, 23 (92%) had comorbidities. Subjective complaints of patients with hepatitis E (with or without cholestasis) were not different from those of patients with hepatitis caused by other hepatotropic viruses. We observed the following symptoms: astheno-adynamia - in 17 (68%), anorexia - in 13 (52%); pruritus - in 17 (68%), dyspeptic symptoms - in 7 (28%) patients. The most common symptoms were astheno-adynamia and itching. Patients with cholestatic form of hepatitis E had longer hospital stay compared to patients with other forms, with an average length of stay of 20.8 days. In addition to the long hospitalisation period, recovery period was longer. **Conclusion:** As with other types of viral hepatitis, cholestasis is commonly observed in hepatitis E. Comorbidities (e.g. diabetesmellitus, alcohol, etc.) are likely to contribute to the development of cholestasis. Hepatitis E with cholestasis syndrome occurred with significant deviations in the biochemical profile that persisted longer than usual and the recovery period is prolonged. Development of cholestasis is associated with prolonged hospitalisation and treatment with greater financial cost.

Key words: hepatitis E, cholestasis syndrome, direct bilirubin, secretory enzymes

INTRODUCTION

Hepatitis E is a viral disease with the clinical and morphological characteristics of acute hepatitis.

The causative agent is the hepatitis E virus (HEV) identified in the 80s of the 20th century. There are more thanten genotypes and so far four of them are known as relevant to human pathology (1,2,3).

In endemic areas the disease is a major health problem.The increase in the number of patients with indigenous hepatitis in developed countries also becomes an issue(1-7).

Hepatitis E clinically manifests most often withicteric disease and subjective complaints do not differ from those of hepatitis caused by other hepatotropic viruses (8, 9).

In Bulgaria hepatitis E is described for the first time by P. Teoharov et al. in 1995 (4). In the following years there is an increase in the number of cases reported for the country (10,11,12).

The term cholestasis was first introduced by pathologists to denote retention of bile and formation of bile thrombi in the bile canaliculi of hepatocytes due to inability to drain in the intestines (13).

Cholestasis syndrome in patients with acute viral hepatitis was first described by Eppingerin the 30s of the 20th century, even before the discovery of hepatotropic viruses (14).

Caroli et al. first described a cholestatic form of acute viral hepatitis in 1942.The name intrahepatic cholestasis was introduced into clinical practice in 1956 and the term cholestasis (cholestatic jaundice) - in 1968 by H. Popper (15). Cholestasis is defined as disorder of bile formation and drainage, resulting in accumulation of its constituents in the liver and blood. This is associated with characteristic clinical, morphological, and biochemical changes (15,16).

From a clinical perspective, cholestasis is a clinical and biochemical syndrome, characterised by itching, jaundice, elevated excretory enzymes and other bile ingredients in the blood (4).

There are three basic factors in the clinical manifestation of cholestasis:

1/ superfluous entry (regurgitation) of bile elements in the blood;

2/ reduced amount or absence of bile in the intestines;

3/ impact of bile components and their toxic metabolites on liver cells and bile ducts (11, 17,18, 19).

The main clinical and biochemical signs of cholestasis are:

1/ **Jaundice.**Increased concentration of conjugated bilirubin is the main feature of cholestasis. Upon resolving of cholestasis, bilirubin may not be detected in urine, while there still might be hyperbilirubinemia in the serum due to the presence of deltabilirubin (13,15).It is believed that jaundice is not an early sign of cholestasis.

2/ **Itching.**It is common in cholestasis and can be a leading or even sole clinical manifestation. A correlation is established between the activity of the enzyme autotaxin (ATX) and the intensity of itching. Apruritogenic factor, excreted with the bile in normal conditions,is assumed to be involved(13).Scratching is the most measurable effect of itching.

3/**Hypercholemia -** increased serum concentration of bile salts and their reduction in the enterohepatic circulation (27).

4/ **Hyperlipidemia.**This is an important feature of certain cholestatic diseases. According to a number of authors, hyperlipidemiais associated with increased level of cholesterol and,to a lesser extent, with the level of triglycerides (17,19,20). Xanthomas are the clinical expression of hyperlipidemia**.**

5/ **Reduced amount or absence of bile in the intestines -** clinically manifested by flatulence, decreased appetite, intolerance to greasy food. Steatorrheaoccurs, accompanied by diarrhoea, dehydration, loss of electrolytes and oil-soluble vitamins (17, 19).

Cholestasis syndrome is observed in all types of viral hepatitis (17, 21-24).According to Caroli, 80% of cases of intrahepatic cholestasis (IHC) are associated with viral hepatitis B and C, and only 20% with other etiological factors (15).

Hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV) can cause serious cholestasis in immunocompromised patients, as well as transplant patients. Theycan develop severe infection known as fibrosingcholestatic hepatitis, which can progress to liver failure if not treated.

Unlike the above mentioned viruses, enterally transmitted hepatotropic viruses hepatitis A virus (HAV) and HEV can cause significant cholestasis in immunocompetent individuals(22, 25).

Cholestasis syndrome associated with acute viral hepatitis (AVH) can progress in two ways:

1/ Acute cytolytic hepatitis with cholestatic components -cytolytic syndrome is dominant with superimposedsymptoms of cholestasis. They are mildly expressed.

2/ Acute viral hepatitis - cholestatic form.It is characterised by less severe symptoms of cytolysis and clearly pronounced cholestatic syndrome, dominant during the disease. According to some authors, typically this form is problematic with a recurrent course and possible

chronification (hepatitis C) (18, 26, 27).
In hepatitis E the cholestatic form is more common and according to different authors varies from 10-25% (25). There are no clear reasons reported so far. A hypothesis suggests HEV replication to occur not only in the cytoplasm of hepatocytes but also in bile structures as well as extrahepatic replication.
Regardless of the complicated course and prolonged jaundice, the cholestatic form in enterally transmitted hepatitis viruses does not progress to liver failure and most patients fully recover (22).
Aim: The aim of this report is to analyse the clinical manifestations in patients with hepatitis E and cholestasis.

MATERIAL AND METHODS

Ninety patients diagnosed with acute hepatitis E were treated at the Infectious Diseases ward of the Regional Hospital -Pazardzhik during the period 1 January 2007 – 30 June 2016, with the majority (83) being hospitalised between 1 January 2014 – 30 June 2016. The first patient in Pazardzhik was diagnosed in 2007. The distribution of patients by year was as follows: 2007-1; 2012-2; 2013-4; 2014-29; 2015-30; 2016-24. Patients were subjected to clinical monitoring and observing the objective condition, combined with paraclinical and instrumental methods of examination.The diagnosis was confirmed at the National Reference Laboratory for hepatitis viruses, National Inspectorate - Sofia, with detection of anti-HEV IgM and anti-HEV IgG antibodies with ELISA methodology (kits HEV IgM, HEV IgG DIA PRO - Milano, Italy). Statistical methods:In processing the data SPSS version 18 was used. Data are presented as absolute number/percentage, as well as averages (arithmetic mean, median) and magnitudes measuring variance (standard deviation, span). A comparison of the average in two related samples was done by t-test and Wilcoxon method, depending on the type of distribution.

RESULTS

Out of 90 patients with hepatitis E, 25 (27.77%) were with signs of cholestasis. Patients with cholestasis were aged between 40-76 years (average 58.04). Gender distribution was 20 (80%) male patients and 5 (20%) female patients. The average age of men was 58.75 years, ranging from 40 to 76, and for women - 55.2, with a range from 47 to 60 years.
Of all patients with cholestasis 23 (92%) had comorbidities, presented in Table 1.

Table1. Comorbidities in 23 patients with cholestasis.

Comorbidities	Number of patients	Percentage	Male	Female
Diabetes mellitus	12	48%	11	1
Cardiacdiseases	13	52%	12	1
Infection with other hepatotropic viruses	7	28%	5	2
Alcohol abuse	12	48%	11	1
Other	5	20%	4	1
More than one disease	17	68%	15	2

Subjective complaints of patients with hepatitis E (with or without cholestasis) were not different from those of patients with hepatitis caused by other hepatotropic viruses. We observed the following symptoms: astheno-adynamia - in 17 (68%), anorexia - in 13 (52%); pruritus - in 17 (68%), dyspeptic symptoms - in 7 (28%)patients. The most common symptoms were asteno-adynamia and itching. Only 7 patients (41.2%) had severe astheno-adynamia, lasting during the first days, and it resolved before the onset of the cholestasis syndrome.
The itching was in varying degree as for 5 patients it was significant even before the onset of jaundice. At the height of disease, itching became more pronounced also for other patients. In all patients itching was most pronounced in the evenings and night hours.Dyspeptic signs were less pronounced - only 7 patients reported nausea and vomiting and these complaints were not leading in the clinical picture.
Upon admission 13 patients (52%) were in satisfactory condition - hemodynamically stable, without intoxication signs. Leading complaints were related to their underlying diseases (diabetes, high blood pressure).
Four patients were admitted inimpaired condition - dyspeptic manifestations, fever, drowsiness, and a state of diabetic ketoacidosis.
In 2 patients leading symptoms were those characteristic of hepatitis - fatigue, lack of appetite, aversion to food, drowsiness, epigastric heaviness and heaviness in the right sub-costal region, itching.
Key parameters in examining the patients were: CBC, prothrombin index, fibrinogen, total bilirubin with fractions, AST, ALAT, GGT,ALP, cholesterol, triglycerides, creatinine, urea, ionogram, proteins, albumin.

Table2. Paraclinical parameters in patients with cholestatic form of hepatitis E – total bilirubin (mmol/l), n=23.

Indicators	Onset	Height	Convalescent period
Median	183.12	191.23	72.5
Min.-max.	39-660	24.0-544.1	17.9-100.9

For all 25 patients total bilirubin was examined at the beginning and at the height of disease, while for 23 patients examination was done also in the convalescent period.At the beginning and at the height of disease the value of total bilirubin remained high, but more important was the ratio conjugated/total bilirubin, presented in Table 3.

Table3. Ratio conjugated/total bilirubin. Comparative testing was made in 15 patients, n = 15.

Indicators		Onset	Height	Conva-lescent period
Total bilirubin	Median	206.86	244.37	52.53
	Min.-max.	54.38-660	24.0-544.1	17.9-100.9
Conjugated bilirubin	Median	127.71	149.49	38.17
	Min.-max.	40.2-450,5	9.2-293.2	6.0-80.6
Ratio conjugated/ total bilirubin	Median	0.594	0.590	0.694

The data obtained showed that the values of both total and conjugated bilirubin rose slightly at the height of disease ($p > 0.05$) as conjugated bilirubin remained high - about 59% in the first and second testing. In the control study,total and conjugated bilirubin during the convalescent period decreased significantly ($p < 0.01$ compared to the first study, 0.001 compared to the second, respectively), but the proportion of conjugated bilirubin increased significantly to 69.4% ($p < 0.05$).Although bilirubin levels were not high during the convalescent period, the percentage of conjugated bilirubin remained significantly elevated.
The values of the cytolytic and excretory enzymes are presented in Table 4.

Table 4. Values of cytolytic and excretory enzymes, n = 24.

Indicators		Onset	Height	Following testing
ASAT U/L	Median	987.73	340	80.95
	Min.-max.	20-3752	29-901	10-216
ALAT U/L	Median	1479.3	580.34	121.52
	Min.-max.	66-4264	79-1485	14-326
GGT U/L	Median	942.5	592.75	
	Min.-max.	238-3865	79-3442	
APH U/L	Median	564.5	519.62	
	Min.-max.	112-1480	126-1243	

ALAT and APH values were examined during the onset, at the height of disease and within a following testing in 14 patients. The ratio of both enzymes is presented in Table 5.

Table 5: APH/ALAT ratio, n = 14.

Indicators	Onset	Height	Following testing
APH/ALAT	0.814	1.67	2.327

APH/ALATratio increasedalong with increasing of the APH value. There was crossing of the curves of the two enzymes. Values above 2 were indicative of cholestasis.

As hyperlipidemia is common in cholestasis, it was observed in the patients of this study (Table 6).

Table 6. Cholesterol and triglycerides in patients with cholestatic form of hepatitis E, n = 19.

Indicators	Median value	Min.-max.
Cholesterol	7.064	2.55-24.9
Triglycerides	3.127	1.3-4.5

Out of 25 patients, elevated cholesterol levels were found in 10 (40%).Elevated triglycerides were found in all 25 patients (100%).In all monitored patients there was deviation in the lipid profile.

Ultrasound was carried out on all patients. In addition to hepatomegaly, steatosis was a common finding. Cholecystitis was diagnosed in 14 (56%) patients, and 7 of them had a calculous cholecystitis, as reported for other types of hepatitis of different etiology (1,26,28). Treatment with antimicrobial agents reduced the subjective symptoms of heaviness, right-sided abdominal pain, and fever.

Hospital stay of patients. Patients with cholestatic form of hepatitis E had longer hospital stay compared to patients with other forms, and their treatment demanded greater financial resources. The average length of stay of the observed patients was 20.8 days.

Financial aspect.In addition to the long hospitalisation period, recovery period was longer and associated with economic costs, as the disease is associated with longer period of temporary disability. Treatment required greater financial resources that exceeded the allocated funds to the clinical pathway and continued in an outpatient setting.

All patients recoveredwithinperiods of different length, but not less than 3 months. In one patient the total bilirubin levels normalised 6 months after the onset of the disease, but the direct fraction was higher than normal for 2 more months.

DISCUSSION

Acutehepatitis E,like hepatitis A,isa disease transmitted via the faecal-oral route.Viral hepatitis A has one of the highest rates of incidence in Bulgaria (31).This disease demonstrates great epidemiological differences between economically developed and developing countries as well as between different social and economic strata within countries (31). Hepatitis E has similar features and inrecentyearsis diagnosed more and more often in Bulgaria (32). In Europe, it is mainly caused by genotype 3. According to studies of P. Teoharov, this genotype is also found among Bulgarian patients.Theclinicalcourseofhepatitis A and E variesfromasymptomaticinfectiontofulminant hepatitis (32).

Cholestasis syndrome is reported with hepatitis E, as with other types of hepatitis, especially type A. 27% of the patients examined in this study had cholestasis.

Twenty-three patients had underlying diseases and 12 patients were alcohol abusers. The proportion of patients with comorbidities was 92%. A pre-existing liver damage was anticipated for alcohol abuse patients or diabetes mellitus patients.

Less pronounced symptoms of intoxication are the hallmark of cholestatic forms (28-30). We also observed this finding in our patients with hepatitis E.

