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CONTENTS

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ACKNOWLEDGEMENTS
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SEROTYPE DISTRIBUTION AND ANTIBIOTIC SUSCEPTIBILITY OF S. PNEUMONIAE CAUSING INVASIVE INFECTIONS AND ACUTE OTITIS MEDIA IN BULGARIA BEFORE INTRODUCTION OF PNEUMOCOCCAL CONJUGATE VACCINE

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SUMMARY:
We report data on serotype distribution and antibiotic susceptibility of 36 invasive S. pneumoniae isolates and 50 isolates from acute otitis media (AOM). The invasive isolates were collected in 14 hospitals located in 10 Bulgarian cities between June 2009 and December 2010. Sixteen isolates were obtained from infections in children ≤5 years and 20 – from children >5 years and adults. The AOM isolates were collected between October 2009 and November 2010 from children aged £ 8 years. The universal infant immunization in Bulgaria with PCV10 was initiated in April 2010, so we considered the time period to the end of 2010 still representative for the pre-vaccine serotype distribution in Bulgaria.
The most frequently encountered serotype among invasive isolates was 19F (13,9%), followed by 1 (11,1%), 4 (8,3%), 6B, 23F, 9V and 3 (5,6% each). Serotypes 6A, 19A, 18C and 7F were represented by one isolate each. Among the 16 IPD isolates from children <5 years the most frequent serotypes were 19F (n=4) and 1 and 4 (n=3 each), followed by 6B, 23F, 9V and 18C (n=1 each). The most commonly encountered serotype among AOM isolates was 19F (24%), followed by 6B (14%), 14 (8%), 9V and 3 (6% each) and 23F and 18C (4% each). According to CLSI breakpoints, among meningitis isolates penicillin-resistance rate was 36,8%. All of the non-meningitis isolates were penicillin susceptible with the exception of one isolate which was intermediate. Among invasive isolates erythromycin, clindamycin and TMP/SMX resistance rates were 17%, 17% and 33%, respectively. The majority of AOM isolates (52%) were penicillin non-susceptible, of which 12% were penicillin-resistant and 40% penicillin-intermediate. AOM isolates resistant to erythromycin, clindamycin and TMP/SMX were 44%, 32% and 56%, respectively. Among both invasive and AOM strains, resistance to levofloxacin and rifampin was not found. Most of the penicillin non-susceptible and polyresistant S. pneumoniae isolates belonged to serotypes 19F, 6B and 14.

Keywords: S. pneumoniae, invasive, acute otitis media (AOM), Bulgaria, serotype, susceptibility, pneumococcal conjugate vaccine (PCV)

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INTRODUCTION

Streptococcus pneumoniae is a leading causative pathogen in acute otitis media (AOM), pneumonia, meningitis and sepsis Those most at risk from infection are infants, children ≤5 years of age and the elderly aged >65 years (1).
Invasive pneumococcal disease (IPD) such as bacteraemia and meningitis develops when infection spreads to normally sterile sites of the body, such as the blood or cerebrospinal fluid. Pneumonia can also become invasive when bacteraemia or empyema develops. Overall, an estimated 800,000 children under the age of 5 years die from pneumococcal disease each year, mostly in developing countries. Predisposing risk factors for IPD and pneumonia include close-contact facilities, poor nutrition, concomitant morbidities (chronic lung disease, immunodeficiency, haematological disease, diabetes) and a history of smoking (1).
In Bulgaria, according to the ECDC Annual epidemiological report on communicable diseases in Europe based on TESSy data for 2006 and 2007 there are 1 and 39 IPD cases reported respectively, for both mainly meningitides (2). According to the yearly epidemiologic analysis of NCIPD (3, 4) in 2005, 2006, 2007 and 2008 are reported 52, 48, 39 and 35 cases of pneumococcal meningitis, respectively. Important point in this reports is the high number of bacterial meningitis with unknown etiology - nearly half of the approximately 250 cases per year for years 2005 – 2007 are with unknown bacterial etiology.
Other local source for epidemiological data is the Bulgarian Surveillance Tracking Antimicrobial Resistance (BulSTAR) - laboratory based surveillance system for annual reporting of the isolation and antimicrobial susceptibility of clinically significant microorganisms in 160 public microbiology laboratories (5). According to BulSTAR annual reports, the number of reported invasive pneumococcal isolates (including blood and CSF isolates) for 2005 is 138, 2006-159, 2007-151 and 2008-121 (6). Until now for Bulgaria only aggregated data are reported and underreporting is quite possible. There is a local need for implementation of case based reporting system for invasive diseases including both epidemiological and laboratory components.

Acute otitis media (AOM) is one of the most common childhood diseases and a frequent reason for antibiotic administration in children, thus acting as an important factor influencing the emergence of antimicrobial resistance. AOM is a common childhood infection, affecting around 75% of children under 3 years of age, and about 40% of these children go on to develop recurrent episodes (7).
Reports concerning the serotype distribution of Streptococcus pneumoniae causing infections in Bulgaria are still limited in number (8). In 2010, universal immunization with 10-valent pneumococcal/H. influenzae protein D conjugate vaccine (Synflorix™, GlaxoSmithKline Biologicals, Rixensart, Belgium; PHiD-CV) was introduced in the national immunization programme. In this regard, monitoring pneumococcal serotype distribution became an important public health issue.
The aim of the present study was to identify the most prevalent S. pneumoniae serotypes causing invasive diseases and AOM before the introduction of universal pneumococcal conjugate vaccination and to analyze antimicrobial susceptibility data on these isolates.

BACTERIAL ISOLATES, MATERIALS AND METHODS

In the present study 36 invasive isolates and 50 AOM isolates were included. The invasive isolates were collected in 14 hospitals located in 10 Bulgarian cities between June 2009 and December 2010. Of these strains 15 were isolated from blood , 19 from CSF, one from pleural fluid and one from peritoneal fluid. Sixteen isolates were obtained from

infections in children ≤5 years and 20 – from children >5 years and adults.

The AOM isolates were collected during a previous study of bacterial etiology of AOM conducted between October 2009 and November 2010 (9) .Twelve otolaryngologists from 4 hospitals and ambulatory clinics in Sofia participated in the study. Middle ear specimens after spontaneous membrane rupture or tympanocentesis were collected from children aged £ 8 years diagnosed with AOM.

The mass infant immunization with pneumococcal conjugate vaccine was initiated in April 2010, so we considered the time period to the end of 2010 still representative for the pre-vaccine serotype distribution in Bulgaria.

The collected isolates were transported to the laboratories of the National Center of Infectious and Parasitic Diseases, identified conventionally and their susceptibility to antibacterial agents was determined by disk diffusion according to CLSI 2010. Penicillin MICs of S. pneumoniae were determined using M.I.C.Evaluator gradient strips (Oxoid, Basingstoke, UK) and interpreted according to CLSI breakpoints for oral and parenteral penicillin according to the type of infection. Pneumococcal serotyping was performed using Pneumococcus 7-10-13 valent latex agglutination kit and quellung reaction with antisera from Statens Serum Institute, Copenhagen.

RESULTS

Serotype distribution

The most frequently encountered serotype among invasive isolates was 19F (13,9%), followed by 1 (11,1%), 4 (8,3%), 6B, 23F, 9V and 3 (5,6% each). Serotypes 6A, 19A, 18C and 7F were represented by one isolate each. The remaining 12 isolates (33,3%) belonged to serotypes not included in the currently available 7-, 10- and 13-valent conjugate vaccines (Table 1). Among the 16 IPD isolates from children <5 years the most frequent serotypes were 19F (n=4) and 1 and 4 (n=3 each), followed by 6B, 23F, 9V and 18C (n=1 each).

The most frequently encountered serotype among AOM isolates was 19F (24%), followed by 6B (14%), 14 (8%), 9V and 3 (6% each), 23F and 18C (4% each) and 1 and 7F (2% each). The remaining 15 isolates (30%) belonged to non-conjugate vaccine related serotypes (Table 2).

Antibiotic susceptibility

According to CLSI breakpoints, among meningitis isolates penicillin-resistance (MIC≥0.12 µg/ml) rate was 36,8%. All of the non-meningitis isolates were penicillin susceptible (MIC<2 µg/ml) with the exception of one isolate which was intermediate (MIC=4 µg/ml). Among invasive isolates erythromycin, clindamycin and trimethoprim/sulfamethoxazole resistance rates were 17%; 17% and 33%, respectively. Isolates resistant to levofloxacin and rifampin were not found.

The majority of S. pneumoniae AOM isolates (n=26; 52%) were penicillin non-susceptible, of which six (12%) were penicillin-resistant (MIC≥2 µg/ml) and 20 (40%) - penicillin-intermediate (MIC 0.12-1 µg/ml). Isolates resistant to erythromycin, clindamycin and trimethoprim/sulfamethoxazole (TMP/SMX) were 44%, 32% and 56%, respectively. Pneumococcal isolates resistant to levofloxacin and rifampin were not found. Among the 26 penicillin non-susceptible isolates, 38% belonged to serotype 19F, 19% to serotype 6B and 15% to serotype 14. Among the six PRSP strains 5 belonged to serotype 19F and one to serotype 14. Most of the penicillin non-susceptible isolates were resistant to macrolides and TMP/SMX

Discussion

In a recently published systematic evaluation of serotypes causing invasive pneumococcal disease among children <5 years before introduction of PCV globally and regionally, six to 11 serotypes accounted for about 70% of IPD. Globally, the most frequently encountered invasive serotypes were 14, 6B, 1, 23F, 5, 19F and 6A – these serotypes accounted for more than half of IPD in every region. (10)

Data on serotype distribution before implementation of PCV in Central Europe showed some variations between countries, but the most prevalent IPD serotypes belonged to the main global serotypes. In Poland, serotypes 14, 6B, 23F and 18C were the most prevalent serotypes causing IPD in 2008. A study of invasive pneumococcal isolates preceding PCV-7 introduction in Turkey showed that serotypes 19F, 6B and 14 were the most common. The main serotypes isolated from IPD in Croatia in 2001, 2005 and 2006 were 14, 6B, 18C and 23F. In the Czech Republic S. pneumoniae serotypes 9V and 14 are the most frequently associated with invasive disease and, along with 6B, 19F, 3 and 23F, were the serotypes most frequently isolated from patients with invasive pneumococcal disease in 2000, 2006 and 2009 (1). Studies regarding serotype distribution of S. pneumoniae clinical isolates in Bulgaria are still limited in number. Setchanova et al. (8) characterized 146 pediatric pneumococcal isolates collected between 1998 and 2009 of which 67 were invasive strains. The most prevalent serotypes among invasive strains were: 19F, 14, 23F, 5, 9V, 6B, 7F and 1.

Among the invasive pneumococcal serotypes found in our study, 19F was also the most frequently isolated serotype in both age groups, followed by 1, 4, 6B, 23F, 9V and 3. When comparing the serotype distribution in the two age groups, serotypes 19F and 1 were more frequently found in the <5 years group and serotypes 4 and 18C were found only in this group. On the other hand, serotypes 3, 6A, 19A and 7F were found only in the >5 group. Non-conjugate vaccine serotypes were more frequently found among isolates in the >5 group (10/20) than in ≤5 group (2/16) (Table 1).

Among the pneumococcal AOM serotypes in this study, 19F was the most prevalent serotype, followed by 6B, 14, 9V, 3, 23F and 18C (Table 2). The serotype distribution of S. pneumoniae was similar to those reported from other European countries and worldwide. Globally, the most commonly reported pneumococcal serotypes causing AOM are 3, 6A, 6B, 9V, 14, 19A, 19F, and 23F (11). Serotype 19F is reported to be the most prevalent among AOM isolates in Germany, Costa Rica, Colombia and Japan (12, 13, 14, 15). Interestingly, in our study the common IPD-associated serotype 14 was found only among AOM isolates and not detected among invasive strains. On the other hand, serotypes 4, 6A and 19A were found only among invasive strains and were not found among AOM isolates. However, the number of isolates was too low to draw any definite conclusion.

Serotype 3 is an important serotype which was found in both invasive and AOM isolates in this study. It is present in PCV13, but not in PCV10. This serotype was included in a prototype 11-valent PCV, in which all serotypes were conjugated to protein D of non-typeable H. influenzae, but was subsequently removed from the final formulation, resulting in the 10-valent final product.(16). The removal of serotype 3 was related to the observation that immune responses after the booster dose to serotype 3 differed from those to other serotypes, and to its failure to demonstrate efficacy against acute otitis media (AOM) (16, 17). Serotype 3 was the only serotype out of the 11 for which post-booster antipneumococcal antibody concentrations were lower than post-primary antibody concentrations after three doses of 11Pn-PD in infancy. Serotype 3 is included in PCV13, but studies to date also show low or no booster responses against this serotype (17).

Penicillin non-susceptibility rate among AOM isolates found in this study (52%) was higher than the average level reported

in Bulgaria. According to the data from Bulgarian surveillance system tracking antimicrobial resistance (BulSTAR), collecting reports from 160 laboratories, penicillin non-susceptible isolates accounted for 21,6 % of all reported pneumococcal isolates in 2008 (6).

In the present study, penicillin non-susceptibility rate among invasive isolates was lower than in AOM isolates: 36,8% in meningitits and 6% in non-meningitis strains. This finding could be explained with the higher prevalence of penicillin-susceptible serotypes (such as 1 and 4) amongst invasive isolates.

As shown previously, penicillin non-susceptibility among pneumococcal isolates in Europe was associated mainly with serogroups 19 and 6 and serotypes 23F and 14 (11). In the present study penicillin non-susceptible and polyresistant S. pneumoniae isolates also belonged mainly to serotypes 19F, 19A, 6B, 9V and 14. Moreover, most of the penicillin-resistant isolates belonged to serotype 19F (Table 1).

In the present study serotype 19F was the most prevalent serotype among both invasive and AOM isolates. Our findings suggest that clonal relationship between 19F isolates could be present among Bulgarian pneumococcal isolates. However, further molecular characterization of the pneumococcal strains causing invasive and respiratory infections is required.

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Table 1. Serotype distribution and number of resistant S. pneumoniae isolates from invasive infections.

Serotype	Total	≤5 years	>5 years	Penicillin	ERY	CLI	TMP/SMX	RIF	LEVO
	n (%)	n	n	MIC (μg/ml)	n	n	n	n	n
19F	5 (13,9%)	4	1	0.03-4	2	2	4	-	-
1	4 (11,1%)	3	1	<0.015-0.03	-	-	-	-	-
4	3 (8,3%)	3	-	0.03-1	1	1	1	-	-
6B	2 (5,6%)	1	1	1-2	1	1	1	-	-
23F	2 (5,6%)	1	1	0.03-1	-	-	1	-	-
9V	2 (5,6%)	1	1	0.25-1	-	-	2	-	-
3	2 (5,6%)	-	2	<0.015	-	-	-	-	-
6A	1 (2,8%)	-	1	1	1	1	1	-	-
19A	1 (2,8%)	-	1	4	1	1	1	-	-
7F	1 (2,8%)	-	1	0.03	-	-	-	-	-
18C	1 (2,8%)	1	-	0.03	-	-	-	-	-
Non-conjugate vaccine serotypes	12 (33,3%)	2	10	0.015-2	-	-	1	-	-

Table 2. Serotype distribution and number of resistant S. pneumoniae isolates from middle ear fluids of Bulgarian children.

Serotype	Total	PISP	PRSP	ERY	CLI	TMP/SMX	RIF	LEVO
	n (%)	n	n	n	n	n	n	n
19F	12 (24%)	5	5	11	8	11	-	-
6B	7 (14%)	5	-	5	5	3	-	-
14	4 (8%)	3	1	3	1	4	-	-
9V	3 (6%)	3	-	-	-	3	-	-
3	3 (6%)	-	-	-	-	-	-	-
23F	2 (4%)	1	-	1	1	1	-	-
18C	2 (4%)	-	-	-	-	2	-	-
1	1 (2%)	-	-	-	-	-	-	-
7F	1 (2%)	-	-	-	-	-	-	-
Non-conjugate vaccine serotypes	15 (30%)	3	-	2	1	4	-	-

REFERENCES
1. Prymula, R et al. Paediatric pneumococcal disease in Central Europe Eur J Clin Microbiol Infect Dis 2011, 30:1311-1320
2. ECDC Annual Epidemiological Report on Communicable Diseases in Europe (2009)http://www.ecdc.europa.eu/en/publications/Pages/Surveillance_Reports.aspx
3. Informational Journal of NCIPD 4-5/2008
4. Informational Journal of NCIPD 3-4/2010
5. Petrov, M. et al. Surveillance of antimicrobial resistance in Bulgaria - a synopsis from BulSTAR 2003 , Eurosurveillance, Volume 10, Issue 6, 01 June 2005
6. Bulgarian surveillance system tracking antimicrobial resistance (BulSTAR). Trends in resistance of key pathogens 2002-2008 . Available from: www.bam-bg.net/index.en.php
7. Vergison, A. Microbiology of otitis media: A moving target. Vaccine, 2008, 26S, G5–G10
8. Setchanova L, Alexandrova A, Haydoushka I, Sredkova M, Bojkova K, Markova B et al. Serotypes and antimicrobial resistance of streptococcus pneumoniae strains isolated from children with invasive and respiratory disease before the introduction of pneumococcal conjugate vaccines in Bulgaria. Pediatriya 2010; 50 (2):39-43
9. Nashev D et al. Study of etiologic structure of acute otitis media in Bulgaria before introduction of pneumococcal conjugate vaccine. Pract. Pediatriya 2011, 1:18-20
10. Johnson et al. Systematic Evaluation of Serotypes Causing Invasive Pneumococcal Disease among Children Under Five: The Pneumococcal Global Serotype Project. PLOS Medicine, 2010, 7, e1000348
11. Rodgers G, Arguedas A, Cohen, R, Dagan R Global serotype distribution among Streptococcus pneumoniae isolates causing otitis media in children: Potential implications for pneumococcal conjugate vaccines. Vaccine 2009; 27:3802–3810
12. Van der Linden M, Reinert RR. Serotype distribution in pneumococcal acute otitis media with ruptured tympanic membrane or sepsis in Germany. Eur J Clin Microbiol Infect Dis. 2010;29(7):749-54
13. Arguedas A, Dagan R, Guevara S, Porat N, Soley C, Pérez A, Brilla R.Middle ear fluid Streptococcus pneumoniae serotype distribution in Costa Rican children with otitis media. Pediatr Infect Dis J. 2005;24(7):631-4
14. Sierra A, Lopez P, Zapata MA, Vanegas B, Castrejon MM, Deantonio R et al. Non-typeable Haemophilus influenzae and Streptococcus pneumoniae as primary causes of acute otitis media in colombian children: a prospective study. BMC Infect Dis. 2011 Jan 5;11:4.
15. Hotomi M, Billal DS, Kamide Y, Kanesada K, Uno Y, Kudo F et al. Serotype distribution and penicillin resistance of Streptococcus pneumoniae isolates from middle ear fluids of pediatric patients with acute otitis media in Japan. J Clin Microbiol. 2008;46(11):3808-10
16. Prymula R, Peeters P, Chrobok V et al. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. Lancet, 2006, 367(9512): 740-8
17. Poolman J et al. Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines 2011, Expert Rev. Vaccines 10(3), 307–322

STUDY OF BACTERIAL ETIOLOGY OF ACUTE OTITIS MEDIA IN BULGARIA BEFORE INTRODUCTION OF PNEUMOCOCCAL CONJUGATE VACCINE

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SUMMARY

We report microbiological data on 119 middle ear specimens collected from children aged ≤ 8 years diagnosed with AOM between October 2009 and November 2010. Among culture positive samples (105/119; 88%) the most frequently isolated pathogen was S.pneumoniae (47,6%), followed by H.influenzae (27,6%), M.catarrhalis (8,6%) and S.pyogenes (6,7%). All H. influenzae isolates, except one, were non-typable (97%). The most commonly encountered serotype among pneumococcal isolates was 19F (24%), followed by 6B (14%), 14 (8%), 9V and 3 (6% each) and 23F and 18C (4% each). The prevalence of penicillin non-susceptible S. pneumoniae was relatively high (52%) and associated mainly with serotypes 19F, 6B and 14.

Keywords: acute otitis media, etiology, S. pneumoniae, serotype, Non-typable H. influenzae, Bulgaria

INTRODUCTION:

Acute otitis media (AOM) is one of the most common childhood diseases and a frequent reason for antibiotic administration in children, thus acting as an important factor influencing the emergence of antimicrobial resistance.

Studies on bacterial etiology of AOM have not been conducted in Bulgaria until now. Furthermore, reports concerning the serotype distribution of Streptococcus pneumoniae causing infections in Bulgaria are still limited in number [1,2]. In 2010, universal immunization with 10-valent pneumococcal/H. influenzae protein D conjugate vaccine (Synflorix™, Glaxo-SmithKline Biologicals, Rixensart, Belgium; PHiD-CV) was introduced in the national immunization programme. In this regard, monitoring vaccine serotype coverage has become an important public health issue.

The aim of the present study was to identify the most prevalent bacterial pathogens in AOM in Bulgarian children, to obtain antimicrobial susceptibility data on major pathogens and to analyze the serotype distribution of S. pneumoniae and Haemophilus influenzae isolates causing AOM before the introduction of universal pneumococcal conjugate vaccination.

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METHODS:

Twelve otolaryngologists from 4 hospitals and ambulatory clinics in Sofia participated in the study. Middle ear specimens after spontaneous membrane rupture or tympanocentesis were collected from children aged £ 8 years diagnosed with AOM between October 2009 and November 2010.
AOM was defined as a visually abnormal tympanic membrane in pneumatic otoscopy suggesting effusion in the middle ear cavity, concomitantly with at least one of the following signs of acute infection: fever, ear ache, irritability, diarrhea, vomiting, acute otorrhea not caused by external otitis, or other symptoms of respiratory infection [3].

Only non-vaccinated with pneumococcal conjugate vaccine children with previously untreated AOM were included in the study.

The mass infant immunization with pneumococcal conjugate vaccine was initiated in April 2010, so we considered the time period between April and November 2010 still representative for the pre-vaccine etiology of AOM in Bulgaria.

The collected ear samples were transported in Amies transport medium in the laboratories of the National Center of Infectious and Parasitic Diseases, cultured on 5% sheep blood agar and chocolate agar and incubated at 35°C in 5% CO2 atmosphere. The bacterial isolates were identified conventionally and their susceptibility to antibacterial agents was determined by disk diffusion according to CLSI 2010. Penicillin MICs of S. pneumoniae were determined using M.I.C.Evaluator gradient strips (Oxoid, Basingstoke, UK) and interpreted according to CLSI oral penicillin V breakpoints. Pneumococcal serotyping was performed using Pneumococcus 7-10-13 valent latex agglutination kit and quellung reaction with antisera from Statens Serum Institute, Copenhagen. Capsular serotyping of H. influenzae was performed by slide agglutination using agglutinating antisera a-f (Remel, Dartford, England). Beta-lactamase production in H. influenzae was tested with nitrocefin disks (Cefinase, BD, Sparks, USA).

RESULTS:

During the study period 119 specimens were collected and investigated. In 14 specimens no aerobic bacteria were isolated. Among 105 culture positive samples most frequently isolated pathogen was S. pneumoniae (n=50; 47,6%), followed by H. influenzae (n=29; 27,6%), M. catarrhalis (n=9; 8,6%) and S. pyogenes (n=7; 6,7%). In the remaining 10 (9,5%) middle ear fluid samples the following microorganisms were isolated: P. aeruginosa–4 isolates, S epidermidis – 2 isolates and S. aureus, S. haemolyticus, P. vulgaris, and A. lwoffii –1 isolate each (Fig.1).
Of the 29 H. influenzae isolates, 28 were non-typable – NTHi (97%). In one isolate capsular serotype a was detected. Among H. influenzae isolates 4/29 (14%) were found positive for beta-lactamase production.
The most frequently encountered serotype among pneumococcal isolates was 19F (24%), followed by 6B (14%), 14 (8%), 9V and 3 (6% each), 23F and 18C (4% each) and 1 and 7F (2% each). The remaining 15 isolates (30%) belonged to serotypes not included in the currently available 7-, 10- and 13-valent conjugate vaccines (Table 1).
The majority of S. pneumoniae isolates (n=26; 52%) were penicillin non-susceptible, of which six (12%) were penicillin-resistant (MIC≥2 µg/ml) and 20 (40%) - penicillin-intermediate (MIC 0.12-1 µg/ml). Isolates resistant to erythromycin, clindamycin and trimethoprim/sulfamethoxazole (TMP/SMX) were 44%, 32% and 56%, respectively. Pneumococcal isolates resistant to levofloxacin and rifampin were not found. (Fig 2).

Among the 26 penicillin non-susceptible isolates, 38% belonged to serotype 19F, 19% to serotype 6B and 15% to serotype 14. Among the six PRSP strains 5 belonged to serotype 19F and one to serotype 14. Most of the penicillin non-susceptible isolates were resistant to macrolides and TMP/SMX (Table 1).

DISCUSSION:

The prevalence of middle ear pathogens reported in the present study is consistent with those found in several European countries prior to pneumococcal conjugate vaccine introduction into routine immunization programs. In the majority of pre-vaccine studies S. pneumoniae is the most frequent pathogen isolated from middle ear fluid, identified in 30–60% of AOM cases worldwide, followed by H. influenzae which represented 20–52% of the pathogens recovered in previously untreated AOM. [4, 5]. M. catarrhalis is usually the third most frequent bacterium isolated (3–20%) and S. pyogenes accounts for 1–5% of cases [4]. The prevalence of the different pathogens may vary between countries, depending on vaccination status of the included children and whether untreated, persistent or recurrent cases of AOM were included.

NTHi was found in 97% of H. influenzae AOM episodes in this study. Virtually all strains of H. influenzae currently causing AOM are nonencapsulated [6]. As previously reported, NTHi is more commonly associated with recurrent and persistent AOM as well as with AOM treatment failures [4, 6].

Among the pneumococcal serotypes found in this study, 19F was the most frequently isolated serotype, followed by 6A, 14, 9V, 3, 23F and 18C (Table 1). The serotype distribution of S. pneumoniae was similar to those reported from other European countries and worldwide. Globally, the most commonly reported pneumococcal serotypes causing AOM are 3, 6A, 6B, 9V, 14, 19A, 19F, and 23F [5]. Serotype 19F is reported to be the most prevalent among AOM isolates in Germany, Costa Rica, Colombia and Japan [7,8,9,10].