According to the available literature data and based on our own observations, intoxication manifestations of hepatitis E are less pronounced if the cholestatic component is not present. This could explain why patients delay seeking medical help and/or often are admitted to other wards depending on their main chronic disease.

The elevated level of triglycerides in all patients does not have a meaningful explanationthough the number of patients is not large. One possible reason may be the presence of comorbidities associated with abnormal lipid profile - namely, diabetes, alcohol use, etc.

All patients were in adult age and male persons were more affected, which is characteristic of hepatitis E in developed countries.

Leading complaints were often related to comorbidities and therefore, some patients were initially treated at wards depending on the underlying disease - endocrinological, cardiac, gastrointestinal.

In some of the patients cholestasis probably was of mixed genesis - related to viral hepatitis and the accompanying disease (steatosis in diabetes or alcohol damage).

CONCLUSIONS

As with other types of viral hepatitis, cholestasis is commonly observed in hepatitis E. Subjective complaints do not differ from those of other types of hepatitis, but intoxication manifestations are less pronounced and of shorter duration.

Itching and asteno-adynamia are leading signs in the clinical picture of cholestasis and hepatitis E.In some patients we observed decompensation of the underlying disease, while hepatitis did not presented withsevere course.Comorbidities are likely to contribute to the development of cholestasis –e.g. diabetes, alcohol use, for which preceding changes in the liver are typical.None of the patients developed fulminant hepatitis even with decompensation of the underlying disease.

Nevertheless, the course of acute hepatitis E had significant deviations in the biochemical profile that persisted longer than usual and the recovery period was prolonged.Development of cholestasis is associated with prolonged hospitalisation and treatment with greater financial cost.

Cholestatic form of hepatitis is a challenge for the physician and the patient's patience. Still, there are unresolved issues regarding treatment and relief of subjective complaints.

REFERENCES:

1.Anderson DA. Hepatitis E, In: Mandell GL., Bennett JE., Dolin R., eds. Principles and Practice of Infectious Diseases, 7th ed, Churchill Livingstone Elsevier, Philadelphia, 2010, 2411-2421.
2.Franco Maria Ruggeri, Ilaria Di Bartolo, EleonoraPonterlo,et al. Zoonotic transmission of Hepatitis E virus in industrialized countries. New Microbiologica.2013;36:331-344.
3.Maria Teresa Perez-Gracia, Mario Garcia, Beatriz Suay and Maria Luisa Mateos-Lindemann.Current Knowledge on hepatitis E. Journal of Translational Hepatology.2015; 3:117-126.

4.Teoharov P, TiholovaM, Draganov P, Lilianova V. First case with hepatitis E in Bulgaria.Infectology.1995; 3:17-18(in Bulgarian).
5.Danielle M. Yugo and Xiang-Jin Meng. Hepatitis E virus: Foodborne, Waterborne and Zoonotic Transmission. Int J Environ Res Public Health. 2013; 10:4507-4533.
6.Franco Maria Ruggeri, BartoloI, OstanelloF,TrevisaniM. Hepatitis E virus- An Emerging Zoonotic and Footborne Pathogen eBook, 2013, 23-25.
7.Ibrahim M Sayed, Ann-SofieVercouter, Sayed F,Abdelwahab, KoenVercauterenand Philip Meuleman – Is Hepatitis E Virus an Emerging Problem in Industrialized Countries? JHepatol.2015; vol 00, N00, 4-10.
8.Aggarwal R, Krawczynski K. Hepatitis E: **Anoverwiev** and recent advances in clinical and laboratory research. J GastroenterolHepatol.2000; 15:9-20.
9.RakeshAggarwal and ShahidJameel.Hepatitis E. Hepatology. 2011; 54(6): 2218-2224.
10. Pishmisheva M, Teoharov P. Clinical course of hepatitis E. Infectology and Parasitology. 2012; 2:13-15 (in Bulgarian).
11. Teoharov P.Hepatitis E viral infection. MedInfo.2013; 10, XIII:1-3 (in Bulgarian).
12. Teoharov P. Study on the epidemiology of viral hepatitis, distribution and specific prophylaxis of the main hepatitis viruses in Bulgaria. Thesis DSc. 2013: 57-63 (in Bulgarian).
13.Krastev Z, Chernev K.Hepatology. 1998, Edition Tilia:95 (in Bulgarian).
14.**Eppinger H.** Die Leberkrankheiten - Allgemeine und SpeziellePathologie und Therapie der Leber-book, 1937.
15.Gancheva D.Cholestasis – clinical, diagnostic and therapeutic aspects. Monography.2015. Edition “Steno”. Varna: 15 (in Bulgarian).
16.Gancheva D, Varbanov G. Cholestatic hepatitis. Bulgarian medicine. 1997; 5 (5-6): 3-5 (in Bulgarian).
17. Zviaginceva T, Chernobai**A**. Intra-hepatic cholestasis: pathogenesis and treatment. Ukraine Medicine.2012; 3 №89, 79-89 (in Russian).
18. Nadinskaia M. Hepatic diseases with cholestasis syndrome. Consiliummedicum, 4,2002, 6, p:9.
19. Nazer H. Cholestasis. Updated Nov 16,2014.emedicine Medscape, article 927624.
20. Eisenburg, J. Cholestasis. Guiding Symptom in Liver Diseases.Pathogenesis and Clinical Pictures.Falf Foundation e.V.,2003,84.
21. Jüngst C, Berg T, Cheng J, Green RM, Jia J, Mason AL, Lammert F. Intrahepatic cholestasis in common chronic liver diseases. Eur J Clin Invest. 2013; 43(10):1069-1083.
22.Khalid S and Jeffrey Crippin.Cholestatic Variants of Viral Disease and Alcohol – in Cholestatic Liver Disease-119-134.
23.Manish Kumar, VivekDewan, Chinmaya Kumar Sahoo, DevkiNandan- Prolonged cholestatic jaundice with very high bilirubin level due to hepatitis e infection - International Journal of Basic and Applied Sciences.2014; 3(2):99-100.
24.Mohammed Sultan Khuroo, Vinod K, Rustgi M, George J Dawson, et al. Spectrum of hepatitis E virus infection in India.J Med Virol. 1994; 43(3):281-286.
25. MounirArroud, Sara Benmiloud, Youssef Bouabdallah, Acute AcalculousCholecystitis Revealing Hepatitis A Virus Infection in Children. J Saudi Gastroenterol. 2009; 15(4):277.
26.Cherveniakova T, Lilianova V, Kancheva L. Acute hepatitis C with cholestasis.Bulgarian hepatology and gastroenterology.2000; 2:23-26.
27.AkagiH, MizukiA, TominagaT,el al. Acute Hepatitis C infection with prolonged intrahepatic cholestasis and remarkable progression of fibrosis mimicking fibrosingcholeststic hepatitis. Nihon ShokakibyoGakkaiZasski.2014; 6:1141-1148.
28. TraunerM, MeierPI, Boyer JL. Molecular Pathogenesis of Cholestasis. N Engl J Med.1998; 339(17):1217-1227.
29.Galev A, Stoyanov P, Kovaliova K, Nedkova K. Clinical course of hepatitis A with cholestasis syndrome. Military Medicine.2005;1:41-45 (in Bulgarian).
30.Ozaras R, Mert A Yilmaz MH, Celik AD, et al., Acute viral cholecystitis due to hepatitis A virus infection. J ClinGastroenterol.2003; 37(1): 79-81.
31. VatevN,PetrovA,TroianchevaM, et al. Hepatitis A in Plovdiv region,2007-2012.Probl Infect Parasit Dis. 2012; 40(2):36-38.
32. Pishmisheva M, Vatev N, Stoycheva M. Clinical forms of hepatitis A in different age groups – analysis for a two-year period, 2011-2012. Probl Infect Parasit Dis. 2013; 41(2):18-23.

COMPARATIVE STUDY OF METHODS FOR DETECTION OF CLOSTRIDIUM DIFFICILE DIRECTLY FROM STOOL

M.Pavlova[1], E.Dobreva[1], K.Ivanova[1], G.Asseva[1], I. N. Ivanov[1], P. Petrov[1], E.Savov[2], I.Todorova[2], A.Trifonova[2], T. Kantardjiev[1]

[1]National Centre of Infectious and Parasitic Diseases, Sofia
[2]Military Medical Academy, Sofia

ABSTRACT:
Objective: In this study, we compared various methods for determination of *Clostridium difficile* antigen and toxins directly from stool.
Material and methods: Thirty-four faecal specimens from patients with diarrhoeal syndrome were examined. The following methods were applied directly on the samples yielding results within a few minutes to a few hours:immunochromatographic assay QuikChek Complete for determination of *glu D*antigen and toxins A/B, EvaGreen Real-Time PCR for *glu D*and *tcdB,C. difficile* Verigene test (Nanosphere, Northbrook, IL, USA) for detecting tcdA, tcdB, and the binary toxin. Culture methods were performed to confirm the results for detection of *C. difficile*from stools.
Results: Twenty-three out of 34 (23/34, 68%) faecal specimens were positive for *C. difficile* by culture methods. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the different methods were as follows: QuikChek Complete - 90.9%, 75%, 95.2%, 60%, and 88.9%; EvaGreen Real-Time PCR – 83.3%, 100%, 100%, 100 %, and 88.9%; Verigene*C.difficile* test – 90.9 %, 100%, 100%, 100%, and 100%.
Conclusion: According to the results obtainedin this study, the most effective method for detection of *C. difficile* antigen and toxins directly from stool samples was the Verigene*C.difficile* test.

ADDRESS FOR CORRESPONDENCE:
M. Pavlova,
National Center of Infectious and Parasitic Diseases,
Department of Microbiology,
26 Yanko Sakazov blvd.
Sofia 1504, Bulgaria
tel. +3592 9446999/ 268, fax: +3592 9433075
e-mail: mimipavlovaa@gmail.com

INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming anaerobic bacillus. It is both a human pathogen and a member of the normal human intestinal flora. Clinically significant *C. difficile*strainsproducetoxins:toxinA(enterotoxin), toxin B (cytotoxin), and binary toxin(CDT) (1). *C. difficile* is the most common cause of diarrhoea in people who develop diarrhoeal symptoms while hospitalised(2, 3).*C. difficile* infections (CDI) can cause symptoms ranging from mild diarrhoea to pseudomembranous colitis, perforation, sepsis, and death. It is a leading cause of nosocomial antibiotic-associated infections with high mortalitycausing significant social financial burden in different regions of the world (4, 5) *C. difficile* toxin is detected in the stools of up to 20–30% of those with antibiotic-associated diarrhoea and more than 95% of those with pseudomembranous colitis. The antibiotics most frequently associated with infection are ampicillin, cephalosporins, clindamycin, and amoxicillin. While the organism is frequently carried by infants, it does not usually cause diarrhoea in this population(6, 7, 8,9).
In recent years, CDI has become more frequent and for this reason rapid diagnosis is very important for the implementation of appropriate therapy(10, 11,12).Unfortunately,little is known about the extent of CDI inBulgariaas most hospitals have only recently adopted diagnostic services (13).
The aim of this study was to compare different rapid methods for detection of C.difficiledirectly fromfaecal clinical samples.

MATERIAL AND METHODS

Stool samples and processing

ThestudywasperformedattheNationalReference Laboratory of Enteric Pathogens(culture and EIA tests), National Reference Laboratory for Controland Monitoringof Antibiotic Resistance, NCIPD(EvaGreen Real-Time PCR), and Military Medical Academy, Sofia (Verigene CDF test). Between February 2014 and March 2015, a total of 34 unformed or liquid faeces samples were obtained from symptomatic patients with risk factors for CDI andhospitalisedat different clinics of Military Medical Academy, Sofia. All samples were tested for tcd A,tcd B, and CDT of which 23/34(68%) were positive for toxigenic *C. difficile*. Duplicate samples of patients wereexcluded from this study.
Four-step algorithm: EIA tests for *C. difficile* toxin, followed by Verigene*C. difficile* test,PCR for *gluD* and *tcdB,* and finallya culture method.

1. EIA tests for *C. difficile* toxin

Samples were tested byrapid membrane enzyme immunoassay for the simultaneous detection of *C.difficile* glutamate dehydrogenase antigen and toxins A and B in faeces(QuikChekComplete, Alere North America, LLC), in accordance with the manufacturer's instructions. Each test took five to ten minutes.

2. EvaGreenReal-Time PCR for *gluD*and *tcdB*

Bacterial DNA was extracted from the sample using QIAmp[R]MiniKit(QIAGEN, Germany),in accordance with the manufacturer's instructions. The extracted DNA was stored at -20ºC until analysis with EvaGreen Real-Time PCRto amplify coding regions of:

gluD -encodes synthesis of the enzyme glutamate dehydrogenase;

tcdB- encodes synthesis of toxin B (cytotoxin), located on the pathogenicityisland (PaLoc).

The primers used and their sequences are presented in Table 1.

EvaGreen Real-Time PCR analysis was optimised to run in a final volume of 25µl reaction with the following primers and conditions: 1X PCR buffer (2.5mM $MgCl_2$, 50 mM TRIS-HCl pH 9.2); dNTP 0.2 mM; 0.5U Hot Start Pol; GluD-s/GluD0.4 µM; NK١٠٤/NK١٠٥ 0.25 µM; EvaGreen 0.8X; initialdenaturation at 95°C– 4 min,followed by 38 cycles of denaturation at 95°C– 30 sec, hybridisation at 58°C– 30 sec, and elongation at 72°C-30sec.

3. Verigene*Clostridium difficile* Nucleic Acid test(Verigene CDF test), (Nanosphere, Northbrook, IL, USA)

CDF is an automated, multiplex test for rapid identificationof toxin-producing *C. difficile*bacteria and differentiation of the PCR ribotype 027 strains. By simultaneously targeting both the toxin A and toxinB genes and differentiating the 027 strain, Verigene CDF deliverscomprehensive results directly from a stool sample.The Verigene CDF test is a multiplex qualitativePCR assay that utilises a nanoparticle-based array hybridisation method to detect *C. difficile*tcdA and tcdB in faecal specimens.

In addition, the assay detects binary toxin gene sequences and the single base-pair deletion at nucleotide 117in *tcdC* to provide a presumptive identification of the epidemic strain ribotype 027(8).

Fresh faecal samples were tested using CDF, in accordance with the manufacturer's instructions. The run time was only 2 hours.