Studies regarding serotype distribution of S. pneumoniae clinical isolates in Bulgaria are still limited in number. Setchanova et al. [1] characterized 146 pediatric pneumococcal isolates collected between 1998 and 2009 of which 67 were invasive strains and 79 - respiratory strains. Among respiratory isolates the most prevalent serotypes were 19F, 19A, 14, 23F, 9V, 6A, 6B, 15B and 3. In the same study, the most prevalent serotypes among invasive strains were: 19F, 14, 23F, 5, 9V, 6B, 7F and 1. In addition, our preliminary data on serotyping of 16 pediatric invasive isolates collected in 2010 showed predominance of serotypes 19F, 4 and 1 among the investigated strains [unpublished data].

Penicillin non-susceptibility rate found in this study (52%) was higher than the average level reported in Bulgaria. According to the data from Bulgarian surveillance system tracking antimicrobial resistance (BulSTAR), collecting reports from 160 laboratories, penicillin non-susceptible isolates accounted for 21,6 % of all reported pneumococcal isolates in 2008 [11].

As shown previously, penicillin non-susceptibility among AOM isolates in Europe was associated mainly with serogroups 19 and 6 and serotypes 23F and 14 [5]. In the present study penicillin non-susceptible and poliresistant S. pneumoniae isolates also belonged mainly to serotypes 19F, 6B and 14. Moreover, most of the penicillin-resistant isolates belonged to serotype 19F (Table 1).

In a study of Arguedas et al. [12] serotype 19F was the predominant serotype causing AOM in Costa Rica, found in 15–75% of cases due to predominance of a penicillin non-susceptible 19F clone from 1999 to 2001. As a result of the dissemination of this clone penicillin non-susceptibility increased from 19.6% in 1992 to approximately 50% in 2001 [12]. Our findings suggest that similar clonal relationship could be present among Bulgarian pneumococcal isolates. However, further molecular characterization of the pneumococcal AOM strains is required.

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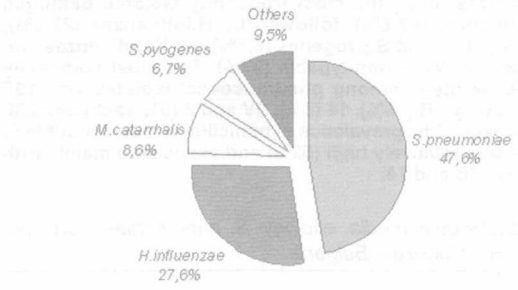

Fig.1. Proportions of the bacterial pathogens isolated from middle ear fluid in this study.

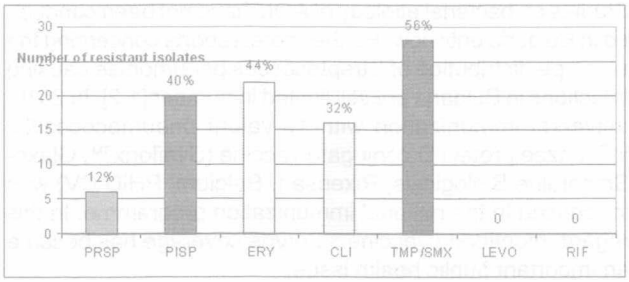

Fig 2. Antimicrobial resistance of S. pneumoniae isolates in this study.

PISP-penicillin-intermediate S. pneumoniae,
PRSP-penicillin-resistant S. pneumoniae,
ERY-erythromycin,
CLI-clindamycin,
TMP/SMX-trimethoprim/sulfamethoxazole,
LEVO-levofloxacin,
RIF-rifampin

Table 1. Serotype distribution and number of resistant S. pneumoniae isolates from middle ear fluids of Bulgarian children

Serotype	Total n (%)	PISP n	PRSP n	ERY n	CLI n	TMP/SMX n	RIF n	LEVO n
19F	12 (24%)	5	5	11	8	11	-	-
6B	7 (14%)	5	-	5	5	3	-	-
14	4 (8%)	3	1	3	1	4	-	-
9V	3 (6%)	3	-	-	-	3	-	-
3	3 (6%)	-	-	-	-	-	-	-
23F	2 (4%)	1	-	1	1	1	-	-
18C	2 (4%)	-	-	-	-	2	-	-
1	1 (2%)	-	-	-	-	-	-	-
7F	1 (2%)	-	-	-	-	-	-	-
Non-conjugate vaccine serotypes	15 (30%)	3	-	2	1	4	-	-

REFERENCES:

1. Setchanova L, Alexandrova A, Haydoushka I, Sredkova M, Bojkova K, Markova B et al. Serotypes and antimicrobial resistance of streptococcus pneumoniae strains isolated from children with invasive and respiratory disease before the introduction of pneumococcal conjugate vaccines in Bulgaria. Pediatriya 2010; 50 (2):39-43
2. Setchanova L, Ouzounova-Raykova V, Zhelezova GZ, Mitov IG. Prevalence and macrolide resistance phenotypes and genotypes among clinical isolates of Streptococcus pneumoniae collected in Sofia, Bulgaria from 2001 to 2005. J Chemother. 2007;19(3):256-62
3. Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, Herva E, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. New Engl J Med 2001;344(6):403—9.
4. Vergison, A. Microbiology of otitis media: A moving target. Vaccine, 2008, 26S, G5–G10
5. Rodgers G, Arguedas A, Cohen, R, Dagan R Global serotype distribution among Streptococcus pneumoniae isolates causing otitis media in children: Potential implications for pneumococcal conjugate vaccines. Vaccine 2009; 27:3802–3810
6. Leibovitz, E, Jacobs, Dagan, R. Haemophilus influenzae. A significant pathogen in Acute Otitis Media. Pediatr Infect Dis J, 2004, 23: 1142-52
7. Van der Linden M, Reinert RR. Serotype distribution in pneumococcal acute otitis media with ruptured tympanic membrane or sepsis in Germany. Eur J Clin Microbiol Infect Dis. 2010;29(7):749-54
8. Arguedas A, Dagan R, Guevara S, Porat N, Soley C, Pérez A, Brilla R.Middle ear fluid Streptococcus pneumoniae serotype distribution in Costa Rican children with otitis media. Pediatr Infect Dis J. 2005;24(7):631-4
9. Sierra A, Lopez P, Zapata MA, Vanegas B, Castrejon MM, Deantonio R et al. Non-typeable Haemophilus influenzae and Streptococcus pneumoniae as primary causes of acute otitis media in colombian children: a prospective study. BMC Infect Dis. 2011 Jan 5;11:4.
10. Hotomi M, Billal DS, Kamide Y, Kanesada K, Uno Y, Kudo F et al. Serotype distribution and penicillin resistance of Streptococcus pneumoniae isolates from middle ear fluids of pediatric patients with acute otitis media in Japan. J Clin Microbiol. 2008;46(11):3808-10
11. Bulgarian surveillance system tracking antimicrobial resistance (BulSTAR). Trends in resistance of key pathogens 2002-2008 . Available from: www.bam-bg.net/index.en.php
12. Arguedas A, Dagan R, Soley C, Loaiza C, Knudsen K, Porat N et al. Microbiology of otitis media in Costa Rican children, 1999 through 2001. Pediatr Infect Dis J. 2003;22:1063–8.

ANTIMICROBIAL ACTIVITY OF TIGECYCLINE AGAINST MULTIDRUG-RESISTANT STRAINS COLLECTED IN BULGARIA.

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SUMMARY

Background: Tigecycline is a new injectable glycylcycline antibacterial product with potent activity and broad spectrum against Gram-positive and Gram-negative bacteria currently used for the treatment of complicated skin and skin structure-infections and intra-abdominal infections. We evaluated the in vitro activity of this compound against clinical strains of Gram-positive and Gram-negative species collected from Bulgarian medical centers in the National Reference Laboratory for "Control and Monitoring of Antimicrobial Resistance" at the NCIPD.

Methods: A total of 187 non-duplicate clinical strains from different medical centers were tested for susceptibility by reference broth microdilution methods according to Clinical and Laboratory Standards Institute guidelines and interpretative criteria.

Results: All S. aureus strains were inhibited at a tigecycline MIC of ≤0.5 µg/ml. Among the tested Enterococcus strains the highest tigecycline MIC value was 0.25 µg/ml. Similarly ESBL producing E. coli and K. pneumoniae exhibited uniformly susceptibility to this compound with MIC values reaching 2 µg/ml only for some strains K. pneumoniae. Among the multiple drug resistant A. baumannii strains collected, our assessment indicated MIC 90 value of 2 µg/ml.

Conclusion: Tigecycline showed highly potent in vitro activity against all clinical strains tested and appears to be an excellent therapeutic option for serious infections caused by difficult-to-treat microorganisms. The present study did not detect any tigecycline-resistant strains with the exception of a single A. baumannii isolate with MIC value of 8 µg/ml.

Key words: Tigecycline, MIC, serious infections, MRSA, ESBL

BACKGROUND /INTRODUCTION

Irrational and inappropriate use of antibiotics has resulted in the emergence of resistant bacteria world-wide. In Bulgaria, the lack of infection control practice and the absence of antibiotic policies in some hospitals has further augmented this problem. There have been reports from all over the country on the rising trend of nosocomial pathogens producing extended spectrum beta lactamases (ESBL) and emergence of methicillin resistant Staphylococcus aureus. Infections due to these organisms have resulted in increased mortality, morbidity and cost of treatment [1]. This increasing resistance among clinical isolates emphasizes the need for new antimicrobial agents with novel mechanisms of action. Currently, there are several agents in development aimed at the treatment of Gram-positive infections. However, new agents with Gram-negative activity are considerably lacking. To compound the issue, therapeutic alternatives are also severely limited for patients with multidrug-resistant Gram-negative infections who exhibit true allergic reactions to penicillins.

Recently, a new antimicrobial class, the glycylcyclines, has been found to treat resistant Gram-positive and Gram-negative infections. These agents are structurally similar to tetracyclines, which gained prescribers' favor because of their broad spectrum of antimicrobial activity, favorable pharmacokinetic profiles, and minimal adverse events. However, their application has been severely limited due to the development of antimicrobial resistance through mechanisms such as efflux pumps and ribosomal protection. Currently, tetracyclines are used for the treatment of Gram-negative infections caused by pathogens with evaluated susceptibility, not for empirical therapy. Tigecycline, former GAR-936, is the first representative of glycylcyclines. This compound is a semisynthetic 9-t-butylglycylamido derivative of the minocycline molecule [2]. Although tigecycline acts on the bacterial ribosome by binding sites similar to those of tetracycline, the radical added to position 9 of the tigecycline molecule has provided additional steric hindrance features that result in a greater spectrum of activity [3]. Thus, tigecycline has documented activity against tetracycline-resistant (tet-R) Gram-positive and Gram-negative pathogens, refractory to both efflux and ribosomal protection mechanisms [4]. Our main objective was to evaluate the in vitro activity of tigecycline in clinical bacterial isolates recently collected at the National Reference Laboratory for "Control and Monitoring of Antimicrobial Resistance" at the NCIPD.

MATERIALS AND METHODS

Bacterial strains

A total of 187 clinical bacterial isolates collected from Bulgarian medical centers in the period of 2008-2010 were evaluated. The distribution of species was as following as: Staphylococcus aureus (36 strains), Enterococcus spp. (27 strains), Escherichia coli (51 strains), Klebsiella pneumoniae (45 strains) and Acinetobacter baumannii (28 strains). Only a single isolate per patient was evaluated. The isolates were identified to the species level by the participating medical center, and sent to the coordinating laboratory for identification confirmation and reference susceptibility testing. The entire collection of strains was stored at –70°C, with two subcultures being made before the organisms were tested.

Medical centers

Clinical isolates of facultatively aerobic bacteria were collected in 11 Bulgarian microbiology laboratories distributed throughout the medical centers of 6 cities: Sofia, Plovdiv, Varna, Burgas, Russe, Targovishte.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using broth microdilution methods, as described by the Clinical Laboratory Standard Institute (CLSI, formerly NCCLS) [5]. Antimicrobial agents were obtained from their respective manufacturers and testing was perfomed using broth microdilution panels. MIC panels were incubated at 35°C in ambient air for 24 h before examination. Minimal inhibitory concentrations (MICs expressed in micrograms per milliliter) results for all isolates were categorized as susceptible or resistant according to the breakpoints published by CLSI. Quality control measures were utilized by testing S. pneumoniae ATCC 49619, S. aureus ATCC 29213, Enterococcus faecalis ATCC 29212, Escherichia coli ATCC 25922, and Pseudomonas aeruginosa ATCC 27853.

RESULTS

Minimum Inhibitory Concentration (MICs) were determined using broth microdilution panels and interpreted according to CLSI guidelines. There is still a lack of universally accepted interpretative MIC breakpoints for Acinetobacter baumannii. The FDA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have issued interpretative breakpoints of susceptibility for tigecycline, though for

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categories of pathogens other than Acinetobacter spp. The breakpoints referring to Enterobacteriaceae established by CLSI have been used as provisional breakpoints for Acinetobacter spp.Species tested in rank order of frequency were as follows: S. aureus (14 % MRSA); Enterococcus spp. (100 % high level aminoglycoside resistant), E. coli (53 % ESBL-producers), Klebsiella pneumoniae (89 % ESBL-producers) and Acinetobacter baumannii (82% meropenem resistant). Our results outlined in Table 1 show uniform susceptibility of all the isolates tested to tigecycline except for a single A. baumannii strain having MIC value of 8 µg/ml. To the exclusion of this strain, the highest MIC value that was reached was 2 µg/ml for some of the A. baumannii and ESBL producing K. pneumoniae isolates. Table 1 indicates the currently established CLSI breakpoints, MIC50/MIC90 (MIC values at which 50 and 90% of the isolates tested are inhibited), percentage of susceptible and resistant strains, and maximum/minimum MICs.

The total of 14 A. baumannii isolates collected exhibited multiple resistance phenotypes towards β-lactam antibiotics (including 82% meropenem resistant), quinolones and aminoglycosides. In addition to tigecycline the MICs of the following antimicrobial agents were determined simultaneously: cefepime, piperacillin/tazobactam and levofloxacin. Tigecycline was the most active drug agent in vitro with MICs ranging from 0.5 to 8 µg/ml. Only a single isolate (3%) was categorized as resistant to tigecycline (MIC value 8 µg/ml). MIC50 and MIC90 values for the three other antibacterial agents tested reached the breakpoint level of resistance - >32 µg/ml for cefepime, >128 µg/ml for piperacillin/tazobactam, and 8 µg/ml for levofloxacin, rendering tigecycline with the highest in vitro activity against A. baumannii.

Enterococcus strains were evaluated as 100% susceptible to tigecycline. Maximum MIC values reached 0.25 µg/ml. MIC50 and MIC90 values were determined as 0.12 µg/ml and 0.25 µg/ml respectively. In addition to tigecycline the MICs of the following antimicrobial agents were also determined simultaneously: levofloxacin, linezolid, and vancomycin. In this study we did not detect resistant to linezolid Enterococcus strains but only 4% (1 isolate) exhibited vancomycin resistance. In regard to levofloxacin the proportion of susceptible strains was measured as 55%. These results arrange tigecycline as the most active drug agent in vitro against Enterococcus spp. followed by vancomycin (92% susceptibility) and linezolid

Table 1. Antimicrobial activity of tigecycline in comparison with selected antimicrobial agents against 187 clinical strains collected in Bulgaria.

Organism/Drug	Breakpoints (S|I|R)	MIC 50 (µg/ml)	MIC 90 (µg/ml)	% Susceptible	% Resistant	Min (µg/ml)	Max (µg/ml)
Acinetobacter baumannii (N = 28)							
Cefepime	≤8 | 16 | ≥32	> 32	> 32	7	93	≤0.5	>32
Piperacillin/Tazobactam	≤16/4 | 32/4-64/4 | ≥128/4	> 128	> 128	7	83	0.06	>128
Levofloxacin							
Tigecycline	≤2 | 4 | ≥8	8	>8	14	71	0.25	>8
	≤2 | 4 | ≥8 [a]	1	2	97	3	0.25	8
Enterococcus spp (N = 27)							
Levofloxacin	≤2 | 4 | ≥8	2	>32	55	33	0.5	> 32
Linezolid	≤2 | 4 | ≥8	2	4	89	0	≤ 0.5	4
Vancomycin	≤4 | 8-16 | ≥32	1	4	92	4	0.5	>32
Tigecycline	≤0.25 | -- | ≥0.5	0.12	0.25	100	0	0.015	0.25
Escherichia coli (N = 51)							
Cefepime	≤8 | 16 | ≥32	32	>32	45	53	≤ 0.5	>32
Levofloxacin	≤2 | 4 | ≥8	8	>8	31	69	0.015	>8
Piperacillin/Tazobactam	≤16/4 | 32/4-64/4 |≥128/4	4	32	82	4	1	>128
Tigecycline E.coli ESBL							
Tigecycline	≤2 | 4 | ≥8	0.25	0.5	100	0	0.12	0.5
E.coli non ESBL							
	≤2 | 4 | ≥8	0.25	1	100	0	0.12	1
Klebsiella pneumoniae (N = 45)							
Cefepime	≤8 | 16 | ≥32	>32	>32	20	80	≤0.5	>32
Levofloxacin	≤2 | 4 | ≥8	8	>8	33	53	0.06	>8
Piperacillin/Tazobactam	≤16/4 | 32/4-64/4 |≥128/4	64	>128	38	35	1	>128
Tigecycline							
K.pn. ESBL	≤2 | 4 | ≥8	0.5	2	100	0	0.25	2
Tigecycline							
K.pn. non ESBL	≤2 | 4 | ≥8	0.5	1	100	0	0.5	1
Staphylococcus aureus (N = 36)							
Levofloxacin	≤1 | 2 | ≥4	0.25	4	86	11	≤0.06	>32
Linezolid	≤4 | -- | ≥8	2	4	97	3	1	8
Vancomycin	≤=2 | 4-8 | ≥16	1	2	100	0	0.5	2
Tigecycline (MRSA)	≤0.5 | -- | ≥1	0.25	0.5	100	0	0.06	0.5
Tigecycline (MSSA)							
	≤0.5 | -- | ≥1	0.12	0.25	100	0	0.06	0.25

a - Provisional breakpoints for Acinetobacter spp.

(89% susceptibility).
Tigecycline was highly active against both methicillin-resistant and –susceptible S. aureus. The observed MIC50 values were 0.12 and 0.25 µg/mL, and MIC90 0.25 and 0.5 µg/mL for MSSA/MRSA respectively. These records recognized all S. aureus strains, irrespective of their methicillin resistance profile, as 100% susceptible to tigecycline in vitro. As with Enterococcus spp., simultaneous MIC evaluation was performed for levofloxacin, linezolid, and vancomycin. MIC distributions revealed high portion of suscebtible isolates to vancomycin (100%), linezolid (97%), and levofloxacin (86%).
E. coli ESBL and non-ESBL producers presented considerable inhibition by tigecycline and none of them reached the upper threshold for susceptibility of 2 µg/mL. MIC values were distributed between 0.5 and 1 µg/mL; MIC50, 0.25 µg/mL for both groups and MIC90, 1 µg/mL and 0.5 µg/mL for non-ESBL and ESBL producers, respectively. In addition to tigecycline, the in vitro activity of three other antibacterial agents was assessed: cefepime, piperacillin/tazobactam and levofloxacin. Of these, piperacillin/tazobactam showed high activity against 82% of the E. coli isolates. 53% of all isolates were resistant to cefepime and 69% - to levofoxacin. While only 4% resistance was detected to piperacillin/tazobactam, not a single resistant strain to tigecycline was observed in this study.
Tigecycline showed high potency also against all K. pneumoniae strains regardless of ESBL production. Nevertheless ESBL producers exhibited higher MIC90 (2 µg/ml) than non ESBL isolates (MIC90 1 µg/ml), whereas MIC50 was evaluated 0.5 µg/ml for both groups. Similarly to E. coli, the in vitro activity of cefepime, piperacillin/tazobactam and levofloxacin was assessed. 80% of the K. pneumoniae strains were determined as cefepime-resistant, which correlates with the higher portion of ESBL producers by comparison with E.coli. MIC values for levofloxacin and piperacillin/tazobactam exhibited 33% and 38% suscebtibility, respectively. From these results tigecycline was considered as the drug agent with the highest in vitro potency against all K. pneumoniae strains (100% susceptible).

DISCUSSION

Tigecycline, a novel glycylcycline antibiotic, exhibits strong activity against Gram-positive, Gram-negative, aerobic, anaerobic, and atypical bacterial species, including many resistant pathogens, i.e., vancomycin-resistant enterococci, methicillin-resistant S. aureus and penicillin-resistant S. pneumoniae [6, 7, 8]. Our results show uniformly the susceptibility of all S. aureus and Enterococcus spp. isolates to tigecycline. We measured maximum MIC levels of 0.5 µg/ml for staphylococci and 0.25 µg/ml for enterococci. In addition, tigecycline has shown excellent activity against most Gram-negative pathogens, including Enterobacteriaceae, Acinetobacter spp., Stenotrophomonas maltophilia [6, 7], Haemophilus influenzae, and Neisseria spp. [9]. Against our collection of E. coli and K. pneumoniae ESBL producing isolates, tigecycline was very potent, inhibiting 100% of them at a concentration of 1 µg/ml for E. coli and 2 µg/ml for K. pneumoniae. Irrespective of beta-lactamase production tigecycline was the most effective drug agent in vitro by comparison with cefepime, piperacillin/tazobactam and levofloxacin. Tigecycline's expanded broad-spectrum activity is further evidenced by its activity against Legionella pneumophila [10], Chlamydia [11], rapidly growing nontuberculosis mycobacteria [12] and anaerobes [13]. On the other hand, tigecycline has demonstrated limited activity against P. aeruginosa and Proteae isolates. It evades acquired efflux and target-mediated resistance to classical tetracyclines, but not chromosomal efflux in Proteae and Pseudomonas [15]. We observed the same trends in our study (data not shown). Tigecycline, a semisynthetic derivative of minocycline is an ideal antimicrobial agent in that it is largely unaffected, as are other glycylcyclines, by a number of defense mechanisms such as protein binding, β-lactamase production (ESBL production, AmpC hyperproducers), the van resistance genes, and others that are used by many of the commonly encountered isolates in community-acquired and nosocomial infections [14]. Regarding A. baumannii, this pathogen has been shown to be susceptible to tigecycline in large-scale microbiological studies. Tigecycline has also shown adequate activity against Acinetobacter species of potential clinical significance other than A. baumannii. Still, whether tigecycline constitutes a potentially effective treatment option against highly resistant Acinetobacter spp. has not been evaluated in a comprehensive manner [16]. According to our results more than 90% of all A. baumannii isolates did not reach the breakpoint level for resistance and exhibited maximum MIC value of 2 µg/ml. A single strain was recognized as resistant to tigecycline with MIC 8 µg/ml. By comparison with the other antibacterial agents tested against A. baumannii (cefepime, piperacillin/tazobactam and levofloxacin), tigecycline showed the highest in vitro activity towards these multiple-drug-resistant strains.
Tissue penetration of tigecycline is excellent and the compound has shown equivalence to imipenem/cilastatin in intra-abdominal infection and to vancomycin plus aztreonam in skin and skin structure infection. Tigecycline may prove particularly useful for treatment of surgical wound infections, where both gut organisms and MRSA are likely pathogens. It is also likely to find a role in the treatment of infections due to multiresistant pathogens, including Acinetobacter spp. and ESBL producers, as well as MRSA and enterococci. On the other hand, the low Cmax in serum must be of some concern if bacteraemia is present [15].
Our results agree with previous in vitro studies [6, 7] and emphasize that tigecycline has excellent activity and spectrum against Gram-positive and some Gram-negative bacteria, including multi-drug resistant strains, and bacterial pathogens causing community-acquired infections or isolated from patients hospitalized in Bulgarian medical centers. All the isolates tested in our study did not exceed the breakpoints for susceptibility to tigecycline with the exception of a single A. baumannii isolate. These results, associated with the tigecycline clinical efficacy data already published, suggest that tigecycline may have an important role in the treatment of severely ill patients, especially in an environment with high antimicrobial drug resistance levels such as in Bulgaria.

REFERENCES
1. Ibrahim EH, Sherman G,Ward S, Fraser VJ, KollefMH. (2000). The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. Chest 118:146-55.
2. Petersen P.J., Jacobus N.V., Weiss W.J., et al. (1999). In vitro and in vivo antibacterial activities of a novel glycylcycline, the 9-t-butylglycylamido derivative of minocycline (GAR-936). Antimicrobial Agents and Chemotherapy 43:738-44.
3. Sum P.E., Petersen P. (1999). Synthesis and structure-activity relationship of novel glycylcycline derivatives leading to the discovery of GAR-936. Bioorg Med Chem Lett 9:1459-62.
4. Bergeron J., Ammirati M., Danley D., et al. (1996). Glycylcyclines bind to the high-affinity tetracycline ribosomal binding site and evade Tet(M)- and Tet(O)-mediated ribosomal protection. Antimicrobial Agents and Chemotherapy 40:2226-8.
5. National Committee for Clinical Laboratory Standards. (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard NCCLS document M7-A6. (6th ed.) Wayne, PA7 NCCLS.
6. Gales A.C., Jones R.N. (2000). Antimicrobial activity and spectrum of the new glycylcycline, GAR-936 tested against 1,203 recent clinical bacterial isolates. Diagn Microbiol Infect Dis 36:19-36.
7. Milatovic D., Schmitz F.J., Verhoef J., Fluit A.C. (2003). Activities of the glycylcycline tigecycline (GAR-936) against 1,924 recent European clinical bacterial isolates. Antimicrobial Agents and Chemotherapy 47:400-4.
8. Fritsche T.R., Kirby J.T., Jones R.N. (2004). In vitro activity of tigecycline (GAR-936) tested against 11,859 recent clinical isolates associated with community-acquired respiratory tract and Gram-positive cutaneous infections. Diagn Microbiol Infect Dis 49:201-9.
9. Deshpande L.M., Gales A.C., Jones R.N. (2001). GAR-936 (9-tbutylglycylamido-minocycline) susceptibility test development for streptococci, Haemophilus influenzae and Neisseria gonorrhoeae: preliminary guidelines and interpretive criteria. Int J Antimicrob Agents 18:29-35.
10. Edelstein P.H., Weiss W.J., Edelstein M.A. (2003). Activities of tigecycline (GAR-936) against Legionella pneumophila in vitro and in guinea pigs with L. pneumophila pneumonia. Antimicrobial Agents and Chemotherapy 47:533-40.
11. Roblin P.M., Hammerschlag M.R. (2000). In vitro activity of GAR-936 against Chlamydia pneumoniae and Chlamydiatrachomatis. Int J Antimicrob Agents 16:61-3.
12. Wallace R.J., Brown-Elliott Jr.B.A., Crist C.J., et al. (2002). Comparison of the in vitro activity of the glycylcycline tigecycline (formerly GAR-936) with those of tetracycline, minocycline, and doxycycline against isolates of nontuberculous mycobacteria. Antimicrobial Agents and Chemotherapy 46:3164-7.
13. Petersen P.J., Weiss W.J., Labthavikul P. (1998). The postantibiotic effect and time-kill kinetics of the glycylcyclines, GAR-936 (TBG-MINO) and (PAMMINO). Program Abstr. 38th Intersci. Conf. Antimicrobial Agents and Chemotherapy abstr. F-132.
14. Daryl J. Hoban,T, Samuel K. Bouchillon, Brian M. Johnson, Jack L. Johnson, Michael J. Dowzicky. (2005). In vitro activity of tigecycline against 6792 Gram-negative and Gram-positive clinical isolates from the global Tigecycline Evaluation and Surveillance Trial. Diagnostic Microbiology and Infectious Disease 52 : 215–227.
15. Livermore DM. (2005). Tigecycline: what is it, and where should it be used? J Antimicrob Chemother 56:611–14.
16. D. E. Karageorgopoulos1, T. Kelesidis, I. Kelesidis1, and M. E. Falagas. (2008). Tigecycline for the treatment of multidrug-resistant (including carbapenem-resistant) Acinetobacter infections: a review of the scientific evidence. Journal of Antimicrobial Chemotherapy 62:45-55.