4. Culture method for *C. difficile*as a "gold standard"

On the day of receipt stool specimens were treated using the alcohol shock method to inhibit normal faecal flora (nonsporulating organisms) and hereby to enhance the isolation of *C. difficile.* A pea-sized portion of stool sample was transferred to 250 ml absolute alcohol (1:1); the suspension was allowed to stay at room temperature for 30 minutes and after that 100 – 150µl of the suspension were inoculated onto Brain Heart Infusion Agar with 10% sheep blood (BulBioLtd, NCIPD,Sofia) and incubated at 37°C for ٤٨hours under anaerobic conditions (gas-pack Tsvetyfirm, Sofia).Recovered isolates were identified as *C. difficile*by performing Gram stainingand latex co-agglutination tests for detection of the common antigen glutamate dehydrogenase (GDH),(Becton Dickinson, USA).

5. Standard statistics

Probability of the patient having a disease when the test is positive - PPV

PPV = a / a+b= a (true positive) / a+b (true positive + false positive)

Probability of the patient not having a disease when the test is negative - NPV

NPV = d / c+d= d (true negative) / c+d (false negative + true negative)

Table 1. Primers used in the EvaGreenReal-Time PCR for *gluD* and *tcdB* of *C. difficile.*

Gene	Sequence	Amplicon size (bp)	Source
GluD-s	5'-GTCTTGGATGGTTGATGAGTAC-3'	158bp	*Paltansing et al., 2007*
GluD-as	5'- TTCCTAATTTAGCAGCAGCTTC-3'		
NK104	5'-GTGTAGCAATGAAAGTCCAAGTTTACGC-3'	204bp	*Kato et al., 1998*
NK105	5'-CACTTAGCTCTTTGATTGCTGCACCT-3'		

RESULTS AND DISCUSSION

In this studywere compared three different methods for rapid diagnosis of *C.difficile* directly from stool. Thirty-four faecal specimens from hospitalised patients with diarrhoeal syndrome were examined applying four-step algorithm: EIA tests for *C. difficile* toxin followed by CDF, PCR for *gluD* and *tcdB,*and finallya culture method.The results from the three rapid tests were compared with the current gold standard method for diagnosis of *C. difficile*. The obtained results are presented in Table 2.

Table 2. Obtained results for different tests.

№	Verigen	PCR	QuikChek	Culture	Latex test
304	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
333	tcdA/B/C wild type	negative	Ag-/tox-	+	+
356	negative	negative	Ag-/tox-	+	+
366	tcdA/B/C wild type	negative	Ag+/tox-	+	+
367	tcdA/B/C wild type	GluD-/tcdB-	Ag+/tox+	+	+
668	tcdA/B/C wild type	negative	Ag+/tox+	+	+
669	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
670	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
671	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox-	+	+
716	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
724	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
726	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox-	+	+
773	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
774	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
775	negative	negative	negative	-	-
776	negative	negative	negative	-	-
777	negative	negative	Ag+/tox+	-	-
798	negative	GluD+/tcdB+	Ag+/tox+	+	+
802	negative	negative	Ag+/tox+	-	-
805	negative	negative	Ag+/tox+	+	+
808	negative	negative	Ag+/tox+	-	-
809	negative	negative	Ag-/tox-	-	-
816	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
822	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox-	+	+
823	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
831	tcdA/B/C wild type	GluD+/tcdB+	Ag+/tox+	+	+
835	negative	negative	Ag-/tox-	-	-
836	negative	GluD+/tcdB-	Ag-/tox-	+	+
841	technical problem	negative	negative	-	-
849	negative	negative	negative	-	-
852	+	GluD+/tcdB+	Ag+/tox+	+	+
853	+	GluD+/tcdB+	Ag+/tox+	+	+
856	negative	negative	negative	-	-
857	negative	negative	negative	-	-

A number of commercially available enzyme immunoassay kits routinely used in most diagnostic laboratories in Bulgariademonstrate poor performance. According tothe results obtained with the kit used in this study, the sensitivity, specificity, and accuracy were only 90.9%, 75%, and 88.9%, respectively;therefore it is not sufficiently sensitive for routine laboratory detection of CDI, even though it is rapid, relatively inexpensive, and specific.

Over the past decade, PCR tests have been developed to provide more accurate diagnosis (8, 1). However, in our study PCR had sensitivity and accuracy of only83.33% and 88.90%, whichcould be explained with low yield of isolatedDNAor the presence of alot of inhibitors in the faecal samples.

The Verigene CDF test is a novel nucleic acid microarray that reliably detects both *C. difficile* toxins A and B in unformed stool specimens and appears to adequately identify ribotype 027 isolates(7).The VerigineCDF test demonstratedthe highest accuracy- 100%, sensitivity – 90.91%, and specificity- 100%, using the direct faecal culture as a reference method (Table 2 and 3). The better performance of the CDF test obtained in this study may be due to comparatively small number of samples.

Table 3. Sensitivity, specificity, PPV, NPV, and accuracy of the different methods.

	Real-timePCR	**Verigene**	**QuikChek**
Sensitivity	*83.33%*	***90.91%***	*90.9%*
Specificity	*100%*	***100%***	*75%*
*PPV **	*100%*	***100%***	*95.20%*
*NPV ***	*100%*	***100%***	*60%*
Accuracy	*88.90%*	***100%***	*88.90%*

*PPV- positive predictive value; ** NPV- negative predictive value.

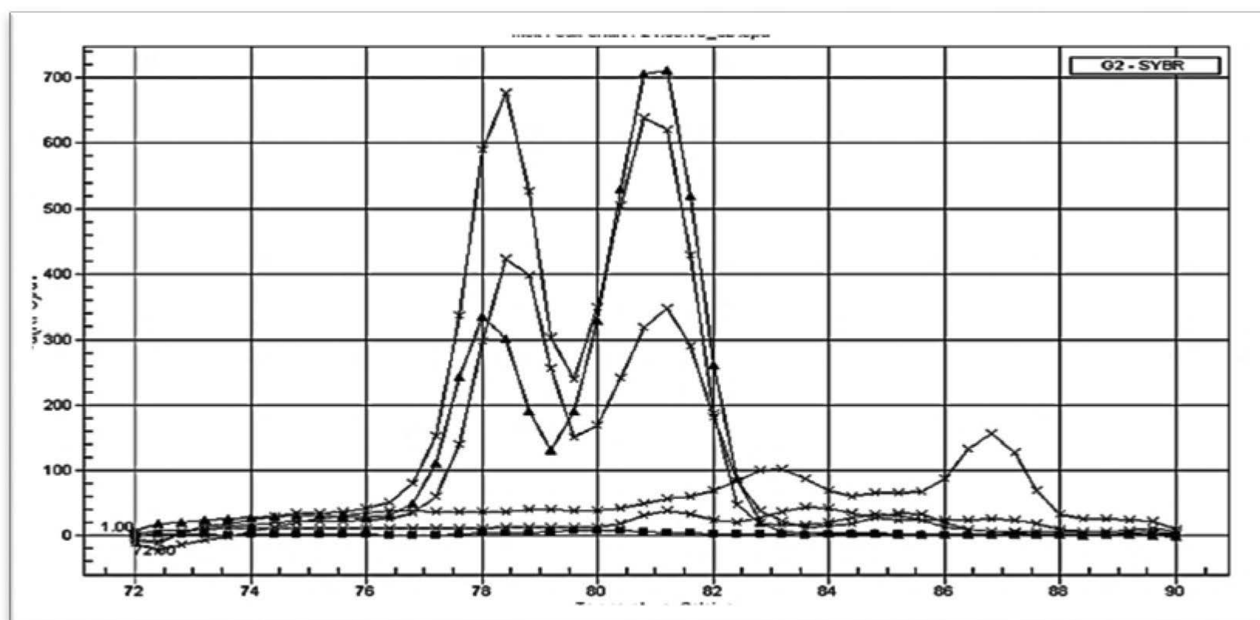

Figure 1.DuplexEvaGreen Real-Time PCR.Melting curve analysis (TmºC): *gluD*(81.2ºC),*tcdB* (78.4ºC).

▲- positive control (ref. strain *C. difficile*, ECDC)

■ - negative control (ddH$_2$O)

X - stool samples

CONCLUSION

C.difficile is the most common cause of nosocomial diarrhoea and is a leading cause of diarrhoea in the elderly.CDIis associated with the use of broad-spectrum antibioticsand place a high burden on the healthcare system.*C. difficile* can lead to severe complications such as pseudomembranous colitis, perforation, sepsis, and death(10).Currently diagnosis of CDI relies upon detection of toxins A/B in stool by enzyme immunoassay. This methodology is unsatisfactory because of low sensitivity resulting in significant false negatives. Screening for glutamate dehydrogenase before confirmation of positives by PCR is cheaper than screening all specimens by PCR and is an effective method for routine use(14). Current EIA tests for CDI are of inadequate sensitivity and should be replaced; however, this may result in apparent changes in CDI rates in national surveillance statistics.

Anaerobic faecal culture is the most sensitive test, but it takes two-three days to provide results. Accurate and timely diagnosis is especially important to preventnosocomial spread (15).

PCR methods are the most recommended test for rapid and accurate diagnosis of CDI by many authors (16, 5, 17,18). Unfortunately, in this study EvaGreen Real-Time PCR method showed lower than the expected results. It demonstratedsensitivity and accuracy of only 83.33 % and 88.90 %,which could be explained with low yield of isolated DNA or the presence of many inhibitors in the faecal samples.

According to the results obtained in this study,the Verigine*C.difficile* test provides the most useful diagnostic information for CDI.The Verigine test showed the highest accuracy 100%, sensitivity- 90.91%, and specificity- 100%, using the direct faecal culture as a reference method.It took only 2 hours and furthermore,the procedure was simple and did not require trained personnel.

We recommend a comprehensive approach for a definitivediagnosis of CDI, with thesimultaneous applicationof several conventional methods.

REFERENCES:

1. Mylonakis E, Ryan ET, Calderwood SB. *Clostridium difficile–Associated Diarrhea*. Arch Intern Med. 2001; 161(4):525-533.
2. Dobreva E, Ivanov I, Dobrevska RV, Ivanova K, Marina M, Petrov P, Kantardjiev T, Kuijper E. *Toxin encoding genes characterization of Bulgarian Clostridium difficile clinical strains.*Comptesrendus de l'Acad´emiebulgare des Sciences. 2012; 65(10): 1365-1372.
3. Wiegand PN, Nathwani D, Wilcox MH, Stephens J, Shelbaya A, Haider S. *Clinical and economic burden of Clostridium difficile infection in Europe: a systematic review of healthcare-facility-acquired infection*. J Hosp Infect. 2012; 81:1-14.
4. Surawicz CM, Brandt LJ, Binion DG, et al. *Guidelines for diagnosis, treatment, and prevention of Clostridium difficile infections.*Am J Gastroenterol. 2013; 108:478- 498.
5. Tolchkov V, Marina M, Kantardjiev T. *Molecular methods in diagnostics of anaerobic infections* (in Bulgarian). Medical Preview. 2010; 46(4):34-41.
6. Ivanova K, Petrov P, Asseva G, Dobreva E, Ivanov I, Vatcheva-Dobrevska R, Marina M, Tolchkov V, Kantardjiev T, Notermans DW, Kuijpef EJ. *First cases of severe hospital-acquired Clostridium difficile infections in Sofia, Bulgaria*. Probl Infect Parasit Dis. 2010; 38(1):22-24.
7. Burke KE, Lamont JT. *Clostridium difficile Infection: A Worldwide Disease*. Gut Liver. 2014; 8(1):1-6.
8. Carroll KC, Buchan BW, Tan S, Multicenter, et al. *Evaluation of the VerigeneClostridiumdifficile Nucleic Acid Assay*. JCM. 2013; 51:4120–4125.
9. Carroll KC, Bartlett JG. *Biology of Clostridium difficile: implications for epidemiology and diagnosis.*Annu Rev Microbiol. 2011; 65:501- 521.
10. Davies KA, Longshaw CM, Davis GL, et al. *Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID).* Lancet Infect Dis. 2014; 14:1208–2019.
11. Bartlett John G. *The case for Vancomycin as the preffered drug for treatment of Clostridium difficile infection*. CID. 2008; 46:1489- 1492.
12. Reller ME, Alcabasa RC, Lema AC, Carroll CK. *Comparison of two rapid assays for Clostridium difficile common antigen and a C. difficile toxin A/B assay with the cell culture neutralization assay*. Am J ClinPathol. 2010; 133:107-109.
13. Dobreva EG, Ivanov IN, Vathcheva-Dobrevska RS, Ivanova KI, et al. *Advances in molecular surveillance of Clostridium difficile in Bulgaria*. J Med Microbiol. 2013; 62:1428- 1434.
14. Goldenberg SD, Cliff PR, Smith S, Milner M, French GL. *Two-step glutamate dehydrogenase antigen real-time polymerase chain reaction assay for detection of toxigenic Clostridium difficile.*J Hosp Infect. 2010; 74(1):48-54.
15. Surawicz CM, Brandt LJ, Binion DG, et al. *Guidelines for diagnosis, treatment, and prevention of Clostridium difficile infections.*Am J Gastroenterol. 2013; 108:478- 498.
16. Dobreva E. *Contemporary methods for diagnostics of Clostridium difficile infections*. Med Review. 2014; 50(2):17-24.
17. Paltansing S, van den Berg RJ, Guseinova RA, Visser CE, van der Vorm ER, Kuijper EJ. *Characteristics and incidence of Clostridium difficile-associated disease in The Netherlands, 2005.*ClinMicrobiol Infect. 2007; 13:1058–1064.
18. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, Suzuki K, Kim SM, Chong Y, Wasito EB. *Identification of toxin A-negative, toxin B-positive Clostridium difficile by PCR*. J ClinMicrobiol. 1998; 36:2178–2182.

CASE OF ICTEROHAEMORRHAGIC LEPTOSPIROSIS WITH PREMORBID LIVER DISORDER

G. Gancheva[1], T. Doichinova[1], I. Pakov[1], I. Christova[2], H. Hristov[1], M. Georgieva[1], I. Petkova[1], E. Taseva[2]

[1] Medical University, Department of Infectious Diseases, Epidemiology, Parasitology and Tropical Medicine, Pleven, Bulgaria
[2] National Centre of Infectious and Parasitic Diseases (NCIPD),
Department of Microbiology, Sofia, Bulgaria

ABSTRACT

Liver involvement is a common feature of leptospirosis. It varies from mild to severe hepatic dysfunction. Our aim was to analyse a case of icterohaemorrhagic leptospirosis and to assess the impact of premorbid liver disorder on severity of leptospirosis. **Case report:** sixty-seven-year-old, frequently fishing veterinary worker was admitted to the Clinic of Infectious Diseases at University Hospital – Pleven with one-week history of fever, myalgia, weakness, vomits, and oliguria. Past history: viral hepatitis A (1974); “hepatic inflammation” (1983). On admission, the patient was in severe condition, afebrile, adequate, with severe adynamia, intensive jaundice, conjunctival suffusions and haemorrhages. Decreased breathing, basal crackles, tachyarrhythmia, hypotension, bloated abdomen, and hepatosplenomegaly were found. Laboratory results: RBC 3.8; Hg 119; WBC 23.7; granulocytes 0.88; platelets 45; total/direct serum bilirubin 153/145 μmol/L; ASAT 42; ALAT 27 IU/L; total protein 61.3; albumins 27.2; fibrinogen 8.0; prothrombin index 82%; C-reactive protein 278; blood-urea-nitrogen 16; creatinine 308. Immunophenotypisation of lymphocytes subsets: granulocytosis with lymphopenia (markedly decreased total T-lymphocytes, T-helpers and T-helpers/cytotoxic T-cells ratio). Abdominal ultrasonography: mild hepatic steatosis; partitioned gallbladder. Microagglutination test: positive for *L. icterohaemorrhagiae* (1:1600) (National Reference Laboratory – NCIPD, Sofia). Prompt intensive treatment was performed, but along with clinical improvement the intensity of jaundice extremely increased. Optical hallucinations and disturbed spatial perceptions appeared. Delirium resolved after increasing the doses of hepatoprotectors. Patient was discharged (on the 14th day) in improved condition with moderate jaundice and hepatomegaly. During the two months follow-up control, favourable recovery was registered. **Conclusions:** Liver involvement in leptospirosis is an important factor for severity. Premorbid liver disorder poses an additional risk for prognosis.

Keywords: *leptospirosis, liver involvement, delirium, acute renal failure, prognosis*

ADDRESS FOR CORRESPONDENCE:
Galya Gancheva,
Clinic of Infectious Diseases, University Hospital
8a “Georgi Kochev” str.
5800 Pleven, Bulgaria
Tel. +359 886 416
E-mail: galya_gancheva@abv.bg

INTRODUCTION

Leptospirosis, the most widespread zoonosis, is emerging as a major public health problem (1). The disease is caused by spirochetes belonging to the genus *Leptospira*, which comprises both saprophytic and pathogenic species (2). The currently used genetically based classification indicates that there are at least 19 species (13 pathogenic and six saprophytic), identified through DNA hybridisation analysis (3). Seven of these species: *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilli*, *L. kirschneri,* and *L. alexanderi* are the main agents of leptospirosis (4).

Leptospirosis has a broad geographical distribution, occurring in both rural and urban areas of tropical, subtropical and temperate regions. The disease outbreaks in developed countries are usually associated with occupational exposure, tourism or sporting events. Developing countries carry the major burden of the disease, with half a million cases reported yearly and a mortality rate ranging from 5 to 10% (2).

The clinical manifestations of human leptospirosis are diverse, ranging from mild, flu-like illness to a severe disease form known as Weil’s syndrome. The severe disease form is characterised by jaundice, acute renal failure, pulmonary distress, and haemorrhage, which can lead to death in 40% of cases (5, 6). In addition, the central nervous system and the cardiovascular system are also affected (7).

Liver involvement is a common feature of leptospirosis. It varies from mild to severe hepatic dysfunction. The jaundice (when is

presented) appears within the initial five to nine days of the clinical onset and lasts up to one month. It has been observed in leptospirosis regardless of the causative Leptospira serovar but *L. icterohaemorrhagiae* is the most common causative agent of icteric leptospirosis (8).
The lesions in the liver histopathological findings reveal disorientation of the hepatic cords and disorganisation of hepatocytes with some degree of dissociation along with fatty infiltration and hyperplasia of Kupffer cells. A high content of bilirubin in the blood is produced by the reticuloendothelial cells of the body, phagocytosing red blood cells at such a rapid rate that the parenchymal cells of the liver cannot effectively excrete all the bilirubin brought to them. The bilirubin in the blood stream increases and jaundice occurs (9). In contrast to the markedly increased bilirubin level, hepatic enzymes are slightly elevated (8-12).
The mortality predictors of leptospirosis published by different authors are quite variable. While some indicators are repeatedly cited as predictors of mortality, the significance of others is questionable. Evidences for hepatic dysfunction are prominent during the clinical course of leptospirosis. However, many of these are not reported as predictors of mortality (13). Some host-related factors are independently associated with severity: history of chronic hypertension, hyperamylasemia, history of chronic alcoholism (9, 10, 14).
Our aim was to analyse a case of icterohaemorrhagic leptospirosis and to assess the impact of premorbid liver disorder on severity of leptospirosis.

CASE PRESENTATION

A sixty-seven-year-old, frequently fishing veterinary worker was admitted after obtained written informed consent to the Clinic of Infectious Diseases at University Hospital – Pleven with one-week history of fever, shivering, pains in the calf, and abdominal muscles, weakness, vomits, anorexia, and decreased urine output. In 1974 he suffered from acute viral hepatitis type A, and in 1983 was treated at the Internal Ward of a regional hospital with diagnosis “hepatic inflammation”.
Upon admission, the patient was in severe condition, afebrile, adequate, with severe adynamia. On the physical examination, there was intensive jaundice, conjunctival suffusions and haemorrhages. Decreased breathing with basal crackles bilaterally, tachyarrhythmia and hypotension were found. Abdominal investigation revealed bloated and painful abdomen, enlarged and tender liver. A splenomegaly also presented. The neurological examination was without abnormal findings.
Initial laboratory investigations revealed RBC 3.8; Hg 119; WBC 23.7; granulocytes 0.88; platelets 45; total/direct serum bilirubin level 153/145 µmol/L; ASAT 42 IU/L; ALAT 27 IU/L; total protein 61.3 g/L; albumins 27.2 g/L; fibrinogen level 8.0 g/L; prothrombin index 82%; C-reactive protein 278 mg/L; blood-urea-nitrogen (BUN) 16 mmol/L; creatinine level 308 µmol/L. The dynamics of laboratory results during the hospital treatment is shown on Table 1.
Immuno-phenotypisation of lymphocytes subsets revealed granulocytosis with lymphopenia (markedly decreased total T-lymphocytes, T-helpers, and T-helpers/cytotoxic T-cells ratio).
The abdominal ultrasonography revealed mild hepatic steatosis and partitioned gallbladder.
Microscopic agglutination test (MAT) was performed at the National Reference Laboratory (National Centre of Infectious and Parasitic Diseases – Sofia) and increased titre of antibodies against *L. icterohaemorrhagiae* (1:1600) was found.
Treatment was performed with Ceftriaxon (6 g/24 h), Methylprednisolone (initial dose 200 mg daily), Hepa-merz, Ademetionin, Human Albumin (3 times to 100 ml), symptomatic drugs, vitamins, glucose and saline infusions. During the first 18 hours, the stimulation with Furozemide was effective and the urine output was 5 500 ml, increasing in the next days to 7 000 ml/24 h. This eliminated the need of dialysis and the nitrogen parameters gradually decreased. Due to critical thrombocytopenia, thrombocyte concentrate was infused during the first three days, followed by elevation of the platelet count. Along with clinical improvement, the intensity of jaundice extremely increased in contrast to normalising of the nitrogen parameters (Figure 1), and optical hallucinations with disturbed spatial perceptions appeared. The delirium resolved after increasing the doses of hepatoprotectors. Subsequently, additional information about regular alcohol consumption was obtained. The patient was discharged on the fourteenth day in improved condition, with moderate jaundice and hepatomegaly. During the following visits of the patient, favourable recovery was registered.

DISCUSSION

Icteric leptospirosis is a severe disease in which the clinical course is often rapidly progressive. Severe cases often present late in the course

Table 1. Laboratory findings of reported patient during hospitalisation

Day / Test	1st	2nd	3rd	4th	5th	6th	7th	8th	10th	12th	14th	Ref. values
Hg	3.8	3.7	3.9	3.5	3.9	3.9	3.4	3.4	3.2	3.2	3.1	120-180
Ht	119	118	123	106	119	120	108	105	95	99	95	0.35-0.5
WBC	23.7	29.7	25.7	20.0	24.0	31.7	34.8	40.0	33.7	22.3	19.8	3.5-10.5
Gra	88	93	89	88	88	91	91	92	91	87	87	50-80
Plt	45	85	88	102	97	144	258	389	639	-	730	150-360
BUN	16	16.4	16.8	14	11	9.9	8.6	8.9	7.5	-	4.9	1.7-8.2
Cr	***308***	***276***	***231***	***156***	***48***	***56***	***93***	***85***	***65***	-	***45***	44-106
K^+	2.9	3.5	3.3	2.5	3.7	3.4	3.5	4.0	4.5	-	4.0	3.5-5.6
Na^+	133	136	138	131	129	129	128	130	131	-	134	130-151
Cl^-	-	96	94	91	88	88	90	93	98	-	97	98-108
TB	***153***	-	***223***	***227***	-	***313.9***	***285***	***276***	***246***	***208***	***129***	3.4-21
DB	145	-	208	210	-	253.1	252	246	216	176	64	0.8-8.5
ASAT	42	-	-	30	-	-	26	25	-	-	23	≤40
ALAT	27	-	-	21.6	-	-	19	20	-	-	31	≤40
GGT	108	-	-	-	-	-	-	102	-	-	-	0-60
AP	99	-	-	-	-	-	-	-	-	-	-	35-129
Amyl	573	333	169	99	-	-	-	91	-	-	-	28-100
CPK	139	-	-	-	-	-	-	-	-	-	-	80-190
TP	61.3	-	66	-	60.5	56.8	-	-	-	60.6	65	66-87
Alb	27.2	-	31	-	31.3	31	-	-	-	29	32	35-52
Fg	8.0	-	7.69	5.0	-	-	-	5.8	-	-	4.73	2.0-4.5
PI	82	-	112	102	-	-	-	94	-	-	102	80-110
CRP	278	304	189	84.5	-	35.6	41.68	-	-	56.9	52.5	0-5.0

Hg – Haemoglobin (g/L); Ht – Haematocrit; WBC – white blood cells (to 10^9 cells/L); Gra – Granulocytes (%); Plt – Platelets (to 10^9 cells/L); BUN – Urea (mmol/L); Cr – Creatinine (μmol/L); TB – Total bilirubin (μmol/L); DB – Direct bilirubin (μmol/L); ASAT – aspartate aminotransferase (IU/L); ALAT – alanine aminotransferase (IU/L); GGT – γ-glutamyltransferase (IU/L); AP – Alkaline phosphatase (IU/L); Amyl – Serum amylase (IU/L); CPK – Creatine phosphokinase (IU/L); TP – Total protein (g/L); Alb – Albumin (g/L); Fg – Fibrinogen (g/L); PI – Prothrombin index (%); CRP – C-reactive protein (mg/L).

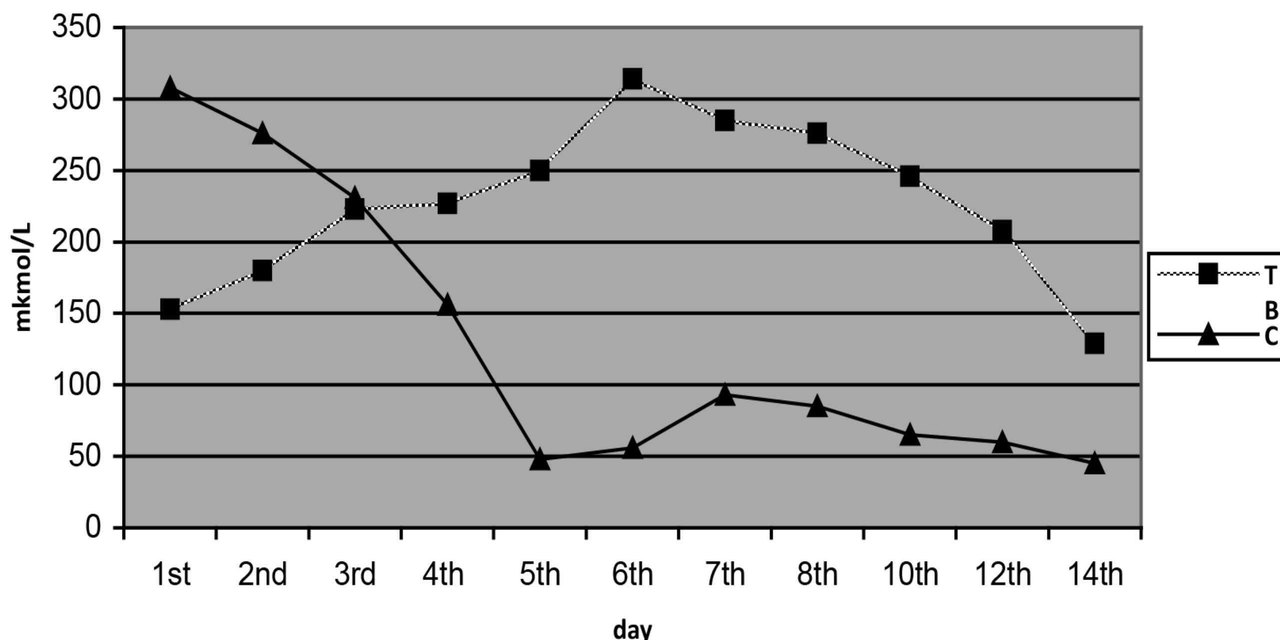

Figure 1. Dynamics of total bilirubin (TB) and creatinine (Cr) levels

of the disease, and this contributes to the high mortality rate, which ranges between 5 and 10% (2), in severe cases to 40% (5, 6). The icteric form of leptospirosis occurs in 5 to 15% of all patients. The complications of severe leptospirosis emphasize the multisystemic nature of the disease. Leptospirosis is a common cause of acute renal failure (ARF), which occurs in 16 to 40% of cases. In patients with ARF, oliguria is a significant predictor of death. Serum amylase levels are often raised significantly in association with ARF, but clinical symptoms of pancreatitis are not a common finding. Necrotising pancreatitis has been detected at autopsy. Cardiac and CNS involvement are common in severe cases and contribute to unfavourable outcome (8). In the presented case here, transitory initial arrhythmia was registered, asymptomatic amylasemia was found and CNS was intact.