PCR- RIBOTYPING AND PCR METHODS FOR DETECTION OF TOXIN CODING GENES IN CLOSTRIDIUM DIFFICILE STRAINS

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SUMMARY

C. difficile infections (CDI) are associated with patients who have contact with health care settings and with antibiotic exposures. This anaerobic bacterium causes asymptomatic colonization to severe diarrhea; pseudomembranous colitis, toxic megacolon, intestinal perforation and death.

C. difficile is recognized as a gut colonizer and a cause of diarrhea in several animal species.

The enteropathogen produces enterotoxin A and cytotoxin B. The majority of strains with changes in coding genes tcdA and tcdB produce a binary toxin CDT.

PCR-ribotyping method and PCR methods for detection of toxin coding genes were presented for characterization of C. difficile strains. Ninety stool samples from patients (65/90) and animals (25/90) were investigated for C. difficile. 20% (18/90) of all samples were positive for C. difficile. 23% (15/65) from clinical samples and 12% (3/25) from horses were positive for C. difficile by culture test. 21, 5% (14/65) of clinical isolates produce toxins A and B by EIA. 86,7% (13/15) from clinical samples were PCR positive for tcdA gene. Deletion in tcdA gene (714bp) was detected in 40% (6/15) of the clinical strains. 93,3% (14/15) C. difficile clinical strains were positive for tcdB gene. Three toxigenic variants C. difficile have been distinguished among clinical strains by PCR: 46,67% (7/15) toxin A+B+; 46,67% (6/15) A–B+ and 6,67% (2/15) A-B-. The binary-toxin genes cdtA and cdtB was PCR detected in one of the A+B+ strains. The genes tcdA, tcdB and cdtA/cdtB were not detected in C. difficile isolates from horses by PCR.

The most prevalent ribotypes among C. difficile clinical strains were: 017- 40% (6/15); 002- 13%; 014/020-13% and 012, 046, 078 were represented by 7% each. Patterns were compared to reference ECDC C. difficile collection. Thirteen percent of

C. difficile clinical strains were corresponded to unknown PCR-ribotypes. PCR-ribotyping patterns of the C. difficile isolates from horses were different from patterns of the clinical strains.

The significant number of cases C. difficile diagnosed with outbreak ribotypes may represent a significant problem in the future.

Key Words: toxin genes, PCR, PCR-ribotyping, ribotypes

INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, motile and spore forming bacterium. The microorganism was isolated from stools of healthy newborn infants by Hall and O'Tool in 1935 (11). It was not known as a pathogen so the "toxin" of the organism was not studied until 1970 (21). Later the investigators have associated

C. difficile with pseudomembranous colitis (PMC). Authors have discovered that the clinical samples from patient with PMC contain high levels of cytotoxic activity.

C. difficile causes disease in humans and animals (10).

C. difficile infections (CDI) has been associated with patients who have contact with health care settings and who have taken antibiotics (3, 32). This anaerobic bacterium transmitted via fecal-oral route and can contaminate hands of health care workers and patients, and patient care environment (29, 32). The organism can be isolated from the clothing and room fixtures of the patient. C. difficile once fall into environment can persist for months, because of spore producing.

In the recent years, CDI are recognized as a cause of diarrhea in outpatients and person with no health care contacts. Community-associated infections have been described among young people and people without antibiotic exposures (7).

C. difficile is associated with asymptomatic colonization to severe diarrhea; pseudomembranous colitis, toxic megacolon, intestinal perforation and death (3, 29). It causes approximately 25% of the cases of antibiotic-associated diarrhea (CDAD) (4, 21). Asymptomatic carriers are an important hidden reservoir of C. difficile and they can spread the infection to other patients. Clinical symptoms develop in one third of colonized patients (27).

The incidence of PMC varied widely between different hospitals and even between different wards in the same hospital. Some investigators reported rates as high as 10% in patients treated with clindamycin (21).

C. difficile is recognized as a gut colonizer and cause of diarrhea in several animal species (horses, dogs, ostriches, rabbits, cats and pigs). The prevalence of this enteropathogen in the faeces of dogs is 6% and in cats to 40%. Reported faecal carriage rates in horses is 2%-29% (1). C. difficile is pathogen in domestic and food animals but there were little investigation regarding transmission of this organism (22).

C. difficile produces two toxins: enterotoxin A (Tcd A, 308 kDa) and cytotoxin B (Tcd B, 270 kDa). Toxin B acts synergistically with toxin A after the epithelium has been injured by TcdA (23). The coding chromosome genes tcdA and tcdB are located within a ≈19,6-kb region of PaLoc (pathogenicity locus) (32, 34). TcdA and TcdB are known as virulence factors and markers for diagnosis of C. difficile disease. Not all toxigenic strains produce both toxins A and B. There are different variant C. difficile strains among people and animals, some of them produce both toxins (A+B+); others produce only TcdB (A-B+) and third produce only parts of the toxin genes (A-B-) (16, 17). The majority of strains with changes in genes tcdA and tcdB produce a binary toxin (8). The role of the binary toxin CDT A/B in enteropathogenecity of

C. difficile is unclear. Toxins can be found in 15%-25% of the stool of patients with CDAD and more than 95% of patients with pseudomembranous colitis (29).

The diagnostic tests for C. difficile are divided into: (i) test based on detection of

C. difficile products; (ii) culture methods; (iii) molecular methods for gene detection (6) . The cell culture cytotoxicity assay (CCA) is regarded as the reference standard. Many laboratories use enzyme immunoassays (EIA) and PCR for detection of C. difficile toxin genes (4, 6).

PCR- ribotyping method have been used to determine the role of the environment; patient-to-patient transmission and for investigation of outbreaks in hospitals and nursing homes (19, 33). This method has a number of advantages over other typing methods: specifically; high discriminatory power; and it is quicker and simplier for performance. PFGE is considered as a "gold standart" for genotyping, but due to DNA degradation in some C. difficile strains (produce endogenous nucleases), other typing technique is preferred (5, 12).

OBJECTIVES

Presentation of PCR-ribotyping method and PCR methods for detection of toxin coding genes for characterization of C. difficile strains isolated from human and animal samples.

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METHODS

Ninety stool samples were investigated for C. difficile. Sixty five were from patients with mild to severe enterocolitis and previous antibiotic treatment, and 25 were from healthy horses.

Laboratory diagnosis of C. difficile was performed by culture test on nonselective media and selective media (selective supplements: Amphotericin B, cycloserine and cefoxitin) (15). Detection of Toxin A and Toxin B was performed by Immuno Card Toxins A&B-EIA (Meridian, Bioscince, USA).

For identification of C. difficile was used Latex-agglutination Culturette TM CDTTM Test Kit (Becton Dickinson, USA) (15). Bacterial DNA was isolated by QIAampR DNA Mini Kit (Qiagen). Detection of toxin genes: tcdA, tcdB, cdtA and cdtB was performed by PCR.

The following primers were used for gene amplification:

- **tcdA gene** (331bp)

Tox-A-s 5'-TGTTGGAATAGGTGCTGAAG-3'

ToxA-as 5'AGATGGAGATGAGAAAAAGTGA- 3' (in house primers, ECDC)

- **deletion in tcdA gene** (2535bp/ 714bp)

NKV 011 5'-TTTTGATCCTATAGAATCTAACTTAGTAAC- 3'

NK 9 5'- CCACCAGCTGCAGCCATA- 3' (16)

- **tcdB** (204bp)

NK104 5'- GTGTAGCAATGAAAGTCCAAGTTTACGC- 3'

NK105 5'-CACTTAGCTCTTTGATTGCTGCACCT- 3' (17)

- **cdtA** (376bp)

cdtA- fw 5'-TGAACCTGGAAAAGGTGATG-3'

cdtA- rev 5'- AGGATTATTTACTGGACCATTTG-3'(8)

- **cdtB** (510bp)

cdtB-fw 5'- CTTATTGCAAGTAAATACTGAG- 3'

ctdB-rev 5'- ACCGGATCTCTTGCTTCAGTC- 3' (8)

PCR- ribotyping was performed with primers:

16S 5'-GTGCGGCTGGATCACCTCCT- 3'

23S 5'-CCCTG CACCCTTAATAACTTGACC- 3' and according Bidet's protocol (2).

PCR products were separated by "HDA-GT12" capillary gel electrophoresis system (Qiagen Corp.) (33).

RESULTS

20% (18/90) of all stool samples were positive for C. difficile. 23% (15/65) from clinical samples and 12% (3/25) from horses were positive for C. difficile by culture test. 21, 5% (14/65) of clinical isolates produce toxins A and B by EIA. The three isolates from horses were negative for toxins by EIA.

Identification of C. difficile strains was confirmed by Gram-staining and latex-agglutination test.

The tcdA gene was detected by PCR with primers Tox-A-s/ Tox-A-as in 86,7% (13/15 clinical isolates) whereas 13,3% (2/15) were negative with these primers (Fig. 1).

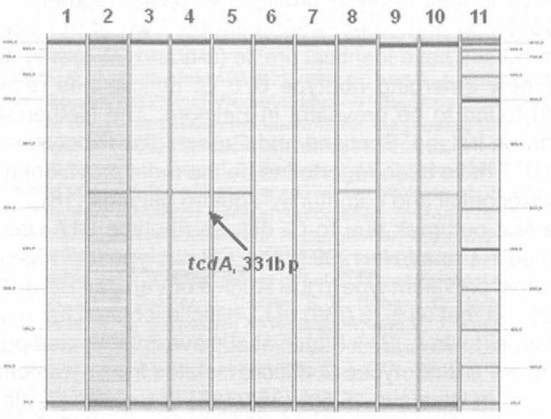

Fig. 1. Detection of tcdA gene in C. difficile clinical strains 1- 36; 2- 181; 3- 1795-9; 4- 1797-15; 5- 217; 6- 223; 7- 239; 8- 253; 9- negative control, ddH20; 10- ribotype 002 (ECDC) possitive for toxA gene; 11- DNA marker, 50-1000 bp

Deletion in tcdA gene (714bp) was detected in 40% (6/15) of the clinical strains and they were toxin A-negative. The intact tcdA gene (2535bp) was amplified in 46,7% (7/15) of the strains, which were considered as toxinA- positive. We don't detect tcdA gene and with primers NKV 011/NK 9 in 13,3% (2/15) C. difficile strains (Fig. 2).

Discrimination of A+/B+ and A-/B+ C. difficile isolates was performed by PCR with primers NKV011/NK9 targeting a specific deletion in tcdA gene (714bp) (16). Presence of this deletion leads to production of inactive toxin A.

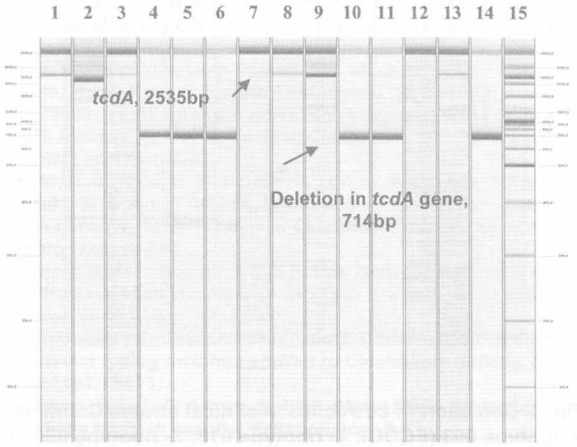

Fig. 2. Detection of deletion in tcdA gene in clinical strains C. difficile
1- 1795-5; 2- 225; 3- 240; 4- 36; 5- 181; 6- 217; 7- 223; 8- 253; 9- 256; 10- 237;
11- 238; 12- negative control, dd H2O; 13- ribotype 001; 14- ribotype 017; 15- DNA marker, 100bp-3000bp

The tcdB gene was detected in 93,3% (14/15) C. difficile clinical strains and only a single strain was negative with primers NK104/ NK105 (Fig. 3).