Our patient developed a febrile illness with jaundice, ARF and conjunctival haemorrhages. Considering his one-week history and the initial laboratory results, supported by information about recreational exposure (regular fishing), leptospirosis was the most probable diagnosis. After the initiated treatment, renal function improved and thrombocytopenia resolved. At the same time, the intensity of jaundice increased and symptoms suggestive of delirium appeared (without neurological abnormalities). Alcoholic hepatitis was discussed in the differential diagnosis, supported by additional information on regular alcohol consumption. Alcoholic hepatitis has been well associated with the development of the above-mentioned clinical manifestations. Fever and/or neutrophilia are also commonly observed. But leptospirosis was strongly suspected in this case and was confirmed serologically by MAT. Serum bilirubin is frequently high in leptospirosis and may persist for several weeks, but transaminases and alkaline phosphatase are usually moderately elevated (8). That constellation was observed in the reported case – serum levels of transaminases were slightly elevated and contrasted to the severe jaundice. Both normal alkaline phosphatase level and abdominal ultrasonography confirmed the lack of cholestasis.

About prognosis, it is likely that alcohol abuse affects the progress of the clinical and laboratory abnormalities, predisposing to more severe clinical course of leptospirosis (14). We consider that regardless of the chronic alcohol abuse in the reported case, the prompt intensive treatment and adequate assessment of neurological symptoms improved prognosis.

MAT is the method of choice for the diagnosis of leptospirosis allowing serologic confirmation after the first week of the disease (8). However, the initiation of treatment is likely to be more effective during the first week (before serological confirmation) and usually begins empirically. The development of antibodies against *Leptospira* in the reported patient occurred in the second week after the onset of the symptoms. This could be related to the immunosuppressive effects of alcohol abuse (15). Some authors speculate that immunodeficient responses against leptospirosis take place in some patients (16).

Early antibiotic treatment of leptospirosis has been associated with a better prognosis. Penicillin has long been considered the drug of choice, though ceftriaxon and cefotaxime are emerging as acceptable (8). Cefotaxime has been suggested to be the drug of choice in alcoholic hepatitis (14), but because of suspected leptospirosis ceftriaxon was preferred in the case we report here.

Weil's disease is likely to be misdiagnosed or overlooked in patients with history of alcohol abuse due to potentially overlapping clinical features. The knowledge of symptoms of these different diagnostic possibilities and interdisciplinary approach to treatment are crucial for good prognosis.

CONCLUSIONS

Liver involvement in leptospirosis is an important factor for severity. Premorbid liver disorder poses an additional risk for prognosis.

CONFLICT OF INTEREST STATEMENT (AUTHORS):

We certify that there is no conflict of interest with any financial organisation regarding the material discussed in the manuscript.

REFERENCES:

1. Vijayachari P, Sugunan AP, Shiram AN. *Leptospirosis: an emerging global public health problem.* J Biosci. 2008; 33(4):557-569.
2. Evangelista KV, Coburn J. *Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune responses.* Future Microbiol. 2010; 5(9):1413-1425.
3. Adler B, Moctezuma A. *Leptospira and leptospirosis.* Vet Microbiol 2010; 140(3–4):287–296.
4. Ahmed N, Devi SM, Valverde M de L, Vijayachari P, Machang'u RS, Ellis WA, Hartskeerl RA. *Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species.* Ann Clin Microbiol Antimicrob 2006; 5:28.
5. Daher EF, Lima RSA, Silva Jr GB, Silva EC, Karbage NNN, Kataoka RS, Carvalho Jr PC, Magalhães MM, Mota RMS, Libório AB. *Clinical presentation of leptospirosis: a retrospective study of 201 patients in a metropolitan city of Brazil.* Braz J Infect Dis. 2010; 14(1):3-10.
6. Daher EF, Soares DS, de Menezes Fernandes ATB, Girão MMV, Sidrim PR, Pereira EDB, Rocha NA, da Silva Jr GB.

Risk factors for intensive care unit admission in patients with severe leptospirosis: a comparative study according to patients' severity. BMC Infect Dis. 2016; 16:40.

7. Spichler AS, Vilaça P, Athanazio DA, Albuquerque JOM, Buzzar M, Castro B, Seguro A, Vinetz JM. *Predictors of lethality in severe leptospirosis in urban Brazil.* Am J Trop Med Hyg. 2008; 79:911-4.
8. Levett P. *Leptospirosis.* Clin Microbiol Rev 2001; 14(2):296–326.
9. DebMandal M, Mandal S, Pal NK. *Is jaundice a prognosis of leptospirosis?* Asian Pacif J of Trop Dis. 2011; 279-281.
10. Herrmann-Storck C, Louis MS, Foucand T, Lamaury I, Deloumeaux J, Baranton G, Simonetti M, Sertour N, Nicolas M, Salin J, Cornet M. *Severe Leptospirosis in Hospitalized Patients, Guadeloupe.* Emerg Infect Dis. 2010; 16(2):331-334.
11. Ananthi B, Sriram L, Sumathi G. *Evaluation of different laboratory parameters in the diagnosis of leptospirosis.* JEMDS. 2013; 24(8):9395-9403.
12. Tailor P, Kapadia M, Modi K, Soni K. *Study of Routine Biochemistry Analytes in Leptospirosis for Evaluating Organ and System Specific Involvement.* NJIRM. 2014; 5(4):44-46.
13. Rajapakse S, Rodrigo C, Haniffa R. *Developing a clinically relevant classification to predict mortality in severe leptospirosis.* J Emerg Trauma Shock [serial online] *2010* [cited 2015 Feb 6]; 3:213-9.
14. Leonidas C, Kalambokis G, Tsianos EV. *Weil's Disease in a Patient with Chronic Viral Hepatitis and History of Alcohol Abuse.* Inter Med 2008; 47:933-7.
15. Alvarado-Esquivel C, Sánchez-Anguiano LF, Hernández-Tinoco J, Ramos-Nevárez A, Cerrillo-Soto SM, Guido-Arreola CA. *Leptospira Exposure and Patients with Liver Diseases: A Case-Control Seroprevalence Study.* Intern J of Biomed Sci. 2016; 12(2):48-52.
16. Rizvi M, Azam M, Ajmal MR, Shukla I, Malik A. *Prevalence of leptospira in acute hepatitis syndrome and assessment of IL-8 and TNF-alpha level in leptospiral Hepatitis.* Ann of Trop Med & Parasit, 2011; 105(7):499–506.

BACTERIOPHAGE THERAPY

A. Trifonova, I. Todorova, E. Savov

Department of Military Epidemiology and Hygiene, Laboratory of Microbiology, Military Medical Academy, Sofia, Bulgaria

ABSTRACT
The increasing problem of antimicrobial resistance is an issue of major concern to physicians and clinical practice, and treatment failure of infections becomes more likely. The prevention of antibiotic-resistant infections requires a multifaceted approach. One of the alternatives in this respect is to use bacteriophage therapy - an “old-new” method, known before the appearance of the first antibiotics. In this review, we present data on the current state and approaches in the use of bacteriophages as a tool for the effective treatment of infections in an era of resistance.

INTRODUCTION
The emergence of pathogen bacteria, resistant to most or even all currently available antimicrobials is an urgent problem for modern medicine. The danger of “post-antibiotic apocalypse” is completely real and the development of alternatives to antibiotic treatment is one of the most important priorities for medicine and biotechnologies.

The rapid acquisition of resistance in many microorganisms and the phenomenon of multidrug resistance are making antibiotics ineffective. This requires the use of more and more powerful antimicrobial agents, which are often the cause of damage to the organism, commensurable with the disease itself. Therefore, the possibilities are rapidly melting away and every year thousands of patients die from infections, caused by multidrug resistant bacteria. Along on the agenda comes, once again, bacteriophage therapy – a method, known before the appearance of the first antibiotics.

Essentially, phages are viruses that infect and replicate within specific bacteria. Depending on the life cycle of the phage, bacterial lysis can occur either soon after the initiation of infection (lytic cycle) or instead, following lengthy periods of delay (lysogenic cycle). In addition to permanently shutting down bacterial metabolism, lysis also releases phage progeny into the surrounding environment, allowing them to infect similar bacteria found nearby. Unlike antibiotics, they have extreme selectivity, affecting only the pathogen, without damage to the normal microflora and no side effects (1).

Even though bacteriophages are discovered by western scientists, active development of phage therapy starts in the former Soviet Union. A world-wide leader in that area is the institute in Tbilisi, founded in 1920, where scientific research on bacteriophages for therapeutic application is being conducted and at present time there is a wide variety of preparations available (2). After the “antibiotic era”, phage therapy has been practically abandoned by western medicine, but in the last few years, there is an increasing interest in it (3).

HISTORY
Despite not being demonstrated at the time, in 1896 Ernest Henkin’s observation on antibacterial activity against *Vibrio cholerae* of the water of the Jumna and Ganges rivers in India, was probably the first experiment due to bacteriophage activity. Later, in the early part of the 20th century, bacteriophages were discovered independently by Frederick Twort and Felix D’Herelle (4).

D’Herelle was the first to observe the bacteriophage phenomenon in 1910 while studying microbiologic means of controlling an epizootic of locusts in Mexico (5). He isolated the bacterium *Coccobaccillus aeridiorum* (presently known as *Enterobacter aerogenes*) (6, 7) and motivated by an ambitious desire to use these bacteria deliberately against plagues of locust, he conducted a number of experiments. During these experiments, d’Herelle observed the appearance of transparent holes in the bacterial growth. He supposed that probably they were caused by a virus. Latter he isolated this virus and used it against locust but without any success. In 1915, the British journal The Lancet published Frederick Twort’s article about “the transmissible bacterial lyses”, in which he described his observation of “the eaten edges of the colonies of *Staphylococcus*”. He observed a clear zone of lysis with the investigated strains once again.

ADDRESS FOR CORRESPONDENCE:
Dr. E. Savov,
Department of Military Epidemiology and Hygiene
Laboratory of Microbiology
Military Medical Academy
1 Georgi Sofiiski blvd.
Sofia, Bulgaria,
tel: +3592 9520337

However, Twort could not explain the event and provided only its description. This was the very first publication on bacteriophages (7). However, Twort did not explore this idea anymore, and it was another 2 years before bacteriophages were "officially" discovered by Felix d'Herelle.

The discovery of bacteriophages by d'Herelle is associated with an outbreak of severe haemorrhagic dysentery among French troops in July-August 1915. During these studies, he used bacterium-free filtrates of the patients' faecal samples, mixed with *Shigella* strains, isolated from the patients. Part of the mixtures was inoculated into experimental animals and another portion was spread on agar medium to investigate the growth of the bacteria. On the agar plates, he observed the appearance of small, clear, transparent areas. In contrast to Hankin and Twort, d'Herelle had little doubt about the nature of the phenomenon, and he proposed that it was caused by a virus capable of parasitizing bacteria. The name ''bacteriophage'' (from 'bacteria' and Greek 'phagein' 'to eat') was also proposed by d'Herelle (5, 6). The main significance of Felix d'Herelle's study is that he launched the idea to use bacteriophages for treatment of human and animal bacterial diseases, the work for which was conducted at the Hopital des Enfants-Malades in Paris in 1919. However, results of these studies were not published immediately and, therefore, the first reported application of phages to treat human infectious diseases came later from Richard Bruynoghe and Joseph Maisin who used bacteriophages to treat staphylococcal skin disease (7). Several similarly promising studies were conducted later (8, 9, 10).

The Institute of Vaccine and Sera in Tbilisi produced the first commercial anticholera phage preparation, which was reported to be used successfully for the control of epidemics threatening the south-eastern territories of the USSR (7). The Oswaldo Cruz Institute in Rio de Janeiro, Brazil, started production of the antidysentery bacteriophages in 1924 to combat dysentery in Latin American countries (11). Phages for therapeutic purposes were also produced in the United States. In the 1940s, the Eli Lilly Company (Indianapolis, IN) produced seven phage products for human use, including preparations targeted against staphylococci, streptococci, *Escherichia coli*, and other bacterial pathogens (5). However, the efficacy of phage preparations was controversial probably due to technical reasons - absence of viable phages, low phage titre or narrow strain range of the phage in these preparations and perhaps this is the main reason the commercial production to be ceased in most Western countries (7). Nevertheless, phages continued to be used therapeutically — together with or instead of antibiotics — in Eastern Europe and in the former Soviet Union. Several institutions were actively involved in this field, with activities centred at the Eliava Institute of Bacteriophage, Microbiology, and Virology (EIBMV) of the Georgian Academy of Sciences, Tbilisi, Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) of the Polish Academy of Sciences, Wroclaw, Poland (5), and mainly in Russia, Ukraine, Belarus, and Azerbaijan in the Soviet Union during the period of 1930s and 1940s. At this time methods and instructions were developed for intramuscular and even intravenous use of phages, which was crucial in cases of generalised infections. The application of mixtures of bacteriophages infecting anaerobes, *Staphylococcus,* and *Streptococcus*, produced by the EIBMV, Tbilisi, Georgia, was reported for treatment of gas gangrene (7). One of the pioneers in the application of phages in surgery was A. P. Tsulukidze who began to use such preparations in 1931 for the treatment of various diseases. Later a wider group of patients was involved in the study (12).

In the 1920s and 1940s the intestinal infections caused by *Salmonella* and *Shigella* species were a huge problem all over the world and clinical studies on phage therapy against *Salmonella* Typhi and *S.* Paratyphi were conducted (7). An article reported a slight improvement in patients' conditions, in particular, shortening of the shivering period. However, a cure was not achieved. In another study, improvement was also observed and the authors concluded that the best result was achieved using a combined intramuscular and oral application of phages (13). Zabrezhinsky and Gorstkina-Shevandrova, 1946, reported a positive effect in 64% of the cases and concluded that early start of phage treatment lead to a higher efficacy of therapy. A significant effect of phage therapy was reported even in cases where treatment was started rather late. Seventy-nine percent of the patients did not show pathological symptoms by the 4th day and 100% by the 6th day after which the stool was normalised. These data were in contrast to results obtained with ''ordinary therapy'', where only 2% (1 case out of 50 patients) showed an improvement on the 2nd day of treatment, 14% on the 4th day, and 46% on the 6th day (14). A later study reported the treatment of 52 cases of typhoid and paratyphoid diseases and compared

the conditions of phage-treated patients with the conditions of 40 patients in the control group. A polyvalent bacteriophage against *S.* Typhi and *S.* Paratyphi was used in this study. The authors reported that fever persisted for a shorter period in the experimental group of patients (38 days) as compared to the control group (52 days) (15). Phages have also been used extensively for prophylaxis in regions with a high incidence of infections. The use of phages for prophylaxis was carried out in 1929 – 1930 against bacterial diseases posing serious problems at that time, such as dysentery, typhoid fever, and staphylococcal infections. The first mass application of dysenteric bacteriophages in the USSR was performed in Alchevsk (Donbas region) in Ukraine in 1930. A similar experiment was conducted successfully later in 1935 on thousands of people in regions with a high incidence of dysentery. Results were reported at scientific conferences in 1934 and 1936 in Kiev and in 1939 in Moscow after which the dysenterial phage preparation was finally approved as a preventive measure for mass application (7).

THERAPY

In spite of the increasing interest in bacteriophages from scientist and biotechnological companies, the western medicine community is poorly informed about this kind of therapy. As possible reasons in this sense can be indicated the insufficient credibility of the phage therapy, patient and physician unfamiliarity and related limited product availability (16). Introducing bacteriophage therapy in the western medical world remains highly problematic as a consequence of few main obstacles. First, the clinical data about the safety and effectiveness of phage therapy are not considered proven and validated by European regulators. Moreover, the regulatory framework for medicinal product development, as present in most countries, requires the drugs to have a fixed chemical composition. Bacteriophages do not fit into this definition (17). Many of the western pharmaceutical companies are interested in this direction, but taking account on the intellectual property (IP) protection is a problem. It is enough for every hypothetical competitor to own a small amount of the drug, to replicate the phages, or easily isolate similar phages from the environment. Given the substantial costs and investment in the development and marketing of conventional medicinal products by the pharmaceutical industry, this is one of the major obstacles for worldwide incorporation in manufacturing of the western phage-preparations. For these reasons, some companies are exploring other possibilities, such as patenting specific phage sequences, using novel concepts related to genetically engineered phage as a vector for lethal genes, or molecules, or modified phage products (17, 18). And finally, there still stands the question of the potential negative coevolutionary consequences of unlimited use of bacteriophage therapy (17, 19). Phage therapy, when used in a flexible and sustainable manner, has the potential for saving thousands of lives every year, but due to the above-mentioned obstacles, access to that therapy remains highly problematic in the western world (17).

Potential advantages of the phages are: each phage affects only a specific type of bacteria, which is its host (this is why phages are completely harmless to the human organism); they are self-replicating but also self-limiting, because they multiply only in the presence of sensitive bacteria, and disappear with little or no trace when the pathogen is no longer present; they have the ability to cross physiological barriers such as the blood-brain barrier and get into the furthest depths of osteomyelitis in a bone; while the occurrence of a strain, resistant to a particular antibiotic takes a few years, the acquisition of resistance to phages takes a much longer period. If there is such a case, however, developing a new phage is incomparably easier and faster (during a few weeks' time), than searching and developing a new antibiotic (7-12 years). Phages were approximately 50% cheaper than antibiotics. This means that a wider application of phage therapy could lead to a significant reduction in healthcare costs (2, 5, 17, 20). Moreover, the application of phages either independently or together with antibiotics could help reduce the development of bacterial resistance (21). Unfortunately, phages have a flaw, which is a continuation of their advantage: their high selectivity is turning out to be a setback for the treatment because the pathogen has to be identified very precisely. Only then can an effective phage be selected. So-called cocktails are developed to overcome the narrow range of a single phage – preparations that include up to 30 virus strains, devoted to targeting five different pathogens (1, 5). Monophage therapy has the advantage of simplifying treatment since only a single phage preparation is needed and there is less potential for complications stemming from phage immunological interaction with the treated subjects. On the other hand, the phage cocktails' use can lead to expansion of the utility of phage formulations, and to a possible reduction of

the development of phage-resistant bacterial mutants during individual treatments, as well (1). Researchers study the different types of phages, the methods of isolation, consider gene engineering modification possibilities, specifics of interaction with pathogens and human organism, the ability of phages to express biofilm-degrading enzymes (19). Despite these efforts, to this moment a very small number of modern, double-blinded, Phase III (efficacy) clinical trials in humans have been recorded. There are a large number of publications on phage therapy, but very few reports in which the pharmacokinetics of therapeutic phage preparations is determined (1, 5, 21). Also, phages are extremely common in the environment (e.g., non-polluted water has been reported to contain ca. $2x10^8$ bacteriophages per ml) and are regularly consumed with foods (22). However, it is very important to ensure the phages' safety before widely using them as therapeutic agents (5). In this sense, the Georgian and Polish phage therapy centres are keeping extensive therapeutic phage collections, which suggests the regularly enrichment with new phages and widening the host range of the collection (3).

During the long history of using phages as therapeutic agents in Eastern Europe and the former Soviet Union (and, before the antibiotic era, in the United States), phages have been administered to humans by different routes - orally, in tablet or liquid formulations, rectally, locally (skin, eye, ear, nasal mucosa, etc.), in tampons, rinses, and creams, as aerosols or intrapleural injections, intravenously, and there are no registered reports of serious complications associated with their use (5).

In spite of pharmaceutical companies, western biotechnological companies and groups of scientists from the science institutes are actively collaborating with the Phage Therapy Centre in Tbilisi, which has opened subsidiary clinics in Canada, USA, and France. The list of treatable diseases by the specialists in these clinics is long and consists of three groups. The first includes acute and chronic skin infections, gingivitis, tracheitis, pneumonia, gastrointestinal infections, dysbacteriosis, prostatitis, urogenital infections. The second group includes diseases, in which the antibiotic action is compromised due to flowed blood circulation – wound infections (including wounds decubitus), diabetic's foot, osteomyelitis, and tropic ulcers. The third group includes the so called "superbugs" – methicillin resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, as well as streptococci, enterococci, *Salmonella*, *Shigella*, *Proteus*, etc., and fungal complications. Statements from the Centre of Phage therapy in Tbilisi assert that there is a "superphage" for every "superbug", and if there is not, they are prepared to develop one (23).

The company Biochimpharm is the manufacturer of biological preparations based on bacteriophages, founded with the separation of the bacteriophage production team of G. Eliava Institute and also located in Tbilisi. The production of various classical bacteriophage preparations continues with renewal of an existing collection of bacterial strains with newly-emerged resistant strains in order to develop additional range of highly effective preparations. Their products are available in pharmacies in Georgia and are exported to other countries. Some of them are: Phagesti (for treatment and prophylaxis of gastrointestinal infections), Phagyo, Phagestaph, and Phagepy (for treatment and prophylaxis of bacterial purulent–inflammatory infections), Phagedys (for treatment and prophylaxis of dysentery, caused by *Shigella*), Phagesal (for treatment and prophylaxis of salmonellosis), etc. (24).

The leader in development and production of phage preparations for healing purposes in Russia is the national company NPO "Mikrogen". Some of the preparations are named after the bacteria against which they are directed: "*E. coli-Proteus* bacteriophage", "*Streptococcus* bacteriohage", "*Klebsiella* purified polyvalent bacteriophage", and others – "Intesti-bacteriophage" (for treatment and prevention of diseases of the gastrointestinal tract caused by dysentery bacteria, *Salmonella*, *E. coli*, *Proteus*, enterococci, staphylococci, *P. aeruginosa* or a combination thereof), "Complex pyobacteriophage"(for treatment and prevention of inflammatory and intestinal diseases caused by staphylococci, enterococci, streptococci, *Proteus*, *P. aeruginosa*, *K. pneumoniae*, *K. oxytoca*, enteropathogenic *E. coli*,), "Sextaphag® polyvalent pyobacteriophage" (treatment and prevention of inflammatory and enteric diseases caused by staphylococci, streptococci, *Proteus*, *Klebsiella*, *P. aeruginosa* and *E. coli*). Recently at the conference "Providing epidemiology safety in surgical hospitals and ICU", scientists from Perm's subsidiary "Perm SIC Biomed" presented their project of producing innovational complex product "Diphage", directed against *Acinetobacter baumannii* and *P. aeruginosa* (two of the most common causative agents of nosocomial infections with extensive resistance to antibiotics of different classes). The specialists

found out some phage races with lytic activity against the infectious agents and also improved the technology of producing “Diphage”, studied its safety, specific activity, range, and other parameters that were necessary for commissioning. Product efficacy studies that took place at the clinical sites, demonstrated high sensitivity to a batch of “Diphage” and at the same time extraordinary antibiotic resistance. This means “Diphage” can be used not only for treatment, but also as means of preventive disinfection. Currently preclinical tests have been successfully completed (25). In Poland, an EU member state, a specific national adaptive regulation based on the Declaration of Helsinki, was issued to regulate phage therapy. A medical doctor is allowed to apply phage therapy where proven therapeutic methods do not exist or have been ineffective (e.g., in MDR infections) and provided that the patient or their legal representative gives informed consent (3). The Hirszfeld Institute of Immunology and Experimental Therapy located in Wroclaw, Poland and the clinicians involved in phage therapy there, are the most experienced group, found outside of the former Soviet Union, working on phage therapy and studying phage physiological effects. In 2005, the institute established its own phage therapy clinic (The Centre for Phage therapy, which accepts patients with drug-resistant bacterial infections), and they are now able to develop more formal trials under European Union guidelines. The institute also explores issues of phage purification, phage therapy economics, phage translocation within bodies, the role of endogenous phages in bacterial control, phage interaction with the animal immune system, and the phage therapy of children and cancer patients. They use also the “phage bank” approach, which means that they choose one or more phages from their collection (over 300 specific bacteriophage strains) which are active against a given bacterial isolate (2, 26).

CONCLUSION

Bacteriophages for therapeutic use are especially helpful in the absence of alternative treatments, as was the case in the pre-antibiotic era, and again are current in the face of declining antibiotic effectiveness and increasing awareness of their often problematic consequences. It is therefore necessary to create the appropriate regulatory and legal framework to stimulate the western companies to invest in phage therapy and make it accessible for patients in need, because when used in a flexible and sustainable manner, it has the potential to save thousands of lives every year.

REFERENCES:

1. Chan BK, Abedon ST, Loc-Carillo C. Phage cocktails and the future of phage therapy. Future Microbiol. 2013; 8(6):769-783.
2. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, Abedon S. Phage therapy in clinical practice: Treatment of human infections. Current Pharmaceutical Biotechnology. 2010; 11:69-86.
3. Pirnay J, Verbeken G, Rose T, Jennes S, Zizi M, Huys I, Lavigne R, Merabishvili M, Vaneechoutte M, Buckling A, De Vos D. Introducing yesterday’s phage therapy in today’s medicine. Future Virol. 2012; 7(4):379-390.
4. Hraiech S, Brégeon F, Rolain JM. Bacteriophage-based therapy in cystic fibrosis-associated *Pseudomonas aeruginosa* infections: rationale and current status. Drug Design, Development and Therapy. 2015; 9:3653–3663.
5. Sulakvelidze A, Alavidze Z, Morris Jr, JG. Bacteriophage therapy. Antimicrob Agents Chemother. 2001; 45(3):649-659.
6. Summers WC. Felix d’Herelle and the Origins of Molecular Biology. 1999, Yale University Press, New Haven, CT.
7. Chanishvili N. Phage therapy – history from Twort and d’Herelle through Soviet experience to current approaches. Adv Virus Res. 2012; 83:3–40.
8. Rice TB. Use of bacteriophage filtrates in treatment of suppurative conditions: Report of 300 cases. Am J Med Sci. 1930; 179:345–360.
9. Schless RA. Staphylococcus aureus meningitis: Treatment with specific bacteriophage. Am J Dis Child. 1932; 44:813–822.
10. Stout BF. Bacteriophage therapy. Texas State J Med. 1933; 29:205–209.
11. Dublanchet A, Bourne S. The epic of phage therapy. Can J Infect Dis Med Microbiol. 2007; 18:15–18.
12. Tsulukidze AP. Phage treatment in surgery. Surgery (‘‘Khirurgia’’), 1940; 12:132–133.
13. Zabrezhinsky LM, Gorstkina-Shevendrova LA (1946). Phage therapy of typhoid fever. J Microbiol Epidemiol Immunol. 4, 25–27. (1940). Experience of phage therapy of dysentery. In ‘‘Selected Articles of the Kuibishev Red Army Military-Medical Academy’’,4, pp. 193–198. KRAMMA, Kuibishev, Russian SSR.
14. Lipkin, Nikolskaya. Experience of phage therapy of dysentery. 1940, In ‘‘Selected Articles of the Kuibishev Red Army Military-Medical Academy’’, Vol. 4, pp. 193–198. KRAMMA, Kuibishev, Russian SSR.
15. Astashkevich H M. Experience of application of phage therapy for treatment of typoid and paratyphoid diseases. J Microb Epidemiol Immun. 1950; 7:42.
16. Kutter E, Kuhl S, Abedon S. Re-establishing a place for phage therapy in western medicine. 2015. Future Microbiol. 2015; 10(5):685-688.
17. Verbeken G, Huys I, Pirnay J, Jennes S, Chanishvili N, Scheres J, Górski A, De Vos D, Ceulemans C. Taking Bacteriophage therapy seriously: A moral argument. BioMed Res Int. 2014; Article ID 621316.
18. Henein A. What are the limitations on the wider therapeutic use of phage? Bacteriophage 2013; 3:2, e24872.
19. Meaden S, Koskella B. Exploring the risks of phage application in the environment. Front Microbiol. 2013; 4(358).
20. Miedzybrodzki R, Fortuna W, Weber-Dabrowska B, Górski A. Phage therapy of the staphylococcal infections (including MRSA) may be less expensive than antibiotic treatment. Postepy Hig Med Dosw. 2007; 61:461-465.
21. Kutter E, Kuhl S, Alavidze Z, Blasdel B. Phage Therapy: Bacteriophages as Natural, Self-Limiting Antibiotics. ResearchGate. 2012; Chapter 112.
22. Bergh O, Borsheim KY, Bratbak, Heldal M. High abundance of viruses found in aquatic environments. Nature. 1989; 340:467-468.
23. www.phagetherapycenter.com
24. www.biochimpharm.ge
25. www.microgen.ru
26. Fortuna W, Miedzybrodzki R, Weber-Dabrowska B, Górski A. A Bacteriophage therapy in children: Facts and prospects. Med Sci Monit. 2008; 14(8):RA126-132.

CELL SURFACE COMPONENTS DETERMINING AGGREGATION IN LACTOBACILLI (MINIREVIEW)

T. Yungareva

LB Bulgaricum PLC

ABSTRACT

The species from the*Lactobacillus*genus are the most common microorganismsexploited as probiotics and food preservatives. Processes such as aggregation, co-aggregation, adhesion, and biofilm formation are important mechanisms that allow colonisation of intestinal and urogenital tract, the oral cavity and different food surfaces. Aggregation is one of the desirable characteristics of probiotic bacteria as it could increase bacterial capacity to compete and it could play an important role in human health. Due to co-aggregation with pathogenic bacteria such as *Escherichia coli*, *Salmonella* Typhimurium,*Campylobactercoli,* and *Campylobacterjejuni,*lactobacilli prevent pathogenic colonisation and assist in the removal of pathogens from the human mucosal surfaces. In some lactobacilli aggregation is the first step of adhesion or biofilm formation. Their ability to form aggregates is a species- and strain-specific feature. The process is a result of a complex interaction between cell surface components, such as proteins, glycoproteins, lipotechoic and techoic acids, secreted factors and enzymes. In some *Lactobacillus* strains the components involved in autoaggregation and adherence or in autoaggregation and co-aggregation are identical, while in other strains each process is determined by a different set of components. The present work presents a brief overview of cell components in lactobacilli considered to be involved in aggregation and co-aggregation.