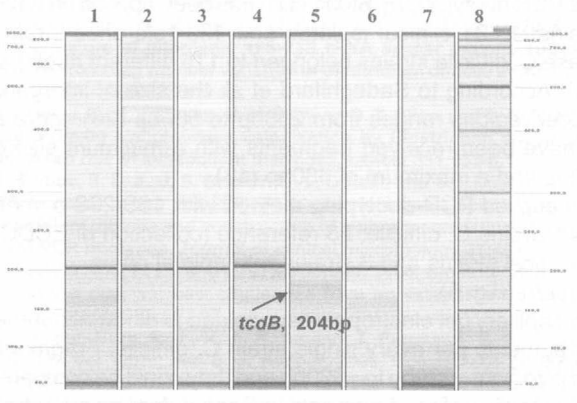

Fig. 3. Detection of tcdB in clinical strains C. difficile
1- 36; 2- 250; 3- 253; 4- 256; 5- 262; 6- ribotype 002 (ECDC);7- negative control, dd H2O; 8- DNA marker, 50-1000 bp

Three toxigenic variants C. difficile have been distinguished among clinical strains by PCR: 46,67% (7/15) toxin A+B+; 46,67% (6/15) A–B+ and 6,67% (2/15) A-B-. In our investigation predominant toxigenic variants were toxin A+B+ and A-B+.

The binary-toxin genes cdtA and cdtB was PCR detected in one of the A+B+ strains (Fig. 4).

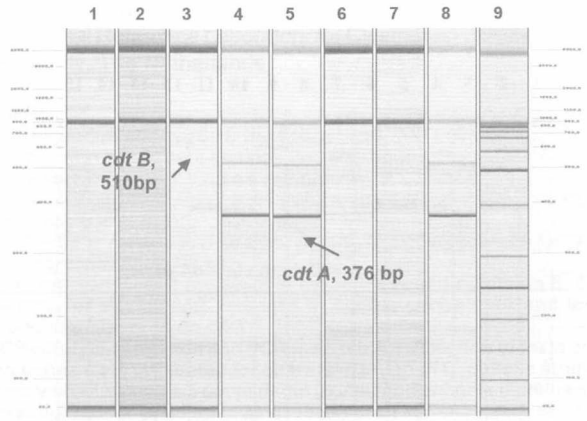

Fig. 4. Detection of cdtA/cdtB in clinical strains C.difficile 1- ribotype 003 (ECDC); 2- ribotype 015; 3- ribotype 056; 4- ribotype 027; 5- ribotype 023; 6- 36; 7- 181; 8- 1795-9; 9- ДНК marker, 100- 3000 bp

The genes tcdA, tcdB and cdtA/cdtB were not detected by PCR in the C. difficile isolates from horses.

The PCR-ribotyping scheme has been applied for typing isolates C. difficile from the United Kingdom since 1995 (28). PCR-ribotyping patterns are based on size variations in the 16S-23S intergenic spacer region of the bacterial rRNA (rrn) operon. Variation in spacer length also observed not only in different isolates, but and between different copies of the operons in the same genome (31).

The PCR ribotyping by Stubs et al. has been applied on 2,030 strains: 1,631 clinical isolates and 133 reference strains. These C. difficile strains belonged to 116 different ribotypes (28). According to Sadeghifard et al. the size of intergenic spacer regions ranges from 238bp to 566bp (26). Indra et al. have been received fragments with a minimum size of 233bp and a maximum of 680bp (13).

We applied PCR-ribotyping method with 16S/23S primers to 43 strains C. difficile: 25 reference (collection of ECDC); 15 clinical strains and 3 strains with animal origin (horses) (Fig. 5).

The capillary gel electrophoresis yielded to different number of fragments per every single strain C. difficile. Fragments had size from ~230bp to ~690bp and they could be compared well with size of the fragments in Sadeghifard's and Indra's approaches (13, 26).

We distinguished six ribotypes among investigated clinical isolates C. difficile:

40 % (6/15) of isolates C. difficile are ribotype 017/ isolates 36, 181, 217, 237, 238, 250 ; 13% (2/15)- ribotype 002/ isolates 240, 262; 13% (2/15)- ribotype 014 (020)/ isolates 253, 256; 7% (1/15)- ribotype 078/ isolate 1795-9; 7% (1/15)- ribotype 046/ isolate 1797-5; 7% (1/15) – ribotype 012/isolate 225 (table 1).

13% (2/15) of clinical isolates C. difficile are not typeble (isolates 223 and 239). Isolate C. difficile 223 gave by capillary gel electrophoresis two fragments ~ 300bp and ~386 bp; isolate 239 - ~210bp and ~470bp.

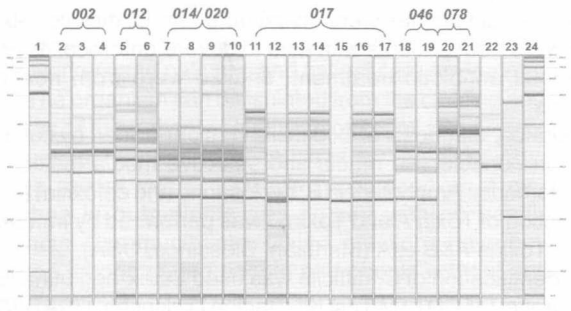

Fig. 5. PCR-ribotyping of C. difficile clinical strains 1- DNA marker, 50-1000bp; 2- ribotype 002 (ECDC); 3- 240; 4- 262; 5- ribotype 012; 6- 225; 7- ribotype 014; 8-ribotype 020; 9- 253; 10- 256; 11- ribotype 017; 12- 36; 13- 181; 14- 217; 15- 237; 16- 238; 17- 250; 18-ribotype 046; 19- 1797-15; 20- ribotype 078; 21- 1795-9; 22- 223; 23- 239; 24- DNA marker, 50-1000bp

Table 1. Prevalent ribotypes in C. difficile clinical isolates

Ribotype (n = 13 isolates)	Percent out of all determined Ribotypes (%)
017 (6)	40
002 (2)	13,3
014/020 (2)	13,3
012 (1)	6,7
046 (1)	6,7
078 (1)	6,7

The prevalent PCR-ribotype in clinical isolates C. difficile is 017. Four isolates ribotype 017 (isolates 217, 237, 238, 250) origin from one hospital in Sofia. Three patients with C. difficile ribotype 017 (isolates 181, 217 and 237) infection had lethal outcomes (33).

The ribotype of 10 clinical isolates C. difficile (36, 181, 1795-9, 1797-15, 217, 225, 237, 238, 240, 250) were confirmed by PCR-ribotyping in C. difficile Reference Laboratory /CDRL/, Leiden University Medical Center, The Netherlands (14).

PCR-ribotyping patterns of the three isolates C. difficile from horses were different from patterns of the clinical strains. We differed in their ribotype profiles two basic fragments: one ~230-240bp and other ~430bp-440bp. Two of the isolates from horses have identical profile (8-h and 22-h) (Fig. 6).

The new emerging ribotype 078 C. difficile has recently been found to be prevalent in Belgium, The Netherlands, Northern Ireland, Scotland and Greece (9). Ribotypes 046 and 017 have been reported to be the most prevalent types in nosocomial and community-aquired settings (18)

The first outbreak due to C. difficile ribotype 017 was described in Canada in 1999 (24). PCR ribotype 017 has been the most prevalent type in the studies of Van den Berg. They improved that 94% (37/39) of C. difficile isolates have been 017 type (toxin A-/B+). Pituch et al. have investigated prevalence of PCR-ribotypes C. difficile isolated from symptomatic patients in Warsaw. 45,5% (357/785) of isolates C. difficile from patients with CDAD have been ribotype 017. Isolates belonging to PCR-ribotype 017 have been found in epidemics among patients with antibiotic–associated diarrhea in internal and surgery units (25).

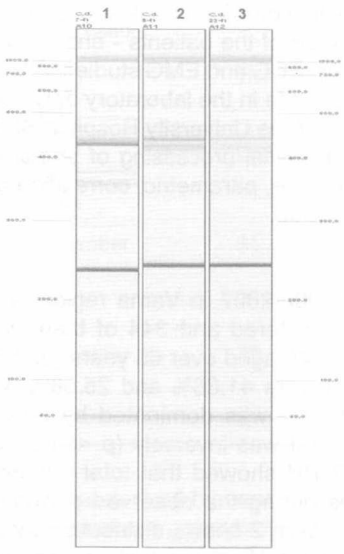

Fig.6. PCR- ribotyping of C. difficile isolates from horses 1- 7; 2- 8; 3- 22

CONCLUSIONS

For the first time in Bulgaria with PCR-ribotyping method and PCR-methods for detection of toxin coding genes were characterized C. difficile isolates with human and animal origin. C. difficile isolates were compared to the most prevalent ones in European countries. (30). The results of the current study would improve the diagnostic and therapeutic preparedness of the Bulgarian hospitals when dealing with C. difficile infections.

There were no data for acquisition of a human CDI as a result of animal contact but animal acquisition of C. difficile from humans has been suggested by some studies (20). The lack of a standard nomenclature and typing system, complicate understanding of common C. difficile strains between animals and humans (10).

REFERENCES

1. Arroyo, L. G., S. A. Kruth, B. M. Willey, H. R. Staempfli, D. E. Low, and J. S. Weese. 2005. PCR ribotyping of Clostridium difficile isolates originating from human and animal sources. Journal of medical microbiology 54:163.

2. Bidet, P., V. Lalande, B. Salauze, B. Burghoffer, V. Avesani, M. Delmee, A. Rossier, F. Barbut, and J. C. Petit. 2000. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing Clostridium difficile. Journal of clinical microbiology 38:2484.

3. Boyanova, L., M. Marina, T. Kantardjiev, I. Mitov. 2008. Clostridium difficile- associated disease- increased alarm after appearence of hypervirulent strain Savr. med. 59:70-78.

4. Boyanova, L., M. Marina, T. Kantardjiev, I. Mitov. 2008. Diagnosis and therapy of Clostridium difficile- associated disease Savr. med. 59:71-80.

5. Cartwright, C. P., F. Stock, S. E. Beekmann, E. C. Williams, and V. J. Gill. 1995. PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of Clostridium difficile. Journal of clinical microbiology 33:184.

6. Crobach, M. J., O. M. Dekkers, M. H. Wilcox, and E. J. Kuijper. 2009. European Society of Clinical Microbiology and Infectious Diseases (ESCMID): data review and recommendations for diagnosing Clostridium difficile-infection (CDI). Clin Microbiol Infect 15:1053-66.

7. Dial, S., A. Kezouh, A. Dascal, A. Barkun, and S. Suissa. 2008. Patterns of antibiotic use and risk of hospital admission because of Clostridium difficile infection. Canadian Medical Association Journal 179:767.

8. Goncalves, C., D. Decre, F. Barbut, B. Burghoffer, and J. C. Petit. 2004. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from Clostridium difficile. J Clin Microbiol 42:1933-9.

9. Goorhuis, A., D. Bakker, J. Corver, S. B. Debast, C. Harmanus, D. W. Notermans, A. A. Bergwerff, F. W. Dekker, and E. J. Kuijper. 2008. Emergence of Clostridium difficile infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clinical Infectious Diseases 47:1162.

10. Gould, L. H., and B. Limbago. Clostridium difficile in food and domestic animals: a new foodborne pathogen? Clinical Infectious Diseases 51:577.

11. HALL, I. C., and E. O'TOOLE. 1935. Intestinal flora in new-born infants: with a description of a new pathogenic anaerobe, Bacillus difficilis. Archives of Pediatrics and Adolescent Medicine 49:390.

12. Hyett, A. P., J. S. Brazier, and G. L. O'Neill. 1997. Pulsed-field gel electrophoresis as a method for typing Clostridium difficile in the routine laboratory. Reviews in Medical Microbiology 8:S64.

13. Indra, A., D. Schmid, S. Huhulescu, M. Hell, R. Gattringer, P. Hasenberger, A. Fiedler, G. Wewalka, and F. Allerberger. 2008. Characterization of clinical Clostridium difficile isolates by PCR ribotyping and detection of toxin genes in Austria, 2006–2007. Journal of medical microbiology 57:702.

14. Ivanova, K., P. Petrov, G. Aseva, E. Dobreva, I. Ivanov, R. Vatcheva-Dobrevska, M. Marina, T. Kantardjiev, D. W. Notermans, E. Kuijper. 2011. Prevalence of Clostridium difficile PCR ribotypes in Bulgaria, 2008-2010. Comptes rendus de l'Academie bulgare des Sciences 64:1051-1058.

15. Ivanova, K., P. Petrov, G. Aseva, E. Dobreva, I. Ivanov, R. Vatcheva-Dobrevska, M. Marina, V. Tolchkov, T. Kantardjiev, D. W. Notermans, E. Kuijper. 2010. First cases of severe hospital-acquired Clostridium difficile. Probl. of Infect.and Parasit. Dis. 38:22-24.

16. Kato, H., N. Kato, S. Katow, T. Maegawa, S. Nakamura, and D. M. Lyerly. 1999. Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive Clostridium difficile strains. FEMS Microbiol Lett 175:197-203.

17. Kato, H., N. Kato, K. Watanabe, N. Iwai, H. Nakamura, T. Yamamoto, K. Suzuki, S. M. Kim, Y. Chong, and E. B. Wasito. 1998. Identification of toxin A-negative, toxin B-positive Clostridium difficile by PCR. J Clin Microbiol 36:2178-82.