Keywords: *Lactobacillus,* aggregation, co-aggregation, pathogenic bacteria

INTRODUCTION

Autoaggregation and co-aggregation are phenomena related to the formation of physical aggregates of bacterial cells of the same strain or of two or more species. The process is considered an essential step in the formation of bacterial biofilms and the interaction of probiotic bacteria with the host and potentially pathogenic bacterial species (1-8).

The aggregation of a probiotic bacterial culture is considered an important property in adhesion to intestinal epithelial cells (1, 2) or colonisation of the oral cavity or the urogenital tract (3, 4, 5, 9, 10). The formed aggregates may then serve as a protective layer against colonisation or adherence of pathogenic bacteria to the mucosal surface (5, 6, 7, 11, 12). And finally the direct co-aggregation with pathogens may facilitate their clearance from the host (6, 8, 13). In addition, aggregation may facilitate genetic exchange and immunomodulation of colonic mucosa (14, 15, 16, 17, 18, 19).

The factors that determine auto- or co-aggregation are not always straightforward to be determined, possibly due to the participation of multiple structures and molecules in the binding process. As the most common genus involved in food industry and probiotic effect studies, the aggregation phenotype in lactobacilli is of special interest. This review has attempted to draw a summary of current data on the factors involved in the aggregation of lactobacilli that may help understanding of the mechanism underlying the aggregation phenotype in these species. Furthermore this knowledge may be useful in the selection of novel probiotic *Lactobacillus* spp. strains.

COMPONENTS DETERMINING AGGREGATION IN *LACTOBACILLUS*

Aggregation mediated by a proteinaceous Aggregation promoting factor and teichoic acids

One of the most common cell components involved in the process of aggregation in lactobacilli are surface or secreted proteins. They are called Aggregation promoting factors (Apf) and mediate direct or indirect cell aggregation.

In the vaginal isolate *Lactobacillus gasseri*2459, for example, there are at least two factors involved in autoaggregation– the first one is a protein attached to the cellsurface and the other one is secreted into the medium as an aggregation promoting factor (Apf). This Apf is a pheromone-like compound with molecular weight less than 2 kDa. It induces the synthesis of adhesion proteins in some Gram–positive bacteria, in particular *Lactobacillus plantarum* LL441 and *Enterococcus faecalis* EF. After 45 minutes of contact with this Apf *L. plantarum* LL441 starts to express a 75-kDa protein. In *E.faecalis* EF the receptor of the Apf is suggested to be a glycoprotein(1).

Many authors provide evidence for the role of cell wall anchored teichoic acids (WTA) or lipoteichoic acids (LTA) as receptors for proteins responsible for cellaggregation. In *L.gasseri*4B2 (previously misclassified as *L. plantarum*)

ADDRESS FOR CORRESPONDENCE:
TsvetelinaYungareva
LB Bulgaricum PLC, R&D Center
14 MalashevskaStr, Sofia 1202, Bulgaria
e-mail: yungareva.ts@lbbulgaricum.bg

Reniero et al. reported an Apf protein of about 32 kDa andimplicated in aggregation. They showed that Apf-promoted aggregation is observed only in strains containing an appropriate receptor, identified as α-1,2-glucose-substitutedLTA andWTA(15). Schachtsiek et al. observed that a nonproteinaceous surface component is involved in the co-aggregation between *L. gasseri* 4B2 and pathogenic bacteria (20).

Subsequent studies, demonstrate that Apf proteins have an important role in cell shape formation/ preservation (21). The Apf proteins of two *L. gasseri* and four *Lactobacillusjohnsonii* strains have recently been described as novel surface proteins. They are encoded by two tandem *apf 1* and *apf 2* genes that have similar gene organisation and characteristics to the S-layer protein genesand influence the shape of cells (22).

The factor, which mediates aggregation in *Lactobacilluscrispatus*M247 is found to be similar to the Apf of *L. gasseri* 4B2 (15,23). However, *L. crispatus* M247 has a homologous non-aggregating mutant Mu5 which still produces its Apf. The non-aggregating phenotype in Mu5 may be a result of a mutation, causing changes in another molecule involved in aggregation, which may have the role of an Apf receptor. One of the putative receptors is 1,2-glucose-substituted WTA or LTA. Therefore the non-aggregating phenotype in *L. crispatus* Mu5 could be a result of a defect in glycosylation of teichoic acids (15, 23).

The putative apf gene of *Lactobacillusacidophilus* NCFM is examined too. This strain shows autoaggregation and co-aggregation with *Clostridium histoliticum* and *Staphylococcus aureus*(24). No direct evidence of a correlation between the expression of the*apf*gene and the aggregative phenotype was found, suggesting that the Apf may not be the only component involved in aggregation. Furthermore, an NCFM mutant, carrying a deletion in one of the genes, involved in the LTA biosynthetic pathway, did not aggregate, which indicates a potential role of LTA in the aggregation phenotype of *L. acidophilus* NCFM.

Notably, other results show that bacterial Apfs could be involved in promoting bile tolerance and interaction with the host epithelium and consequently may support the fitness and adaptation of microorganisms to the gastrointestinal tract (25).

Role of S-layer proteins in the aggregation phenotype

Other components involved in the aggregation phenomenon are proteins from the bacterial surface layer (S-layer).

L.crispatus ZJ001, isolated from pig intestines has an S-layer protein of approximately 42 kDa, which plays a key role in autoaggregation and adhesion to HeLa cells. The S-layer proteins from *L. crispatus* ZJ001 also inhibited adhesion of*S.*Typhimurium and *E. coli* O157:H7 to HeLa cells (26).

Autoaggregation of *Lactobacillus kefiri*CIDCA 8321 and its co-aggregation with the yeast *Saccharomyces lipolytica* CIDCA 812are mediated by thermolabilesurface molecules with a lectin-like activity non-covalently bound to the S-layer. This strain also agglutinates human red blood cells, but has poor adhesion to Caco-2 cells(27, 28, 29).

In*L. acidophilus* M 92 a 45-kDa S-layer protein is responsible for aggregation. This strain is shown to have a strong autoaggregation phenotype. It also demonstrates co-aggregation with another potential probiotic strain *E.faecium* L3 and also with *E.coli* and *S.* Typhimurium. The co-aggregation with other probiotic strains could increase the colonisation potential of *L. acidophilus*M 92 in a mixed probiotic culture (30).

Other cellsurface proteins involved in aggregation

Other cellsurface proteins mediating aggregation are also described in lactobacilli. They are in most cases novel surface proteins and some of them are studied in detail.

One such novel protein is the co-aggregationpromoting factor (Cpf), described in *Lactobacillus coryniformis*DSM 20001^T. Unlike earlier studies, limited to lactobacilli of human and animal origin (15, 31), this is the first example of a *Lactobacillus* species of agricultural origin which has aggregative phenotype (6). Strains of *L. coryniformis* are commonly found in silage, cow dung, dairy barn air (32), as well as in cheese, salami, and Turkish boza (33, 34, 35).Futhermore, Cpf is the first described surface protein which mediates co-aggregation with the major species involved in food-borne *Campylobacter* infections - *C. coli* and *C. jejuni*(36). It also co-aggregates with *E.coli* K88. Cpf is a novel surface protein which is a member of the Csc family of proteins. The proteins from this family have cellsurface location(37). All of them - CscA, CscB, CscC,andCscD - have a signal peptide for secretion by the Sec-dependent pathway, while some of them have cell surface anchors, novel WxL domains, and putative domains for sugar binding and degradation. Cpf is a product of the*cscB* gene. CscB proteins are non-covalently bound to the bacterial cell surface with WxL domains which are involved in binding to peptidoglycan (6, 38, 39 ,40).

Several cell surface proteins are found and studied for other *Lactobacillus* species.*Lactobacillus sakei*23Khas a set of components involved in aggregation and biofilm formation, which is responsible for meat surface colonisation. The first set contains two proteins with a cell wall-binding Lys M domain in their N-terminal region and a C-terminal domain similar to Apf of intestinal lactobacilli and enterococci (21). The

proteins from the other set have two repeated GW-like modules in their N-terminal sequence involved in LTA binding and a C-terminal cell wall-binding P60 domain (PF00877) (41)**.** All of these proteins share a central region rich in Ser/Thr/Gln and demonstrate physical properties similar to S–layer proteins. Furthermore, fifteen proteins from several gene clusters have a C-terminal WXL-like domain (42) with a YxxT(L/I)TW(T/S)L motif which is observed in Cpf surface protein, mediating co-aggregation of *L. coryneformis* DSM 20001T with *E. coli* (22)**.** These proteins are probably a part of multicomponent complex on the surface of the bacterium (43).

Recently, the largest aggregation promoting protein in LAB (AggLb) is described in *Lactobacillus paracasei*subsp.*paracasei*BGNJ1-64. Its molecular weight is 318.6 kDa (44). AggLb is a collagen-binding protein and has a similar structure to lactococcalAggL. The genes encoding both AggLb and AggLare located on plasmids (45)**.** AggLb contains a conservative C-terminal LPXTG motif (46)for cell wall anchoring, six collagen-binding domains and20 repeats of the CnaB-like domain. The autoaggregationability of strains depends on the collagen-binding domainsand the first two CnaB-like domains, while the last 18 C-terminal CnaB-like domains are not required for autoaggregation. They likely function as an antenna which exposes the collagen-binding domains to the surface to improve target protein interactions (47). Another *L. paracasei*subsp.*paracasei*strain, BGNJ2-8, has a large self-aggregationpromoting protein with molecular weight >200 kDa which shares some similarity with biofilm-associated proteins (48).

The extracellular protein D1 from *L.plantarum* NCIMB 8826 plays a role as an aggregation promoting factor and an adhesin, displayed affinity to mucin (mucin type III, from porcine stomach) and fibronectin (49). This protein has an internal region characterised by an abundance of uncharged polar amino acids, notably, serine and threonine. This serine/threonine-rich protein is related to the autoaggregative phenotype and the attachment to host surfaces. It mediates the molecular cross talk with the human host. The purified ST peptide is able to interact with human dendritic cells, promoting a tolerogenic and anti-inflammatory phenotype (50, 51).

Exopolysaccharides and enzymes contributing to cell aggregation

Other cell components which influence forming of cell aggregatesareextracellular polysaccharides (EPSs) (52)**.**Aslim et al. reported that aggregation in some *Lactobacillusdelbruekii*ssp. *bulgaricus*strains correlates with production of EPSs. *L. delbruekii*ssp.*bulgaricus*B3 and G12 which are high EPS-producing strains showed greater autoaggregation and co-aggregation with *E. coli* ATCC 11230 in contrast with low EPS-producing strains *L. delbruekii*ssp.*bulgaricus*22 and B2 (53).

Enzymes of some *Lactobacillus reuteri* strains participate in cell aggregation. In a pig intestinal isolate *L. reuteri*1063aputative ATP-dependent RNA helicase participates as a mediator of cell aggregation. This enzyme belongs to the DEAD-box helicases family and contains all eight motifs from the conservative region typical for this family, including the D-E-A-D box (54, 55).

Glucosyltransferase A (GtfA) of *L. reuteri* TMW1.106 is also implicated in the process of aggregation. Glucan produced from sucrose by GtfA is responsible for cell aggregation. It is observed that production of glucan has a key role in aggregation of Gram-positive bacteria (56, 57,58, 59, 60).

In a vaginal isolate *L. plantarum* CMPG5300 the highly autoaggregative and adhesive phenotype dependent on its *srtA* gene, encodes the housekeeping sortaseA (61).

CONCLUSION

A great variety of components involved in the aggregation processamong *Lactobacillus* species evenamong *Lactobacillus*strainsis observed. The study of interactions leading to formation of aggregates is important for selection of lactobacilli which could be used in food preservation or in manufacture of probiotic products and functional foods with healthy benefits.

REFERENCES

1. Boris S, Suarez JE, Barbes C.*Characterization of the aggregation promoting factor from Lactobacillus gasseri, a vaginal isolate*. J ApplMicrobiol. 1997; 83:413-420.
2. Reid G, Hawthorn L-A, Mandatori R, Cook RL, Beg HS. *Adhesion of lactobacilli to polymer surfaces in vivo and vitro.* Microbial Ecology. 1988; 16: 241-251.
3. Kolenbrander, PE. *Oral microbial communities: biofilms, interactions, and genetic systems.* Annu. Rev. Microbiol. 2000; 54:413-439.
4. Mastromarino P, Brigidi P, Macchia S, Maggi L, Pirovano F, Trinchieri V, Conte U, Matteuzzi D. *Characterization and selection of vaginal Lactobacillus strains for the preparation of vaginal tablets.* J. Appl. Microbiol. 2002; 93:884-893.
5. Reid G, McGroarty JA, Domingue PAG, Chow AW, Bruce AW, Eisen A, Costerton JW. *Coaggregation of urogenital bacteria in vitro and in vivo.*Curr. Microbiol. 1990; 20:47-52.
6. Schachtsiek M, Hammes WP, Hertel C. *Characterization of Lactobacillus cornyformis DSM 20001T surface protein Cpf mediating coaggregation with and aggregation among pathogens.* Appl Environ Microbiol 2004; 70: 7078–7085.
7. Younes JA, van der Mei HC, van den Heuvel E, Busscher HJ, Reid G (2012) *Adhesion forces and coaggregation between vaginal staphylococci and lactobacilli.*PLoS One 7: e36917.
8. Bujnakova D, Kmet V. *Aggregation of animal lactobacilli with O157 enterohemorrhagic Escherichia coli.* J. Vet. Med. B Infect. Dis. Vet. Public Health. 2002; 49:152–154.
9. Verdenelli MC, Coman MM, Cecchini C, Silvi S, Orpianesi C, Cresci A. *Evaluation of antipathogenic activity and adherence properties of human Lactobacillus strains for vaginal formulations.* J. Appl. Microbiol. 2014; 116;1297–1307.
10. Kaewnopparat S, Dangmanee N, Kaewnopparat N, Srichana T, Chulasiri M, Settharaksa S. *In vitro probiotic properties of Lactobacillus fermentum SK5 isolated from vagina of a healthy woman.* Anaerobe. 2013; 22:6–13.
11. Gorska S, Buda B, Brzozowska E, Schwarzer M, Srutkova D, Kozakova H, et al. *Identification of Lactobacillus proteins with different recognition patterns between immune rabbit sera and nonimmune mice or human sera.* BMC Microbiol. 2016; 16:17 10.
12. Nami Y, Abdullah N, Haghshenas B, Radiah D, Rosli R, Khosroushahi AY. *Probiotic potential and biotherapeuticeffects of newly isolated vaginal Lactobacillus acidophilus 36YL strain on cancer cells.* Anaerobe. 2014; 28;29–36.
13. do Carmo MS, Noronha FMF, Arruda MO, da Silva Costa ÊP, Bomfim MRQ, Monteiro AS, Ferro TAF, Fernandes ES, Girón JA, Monteiro-Neto V. *Lactobacillus fermentum ATCC 23271 displays*

in vitro inhibitory activities against Candida spp. Front Microbiol. 2016; 7: 1722.