18. Kuijper, E. J., B. Coignard, and P. Tull. 2006. Emergence of Clostridium difficile-associated disease in North America and Europe. Clin Microbiol Infect 12 Suppl 6:2-18.

19. Kuijper, E. J., R. J. van den Berg, and J. S. Brazier. 2009. Comparison of molecular typing methods applied to Clostridium difficile. Methods Mol Biol 551:159-71.

20. Lefebvre, S. L., L. G. Arroyo, and J. S. Weese. 2006. Epidemic Clostridium difficile strain in hospital visitation dog. 1.

21. Lyerly, D. M., H. C. Krivan, and T. D. Wilkins. 1988. Clostridium difficile: its disease and toxins. Clinical microbiology reviews 1:1-18.

22. Marks, S. L., E. J. Kather, P. H. Kass, and A. C. Melli. 2002. Genotypic and phenotypic characterization of Clostridium perfringens and Clostridium difficile in diarrheic and healthy dogs. Journal of veterinary internal medicine 16:533-540.

23. Mitchell, T., J. Ketley, S. Haslam, J. Stephen, D. Burdon, D. Candy, and R. Daniel. 1986. Effect of toxin A and B of Clostridium difficile on rabbit ileum and colon. Gut 27:78.

24. Pepin, J., L. Valiquette, M. E. Alary, P. Villemure, A. Pelletier, K. Forget, K. Pepin, and D. Chouinard. 2004. Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. Canadian Medical Association Journal 171:466.

25. Pituch, H., J. S. Brazier, P. Obuch-Woszczaty ski, D. Wulta ska, F. Meisel-Miko ajczyk, and M. uczak. 2006. Prevalence and association of PCR ribotypes of Clostridium difficile isolated from symptomatic patients from Warsaw with macrolide-lincosamide-streptogramin B (MLSB) type resistance. Journal of medical microbiology 55:207.

26. Sadeghifard, N., V. Gurtler, M. Beer, and R. J. Seviour. 2006. The mosaic nature of intergenic 16S-23S rRNA spacer regions suggests rRNA operon copy number variation in Clostridium difficile strains. Appl Environ Microbiol 72:7311-23.

27. Shim, J. K., S. Johnson, M. H. Samore, D. Z. Bliss, and D. N. Gerding. 1998. Primary symptomless colonisation by Clostridium difficile and decreased risk of subsequent diarrhoea. Lancet 351:633-6.

28. Stubbs, S. L. J., J. S. Brazier, G. L. O'Neill, and B. I. Duerden. 1999. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of Clostridium difficile and construction of a library consisting of 116 different PCR ribotypes. Journal of clinical microbiology 37:461.

29. Sunenshine, R. H., and L. C. McDonald. 2006. Clostridium difficile-associated disease: new challenges from an established pathogen. Cleve Clin J Med 73:187-97.

30. van den Berg, R. J., H. A. A. Ameen, T. Furusawa, E. C. J. Claas, E. R. van der Vorm, and E. J. Kuijper. 2005. Coexistence of multiple PCR-ribotype strains of Clostridium difficile in faecal samples limits epidemiological studies. Journal of medical microbiology 54:173.

31. van den Berg, R. J., E. C. Claas, D. H. Oyib, C. H. Klaassen, L. Dijkshoorn, J. S. Brazier, and E. J. Kuijper. 2004. Characterization of toxin A-negative, toxin B-positive Clostridium difficile isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. J Clin Microbiol 42:1035-41.

32. Vatcheva-Dobrevska, R. 2009. Methods for prevention and control of infection in patients with Clostridium difficile- associated disease. Nosocomial infections:11-16.

33. Vatcheva-Dobrevska, R., E. Dobreva, I. Ivanov, K. Ivanova, M. Marina,T. Kantardjiev. 2010. PCR- ribotyping as a method for characterization of clinical isolates Clostridium difficile Milit. med. 3:13-18.

34. Voth, D. E., and J. D. Ballard. 2005. Clostridium difficile toxins: mechanism of action and role in disease. Clin Microbiol Rev 18:247-63.

LYME BORRELIOSIS IN PATIENTS OVER 60-YEARS-OLD

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SUMMARY:
Lyme borreliosis is the most common tick-borne infection in Europe. All age groups are susceptible. Problems of persons over 60 years (60 +) are the focus of WHO and relative institutions. Various aspects of infectious diseases in patients over 60s are not investigated. Our goal was to investigate the course of Lyme borreliosis (LB) in elderly patients. 74 patients with Lyme borreliosis aged 61 to 85 years and 89 controls aged 20 to 57 years for the period 2000 – 2007 were examined. Routine clinical and epidemiological methods, hematological and biochemical tests, serological methods for detection of anti-borrelial antibodies in serum and CSF of patients were used. Variation, alternative, parametric, correlation and graphical analysis were used for statistical analysis. Within this period in Varna region, 497 people were infected with LB, of whom 344 were hospitalized. Of these, 149 were aged over 60 years (32.55 percent). 77.02% of the adults have accompanying illnesses. Clinical course, laboratory findings, seropositivity and therapeutic approaches in patients with LB at 60 + are described. Conclusions: Lyme borreliosis proceeds as normal in patients over 60 years; in patients at 60 + late LB was not observed; the most common form of early LB in elderly patients was EM; in 44.59% of the patients wound, crusta, necrosis or bula were found in the central part of EM; regional lymphonodulitis was established in 48.64% of the patients over 60 years with LB; initial seropositivity was present in 18.90% of the patients at 60 +. Early and adequate treatment of the old patients conducted in the early stage of LB is beneficial and a guarantee of a good prognosis.

Keywords: Lyme borreliosis, elderly, erythema migrans

INTRODUCTION:

Lyme borreliosis (LB) is the most common tick-borne disease in Europe. The most affected are Veliko Tarnovo and Varna regions, but in recent years the disease is widespread. All age groups are affected. Problems of patients over 60 years (60 +) are the focus of WHO and its sister institutions. This age group is associated with a number of socially significant diseases. Problems of atherosclerosis, cardiovascular diseases, diabetes, dementia and others are studied in detail. Information in the literature about course of infectious diseases in old and elderly patients is scarce. A number of issues of infectious diseases in patients over 60-year-old are not investigated.

OBJECTIVE:

To investigate course of Lyme borreliosis (LB) in old and elderly patients.

MATERIAL AND METHODS:

74 patients with Lyme borreliosis aged 61 to 85 years (mean age - 73,24 ± 1,83 years old) and 89 controls aged 20 to 57 years (mean age - 37,46 ± 4 62 years) for the period 2000 - 2007 were studied. Routine clinical and epidemiological methods were used - targeted epidemiological history, physical examination and monitoring of patients, haematological and biochemical tests - blood count, blood sugar, urea, total bilirubin, GPT, GOT, urine and CSF; CRP, serological methods for detection of antibodies to Borrelia burgdorferi in serum and CSF of the patients - enzyme immunoassay method (ELISA); ECG and EMG studies. All clinical laboratory tests were made in the laboratory of Clinical Infectious Diseases Clinic of the University Hospital "St. Marina" EAD - Varna. For statistical processing of primary information, variation, alternative, parametric, correlation and graphical analysis were made.

RESULTS:

Within period 2000-2007 in Varna region, a total of 497 people were registered and 344 of them were hospitalized. Of these, 149 aged over 60 years (32.55%). Patients of 18-59 years were 41.86% and 25.58% were children. The group of 60 + was dominated by rural inhabitants, while in controls it was inversely ($p < 0,05$). Official registration of RIPCPH showed that total number of infected from the cities during the observed period was greater. Figure 1 and Figure 2 shows distribution by sex and residence of the patients. Women in both groups were more often infected than men. Similar data have been provided by other Bulgarian as well as European authors (2,3,4,6,9,10,12).

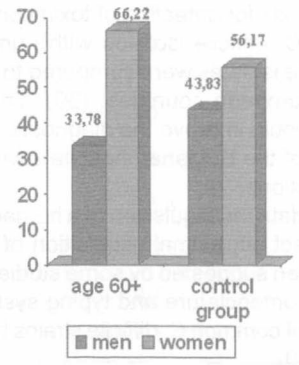

Fig 1. Distribution of patients with LB by gender

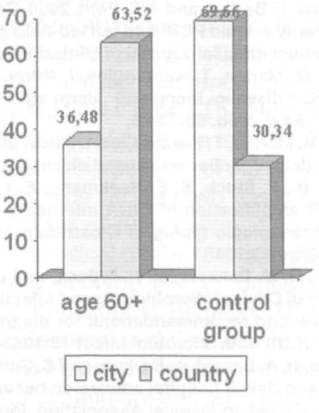

Fig 2. Distribution of patients with LB by residence

All 74 patients at age 60 + reported a tick bite, and similar is situation in the control group - only 5 (5.61%) had no recollection of tick bites but an opportunity for tick attack - farming, working in the field and others (Table 1). According to the current knowledge, LB without a tick bite does not exist (12). When tick infestation is about 20%, estimated risk is that one of 100 bites causes EM case and one of 400 - a case of LB in stage II-III (12).

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Table 1. Tick bites location

		Tick bite					
		Leg	Hand	Body	Head	Neck	No data for tick bite
Controls n=89	number	31	8	22	15	0	5
	%	34,83	8,98	24,71	16,85	0,00	5,61
„60+"	number	20	4	36	11	3	0
n=74	%	27,02	5,40	48,64	14,86	4,05	0,00

The disease is characterized by seasonality, and the largest number of hospitalized patients with LB over 60 years were in May, April and June (Fig. 3). Similar was situation with the controls. Actually, there were infected patients from February to November, corresponding to the seasonal peak in number of tick populations in the region. Seasonal peak of ticks is from April to July, but circulation is up to October (1). During the observed period, entering of species I. ricinus in settlements, gardens, parks, green spaces around dwellings, was reported. About Varna district according to our authors (1), leading among tick populations is that of I. ricinus – up to 83% and it is the main vector of LB. I.ricinus is the dominant species in the whole country and in Europe, which explains why LB is the most common tick-transmitted infection. In Varna region, estimated tick infestation is 15.05% (1).

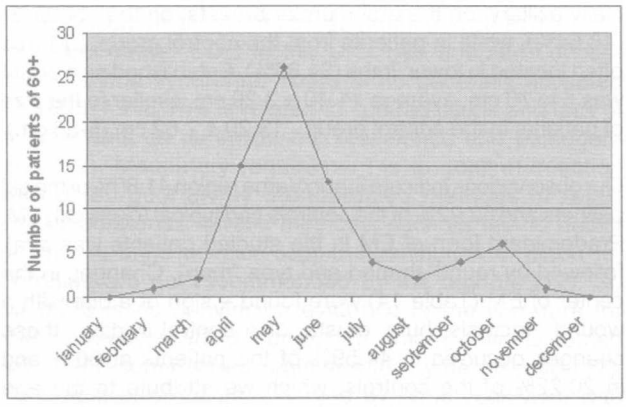

Fig. 3. Number of patients of 60+ with Lyme disease according to season

The incubation period of LB is from 3 days to 4 weeks. In investigated by us patients, it was 10.05.± 2.68 days and in controls it was 8.32 ±0.90 days (from 2-30 days). Table 2 shows that 77.02 % of adults had accompanying illnesses and in the control group - only 5.61% ($p < 0.05$). The most numerous was group of patients over 60 + with hypertension.The sum of percentages does not equal 100 because some patients had more than one illness.

Table 2. Presence of accompanying illnesses in patients with Lyme disease ($p<0.05$)

Accompanying illnesses	Patients 60+ n=74		Controls (18-59 y) n=89	
	Number	%	number	%
Yes	57	77,02	5	5,61
No	17	22,97	84	94,38
Hypertension	49	66,21	2	2,24
Ischemic heart disease	15	20,27	0	0,00
Cerebral vascular disease	4	5,40	0	0,00
Heart failure	6	8,10	0	0,00
Bronchial asthma	2	2,70	0	0,00
Rheumatoid arthritis	2	2,70	0	0,00
Ulcer	2	2,70	1	1,12
Chronic gastritis	1	1,35	0	0,00
Neoplasm	5	6,75	0	0,00
ХВБСН	1	1,35	1	1,12
ХОББ	3	4,05	0	0,00
Parkinson syndrome	1	1,35	0	0,00
Diabetes mellitus	4	5,40	0	0,00
Renal stone disease	1	1,35	0	0,00
Chronic lymphatic leucosis	1	1,35	0	0,00
Neurosis, neurasthenia	0	0,00	1	1,12

Data from the literature on the clinical manifestation suggest that it varies in different regions according to antigenic mosaic of the etiological agent (12). Transition from one stage to another is sometimes smooth, but sometimes there are symptoms characteristic of various stages.The disease can appear at every stage. Table 3 shows clinical characteristics of LB observed in our patients. Typical manifestation of the first stage of the disease is Erythema migrans (EM) - erythematous skin lesion - an expanding ring or arch-shaped gradually fade into the center. It starts as red papula or macula, which for several days to weeks, extends to a large, red plaque measuring 5 to 50 cm (3,12). EM is the only manifestation of Lyme disease, which is sufficient for diagnosis without need of laboratory confirmation (12). EM was observed in 95.94% of the adult patients (71) and in 83.14% of the controls. EM in patients at 60 + was most frequently localized in the body and especially axillary, on the groin, under breasts, on the abdomen (48.64%), while in patients from the control group, EM was often located in lower limbs (34.83%). Established size of EM was 5 to 70 cm, average 11.40.± 3.29 cm, similar to the size of patients in the control group - 13,20 ± 2,62 cm (5-50 cm).