14. Cesena, C, Morelli L, Alander M, Siljander T, Tuomola E, Salminen S, Mattila-Sandholm T, Vilpponen-Salmela T, von Wright A. *Lactobacillus crispatus and its nonaggregating mutant in human colonization trials*. J. Dairy Sci. 2001; 84:1001–1010.
15. Reniero, R, Cocconcelli P, Bottazzi V, Morelli L. *High frequency of conjugation in Lactobacillus mediated by an aggregation-promoting factor.* J. Gen. Microbiol. 1992; 138:763–768.
16. Voltan S, Castagliuolo I, Elli M, Longo S, Brun P, D'Inca R, Porzionato A, Macchi V, Palu G, Sturniolo GC, Morelli L, Martines D. *Aggregating phenotype in Lactobacillus crispatus determines intestinal colonization and TLR2 and TLR4 modulation in murine colonic mucosa.*Clin. Vaccine Immunol. 2007; 14:1138–1148.
17. Yamaguchi T, Kasamo K, Chuman M, Machigashira M, Inoue M, Sueda T. *Preparation and characterization of an Actinomycesnaeslundii aggregation factor that mediates coaggregation with Porphyromonasgingivalis.* J. Periodontal Res. 1998; 33:460–468.
18. Bensing BA, Dunny GM. *Cloning and molecular analysis of genes affecting expression of binding substances, the recipient-encoded receptor(s) mediating mating aggregate formation in Enterococcus faecalis.* J. Bacteriol. 1993; 175:7421-7429.
19. Gasson, MJ, Swindell S, Maeda S, Dodd HM. *Molecular rearrangement of lactose plasmid DNA associated with high-frequency transfer and cell aggregation in Lactococcuslactis 712.* Mol. Microbiol. 1992; 6:3213-3223.
20. Schachtsiek M, Hammes W, Hertel C. *Characterization of Lactobacillus coryniformis DSM20001T surface protein Cpf mediating coaggregation with and aggregation among pathogens.* Appl. Environ. Microbiol. 2004; 70, 7078–7085.
21. Jankovic I, Ventura M, Meylan V, Rouvet M, Elli M, Zink R. *Contribution of aggregation-promoting factor to maintenance of cell shape in Lactobacillus gasseri 4B2.* J Bacteriol. 2003; 185:3288-3296.
22. Ventura M, Jankovic I, Walker D C, Pridmore R D, Zink R. *Identification and characterization of novel surface proteins in Lactobacillus johnsonii and Lactobacillus gasseri.*Appl Environ Microbiol. 2002; 68:6172-6181.
23. Marcotte H, Ferrari S, Cesena C, Hammarstrom L, Morelli L, Pozzi G, Oggioni MR. *The aggregation-promoting factor of Lactobacillus crispatus M247 and its genetic locus.* J ApplMicrobiol. 2004; 97:749-756.
24. Collado MC, Meriluoto J, Salminen S. *Adhesion and aggregation properties of probiotic and pathogen strains.* Eur. Food Res. Technol.2008; *226:1065*-1073.
25. Goh YJ, Klaenhammer TR. *Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of Lactobacillus acidophilus NCFM.*Appl Environ Microbiol. 2010; 76: 5005–5012.
26. Chen X, Xu J , Shuai J, Chen J, Zhang Z, Fang W. *The S-layer proteins of Lactobacillus crispatus strain ZJ001 is responsible for competitive exclusion against Escherichia coli O157:H7 and Salmonella typhimurium.* International Journal of Food Microbiology. 2007; 115: 307–312.
27. Garrote GL, Delfederico L, Bibiloni R, Abraham AG. Pérez PF, Semorile L, De Antoni GL. *Lactobacilli isolated from kefir grains: Evidence of the presence of S-layer proteins.* Journal of Dairy Research. 2004; 71: 222-230.
28. Golowczyc MA, Mobili P, Garrote GL, Serradell MA, Abraham AG, De Antoni, GL. *Interaction between Lactobacillus kefir and Saccharomyces lipolytica isolated from kefir grains: Evidence for lectin-like activity of bacterial surface proteins.* Journal of Dairy Research. 2009; 76: 111-116.
29. Mobili P,Gerbino E, Tymczyszyn EE, Gómez-Zavaglia A. *S- layers in lactobacilli: structural characteristics and putative role in surface and probiotic properties of whole bacteria.* In: Méndez-Vilas, A. (Ed.), Current Research. Technology and Education Topics in Applied Microbiology and Microbial Biotechnology Formatex Research Center, Spain, 2010, 1224–1243.
30. Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S. *Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92.* J ApplMicrobiol. 2003; 94: 981–987.
31. Boris S, Suarez JE, Vazquez F, Barbes C.*Adherence of human lactobacilli to vaginal epithelial cells and interaction with uropathogens.*Infect. Immun. 1998; 66**:**1985-1989.
32. Abo-Elnaga IG, Kandler O. *ZurTaxonomie der Gattung Lactobacillus Beijerinck.*I. Das Subgenus StreptobacteriumOrla Jensen. Zentbl. Bakteriol. Parasitenkd. Infektionskr,1965, Abt. II 119:1-36.
33. Hegazi FZ, Abo-Elnaga IG. *Characters of Lactobacillus coryniformis, isolated from an Iraqi cheese.*Zentrbl. Bakteriol. Naturwiss. 1980; 135:205-211.
34. Samelis J, Maurogenakis F, Metaxopoulos J. *Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami.* Int. J. Food Microbiol. 1994; 23:179-196.
35. Hancioglu, O, Karapinar M. *Microflora of Boza, a traditional fermented Turkish beverage.* Int. J. Food Microbiol. 1997; 35:271-274.
36. Nachamkin I, Engberg J, Moller F Aarestrup. *Diagnoses and antimicrobial susceptibility of Campylobacter species.* In I. Nachamkin and M. J. Blaser (ed.), Campylobacter. American Society for Microbiology, Washington, D.C, 2000, 45-66.
37. Sabet C, Lecuit M, Cabanes D, Cossart P, Bierne H. *LPXTG protein InlJ, a newly identified internalin involved in Listeria monocytogenes virulence.* Infect. Immun. 2005; 73:6912–6922.
38. Brinster S, Furlan S, Serror P.*C-Terminal WxL Domain Mediates Cell Wall Binding in Enterococcus faecalisand Other Gram-Positive Bacteria*.J. Bacteriol. 2007; 189: (4) 1244-1253.
39. Siezen R, Boekhorst J, Muscariello L, Molenaar D,Renckens B, Kleerebezem M. *Lactobacillus plantarumgene clusters encoding putative cell-surface protein complexes for carbohydrate utilization are conserved in specific gram-positive bacteria.*BMC Genomic. 2006; 7:126.
40. Galloway-Peña JR, Liang X, Singh KV, Yadav P, Chang C, La Rosa SL, Shelburne S, Ton-That H, Höök M, Murray BE. *The identification and functional characterization of WxL proteins from Enterococcus faecium reveal surface proteins involved in extracellular matrix interactions.*J Bacteriol. 2015; 197(5): 882–892.
41. Jonquières R, Bierne H, Fiedler F, Gounon P, Cossart P. *Interaction between the protein InlB of Listeria monocytogenes and lipoteichoic acid: a novel mechanism of protein association at the surface of Gram-positive bacteria.* Mol. Microbiol. 1999; 34, 902–914.
42. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, SandbrinkHM, Fiers MW, Stiekema W, Lankhorst RM, Bron PA, Hoffer SM, Groot MN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ. *Complete genome sequence of Lactobacillus plantarum WCFS1.* Proc. Natl. Acad. Sci. USA.2004; 100: 1900–1995
43. Chaillou S, Champomier-Vergès MC, Cornet M, Crutz-Le Coq AM, Dudez AM, Martin V, Beaufils S, Darbon-Rongère E, Bossy R, Loux V, Zagorec M. *The complete genome sequence of the meat-borne lactic acid bacterium Lactobacillus sakei 23K.* Nature Biotechnology.2005; 23, 1527 - 1533
44. Miljkovic M, Strahinic I, Tolinacki M, Zivkovic M, Kojic S, Golic N, Kojic M.*AggLb is the largest cell-aggregation factor from Lactobacillus paracasei subsp. paracasei BGNJ1-64, functions in collagen adhesion, and pathogen exclusion in vitro.*PLoS One. 2015; 10(5): e0126387.
45. Kojic M, Jovcic B, Strahinic I, Begovic J, Lozo J, Veljovic K, et al. *Cloning and expression of novel lactococcal aggregation factor from Lactococcuslactis subsp. lactis BGKP1.* BMC Microbiol. 2011; 11: 265.
46. Ton-That H, Lui G, Mazmanian SK, Faul KF, Schneewind O. *Purification and characterization of sortase, the trans-peptidase that cleaves surface proteins of* Staphylococcus aureus *at the LPXTG motif.* ProcNatlAcadSci U S A. 1999; 96:12424–12429.
47. Miljkovic M, Bertani I, Fira D, Jovcic B, Novovic K, Venturi V, KojicM.*Shortening of the Lactobacillus paracasei subsp. paracasei BGNJ1-64 AggLb protein switches Its activity from auto-aggregation to biofilm formation.* Front Microbiol. 2016; 8;7:1422.
48. Lozo J, Jovcic B, Kojic M, Dalgalarrondo M, Chobert JM, Haertlé T, et al. *Molecular characterization of a novel bacteriocin and an unusually large aggregation factor of Lactobacillus paracasei subsp. paracasei BGSJ2-8, a natural isolate from homemade cheese.*CurrMicrobiol. 2007; 55: 266–271.
49. Sanchez B, Schmitter JM, Urdaci MC. Identification of novel proteins secreted by Lactobacillus plantarum that bind to mucin and fibronectin. J. Mol. Microbiol. Biotechnol. 2009; 17:158–162.
50. Bernardo D, Sanchez B, Al-Hassi HO, Mann ER, Urdaci MC, Knight SC, Margolles A. Microbiota/host crosstalk biomarkers: regulatory response of human intestinal dendritic cells exposed to Lactobacillus extracellular encrypted peptide. PLoS One. 2012; 7:e36262.
51. Hevia A, Martínez N, Ladero V, Alvarez MA, Margolles A, Sánchez B. An extracellular serine/threonine-rich protein from Lactobacillus plantarum NCIMB 8826 is a novel aggregation-promoting factor with affinity to mucin. Appl EnvironMicrobiol. 2013; 79, 6059–6066.)
52. Kumar CG, Anad SK. *Significance of microbial biofilms in food industry: a review.* Int. J. Food Microbiol. 1998; 42:9–27.
53. Aslim B, Onail D, Beyatli Y. *Factors Influencing Autoaggregation and Aggregation of Lactobacillus delbrueckii subsp. bulgaricus Isolated from Handmade Yogurt*. Journal of Food Protection. 2007;70(1): 223–227
54. Schmid, SR, Linder, P.*D-E-A-D protein family of putative RNA helicases.* MolMicrobiol. 1992*;* 6*:* 283–292.
55. Roos S, Lindgren S, Jonsson H. *Autoaggregation of Lactobacillus reuteri is mediated by a putative DEAD-box helicase.*MolMicrobiol. 1999; 32(2):427-36.
56. Banas JA, Vickerman MM. *Glucan-binding proteins of the oral streptococci.* Crit Rev Oral Biol Med. 2003; 14, 89–99.
57. Gibbons RJ, Fitzgerald RJ. *Dextran-induced agglutination of Streptococcus mutans , and its potential role in the formation of microbial dental plaques.* J Bacteriol. 1969, 98:341–346.
58. Lynch DJ, Fountain TL, Mazurkiewicz JE, Banas, JA. *Glucan-binding proteins are essential for shaping Streptococcus Mutans biofilm architecture.* FEMS MicrobiolLett. 2007; 268: 158–165.
59. Walter J, Schwab C, Loach DM, Gänzle MG , Tannock GW. *Glucosyltransferase A (GtfA) and inulosucrase (Inu) of Lactobacillus reuteri TMW1.106 contribute to cell aggregation, in vitro biofilm formation, and colonization of the mouse gastrointestinal tract.* Microbiology. 2008; 154: 72-80.
60. Narimatsu M, Noiri Y, Itoh S, Noguchi N, Kawahara T, Ebisu S. *Essential role for the gtfA gene encoding a putative glycosyltransferasein the adherence of Porphyromonasgingivalis.* Infect. Immun. 2004; 72:2698–2702.
61. Malik S, Petrova MI, Claes IJ, Verhoeven TL, Busschaert P, Vaneechoutte M, et al. *The highly autoaggregative and adhesive phenotype of the vaginal Lactobacillus plantarum strain CMPG5300 is sortase dependent.*Appl Environ Microbiol. 2013; 79, 4576–4685.

CONFLICT OF INTEREST STATEMENT (AUTHORS)

I certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Author name Date Signature

When there is conflict of interest, specify the company title and the relationship with the Author.

CONFLICT OF INTEREST STATEMENT (REVIEWERS)

I certify that have no personal or financial conflict of interest with authors of the manuscript provided me for review.

Reviewer name Date Signature

When there is conflict of interest, please specify the relationship with the Author.

STATEMENT ABOUT PROTECTION OF HUMAN SUBJECTS AND ANIMALS IN RESEARCH

I certify that this study involving human subjects is in accordance with the Helsinky declaration of 1975 as revised in 2000 and that it has been approved by the relevant institutional Ethical Committee.

Author name Date Signature

I certify that this study involving animals followed the institutional and national guide for the care and use of laboratory animals.

Author name Date Signature