Our observations indicate that in Varna region 41.89% of the old patinets and 62.92% of the controls had typical (classical) EM. Predominant form of EM in the studied patients was oval, followed by round-shaped and type "map". Changes in the center of EM (Table 14) were found – sign of a bite with a wound, necrosis, bula, crusta, or a central lividity - these changes occurred in 44.59% of the patients at 60 + and in 20.22% of the controls, which we attribute to the age changes in the wall of blood vesselsl and reduced regenerative capacity of the elder organism. Recovery period for complete clearing of the EM ranged from 3 to 35 days, average 13.26 ± 2.63 days. In 42% of patients at 60 + it was approximately two weeks. In a 69 year-old woman, skin changes occurring within the incubation period after tick bite were the type of Erythaema nodosum. Such skin changes were also described by Zarcheva in her study (2). Common complaints. Single patients in the 60 + group reported itching, burning or mild soreness. In others, transient fatigue, headache, aching muscles and joints were observed. In 5 patients of 60 + (6.75%) and 9 controls (10.11%) short subfebrile temperature was found (Tabl.4). The literature rarely describes common complaints. Short subfebrile temperature, myalgia, arthralgia, headache, fatigue may occur. Local pain, burning and manifested itching are not typical for EM (2,3,4,10). Most researchers agree that high fever in patients with EM is rarely recorded and suggests for coinfection with another pathogen (6,8,9,10,12).

There were also various limfangitis and regional limfonodulitis in the studied patients. In 36 (48.64%) of the 60+ group and in 26.96% of the controls regional limfonodulitis was established. Regional lymph nodes were enlarged to 2 cm in diameter, in most patients – painless, in isolated cases with moderate pain, not involving skin. By clearing the EM lesion, changes in the lymph nodes also went to normal. Lymph nodes decreased 30-35 days after completion of treatment. Arthralgia in 5 of the elder patients could not be interpreted as Lyme arthritis, as it preceded the onset of EM. Similar arthralgia were found in 8 of the controls. Lyme arthritis is characterized by recurrent pain and swelling of large joints, most often knee (in 80%). In chronic Lyme arthritis, there is permanent swelling of the joints more than 1 year. It occurs more frequently in the U.S. (60% of LB), in Europe it is usually milder and affects fewer patients (in 47% of cases) (2,6,9,11,12). In five patients from group 60 +, gonarthrosis and other changes in the locomotor system were found. Lyme-carditis is a manifestation of the second stage, occurring in 4-8% of patients with LB. Most commonly appears as A-V block and other rhythm disorders (12). In all patients studied by us ECG was held. We registered single extrasystoles in only one of the old patients in the

Table 3. Patients with erythema migrans

		Erythema migrans						
		Typical	With wound	With necrosis	With bula	With central lividity	With crusta	none
Controls n=89	Number	56	13	3	1	1	0	15
	%	62,92	14,60	3,37	1,12	1,12	0,00	16,85
„60+" n=74	number	31	11	14	3	5	7	3
	%	41,89	14,86	18,91	4,05	6,75	9,45	4,05

Table 4. Other clinical manifestations in patients with LB

		Lymphadeno-pathy	Fever	Arthralgia	Myalgia	Neuritis, polineuritis
Controls n=89	Number	24	9	8	5	11
	%	26,96	10,11	8,98	5,61	12,35
„ 60+" n=74	Number	36	5	5	5	0
	%	48,64	6,75	6,75	6,75	0,00

first days of hospitalization on the occasion of the EM. In the studied old patients, no case of neuroborreliosis was observed. Their neurological symptoms, most often trembling and tingling in the extremities have preceded the tick attack. We detected age-connected changes (osteochondrosis) in particular sites of the spine. None of the patients at 60 + could connect memory reduction with the tick bite. There was no deterioration of the memory disorders after the tick bite. In 11 patients of the control group (12.35%) polyneuritis was diagnosed. All of them, months after disappearance of EM, reported tingling and trembling of the limbs. EMG-studies have found evidence of polyneuritis with predominantly sensory disorders.

In a 30-year-old woman, hospitalized in the clinic in 2003 for the second time, nevroboreliosis – chronic recurrent poliradiculonevritis, was diagnosed. Months after treatment with penicillin for EM in 2001, "trembling" of the four limbs appeared. LB was serologically confirmed. In 2002, due to the persistence of complaints, IgM antibodies in CSF were detected. In 2003, serum IgM and IgG antibodies persisted. EMG conducted repeatedly throughout the complaints recorded damage of anterior roots of C 6-7-8, L5 and S1.

In two men from the control group, aged 51 and 54 years, neurological complaints without objective findings persisted. This reason – lack of objective finding and adequately conducted treatment of LB manifestations at an earlier stage, led us to discuss their status as post-Lyme syndrome. Most authors agree that in these cases, the patients have unexplained chronic subjective symptoms after treatment of objectively manifested LB (e.g. erythema migrans) (12). Laboratory studies at all stages of the disease are nonspecific (6,12). Table 5 presents deviations in clinical and laboratory findings in the studied patients at 60+ and those in the control group with Lyme disease.

thors are found in 40-60% of patients with early LB (5.12). A higher percentage of negative serological tests in our patients could be explained by earlier time of drawing the first serum sample. Control examination 1 month later showed positive 52.70% of the old patients (39 people). Similar data for increasing the rate of seropositivity in the later samples have been shown by other authors (2,3,5). After 2-3 months, IgG antibodies were established in 55.40% (41) of the old patients. Negative serological result does not reject the diagnosis. Over the time, antibody levels gradually decrease, but IgG and even IgM can persistit for years. About 9.45% of our elderly patients and 11.23% of the controls (followed up to 1 year after hospitalization) continue to maintain high levels of IgG and IgM at least one year after the treatment. Similar data for about 10 % are referred by other authors (7). Persistence of antibodies without clinical signs is not a requirement to repeat the treatment. Suspected for LB patients are recommended for high-tech diagnostic methods (Immunoblot, PCR, etc.). In recent years, CDC in the U.S. has adopted concept of post-Lyme syndrome. There is no generally accepted definition for it. This is related to contradictions and lack of reliable data on its incidence, prevalence and pathogenesis (12). Mandatory requirement is to confirm infection with B. burgdorferi by standardized methods in reference laboratories. Invalid methods, such as test to detect antigen in urine or microscopic examination of blood for borreliae are not recommended by the authors (12). So far there is no convincing evidence in the literature for chronic infection with B. burgdorferi among patients treated with the recommended regimens for Lyme disease (12). Treatment of probants observed by us is consistent with the recommendations of EUCALB, CDC, the consensus of BSID and clinical stage of the disease. In 90.54% of adults and 76.40% of the controls i.v. penicillin is applied, which

Table. 5. Clinical, laboratory and serological findings in patients with LB

		Deviations in laboratory findings		Serological investigations				
				Isample				
		Erythrocyte Sedimentation rate	Liver function tests	Positive	Negative	Not investigated	II sample (positive)	III samle (positive)
Patients 60+	number	21	4	14	25	35	39	7
	%	28,37	5,40	18,90	33,78	47,29	52,70	9,45
Controls	number	0	0	53	26	10	53	10
	%	0,00	0,00	59,55	29,21	11,23	59,55	11,23

Laboratory tests showed no abnormalities except moderately accelerated erythrocyte sedimentation rate in 21 old patients (28.37%). In 4 patients over 60 years (5.40%) slight increase in transaminases and in two patients mild direct bilirubinemia, suggesting involvement of the liver in the infectious process, were recorded. Similar reports give Bulgarian and other authors (2,3). In our study, from 52.70 % studied elder patients (Table 5) IgM antibodies were detected in 18.9%, 33.78% were negative. In the control group, positive were 59.55%, and 29.21% were negative. Specific antibodies according to various au-

is consistent with the greater frequency of EM. In cases of other than skin involvement cephalosporins III generation were applied. Taking into account the terrain and side-effects, treatment with doxycycline p.o. was performed in 6.75% of the patients at 60+ and in 7.86% of the controls. In three of the controls, because of accompanying illness, combinations of other antibiotics were used (Fig. 4). Timely and adequate treatment is a guarantee of good prognosis of LB. This view is confirmed by the fact that none of our elder patients were hospitalized with arthritic or polineuritic complaints after completing the treatment (ffollowed 1,3,6, and 12 months).

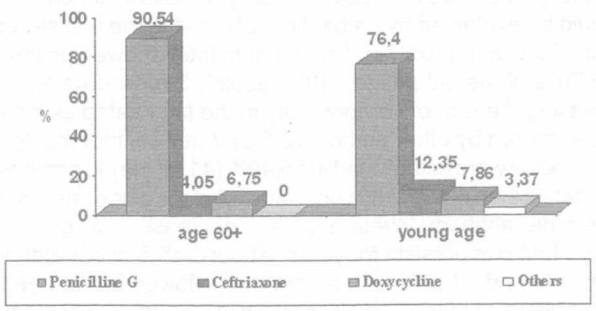

Fig.4. Etiological treatment of patients with LB in %

Conclusions:

1. Lyme borreliosis proceeds as normal in patients over 60 years.
2. We do not observed late Lyme borreliosis in patients at 60 +.
3. The most common form of early LB in elder patients is EM. In 44.59% of these patients, in the central part of the EM lesion, wound, crusta, necrosis or bula could be detected.
4. In 48.64% of the patients over 60 years LB regional limfonodulitis was established.
5. Early seropositivity is present in 18.90% of the patients with LB at 60 +, i.e. their humoral immune response is delayed. This inflicts use of immunoblot method in doubtful cases.
6. Timely and adequate treatment of the old patients conducted in early stage of LB is beneficial and a guarantee of good prognosis.

REFERENCES:

1. Valkanova N et al. Tick populations in Norteast Bulgaria and related transmissive infections. Scientific reports of USB – Dobrich, vol. 8, web-based, Feb-Mar, 2006.
2. Zarcheva V. and K. Kostov. Lyme borreliosis. Medical department – Glaxo, Bulgaria, 1994.
3. Komitova R, L. Angelov, I. Christova et al. Erythema migrans in Bulgaria – main characteristics, Infectology 35, 2003, 25-29.
4. Savova J, L. Angelov, I. Vaptzarov. Erythema chronicum migrans and Lyme disease, Dermatol. Venerol., 37, 1988, 62 – 65.
5. Christova I., M. Nikolova, H. Manev. IgM and IgG immune response in the early Lyme borreliosis stage, Immun. 27, 1995, 22 – 23.
6. Brouqui P., F. Bacellar, G. Baranton et al.(ESCMID STUDY GROUP REPORT). Guidelines for diagnosis of tick-borne bacterial diseases in Europe, Clin.Microbiol. Infect. 2004; 10:1108-1132
7. Christova I., R. Komitova. Serological follow – up of patients with erythema migrans: persistence of antibody response to Borrelia burgdorferi in Lyme disease patient, Eur. J. Inflam., 1, 2003, 73 – 77
8. Cimperman J., Maraspin V., Lotric – Furlan S., Ruzic – Sablji c E., Avsi c – Zupanc T., Picken RN., Strle F. Concomitant infection with tick – borne encephalitis virus and Borrelia burgdorferi sensu lato in patients with acute meningoencephalitis.MEDLINE – DISC 2 (10/1997 – 08/1998)
9. Dattwyler R., et al. Ceftryiaxone compared with doxycycline for the treatment of acute disseminated Lyme disease. N Engl J Med, 1997, 337, 289 – 294
10. Smith, R. et al. Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. – Ann. Intern. Med., 136, 2002, 421 – 479
11. Weber, K. et H. Pfister. History of Lyme borreliosis in Europe. – In: Aspect of Lyme borreliosis. K. Weber et W. Burgdorfer (eds). Berlin, Springer – Verlag, 1993, 1 – 20
12. Wormser G.,Dattwyer J, Shapiro D. et al., The Clinical assessment, treatment and prevention of Layme Disease, Human Granulocytic Anaplasmosis and Babesiosis: Clinical Practice Guidelines by the Infection Disease Society of America, Clin I Dis. 2006, 43:1089-134.

CHLAMYDIA TRACHOMATIS IN SYMPTOMATIC STD PATIENTS FROM SOFIA, BULGARIA: A FOUR YEAR SURVEY, 2006-2009.

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SUMMARY:
In total 512 samples were examined for Chlamydia trachomatis. All samples were analyzed by nucleic acid amplification (PCR) test. The specimen of choice for the men was first-catch urine and cervical swab sample for the women, as recommended. We identified prevalence of C. trachomatis of 3.71% in the examined samples. The prevalence of Chlamydia infection for the town of Sofia could not be deduced from these figures. We identified higher prevalence of C. trachomatis among men, thus men possibly provide a reservoir for continued transmission to women. Screening more men with nucleic acid amplification test assays may provide the possibility of reducing the overall burden of Chlamydia in both men and women.

Key words: Chlamydia, PCR

INTRODUCTION

Chlamydiae are Gram-negative, obligately intracellular eubacteria, showing a two-stage developmental life cycle. C. trachomatis infection of the lower genital tract is one of the most prevalent sexually transmitted diseases (STDs) worldwide (3). Chlamydia is by far the most frequently reported sexually transmitted infection (STI) in Europe. In contrast to other STIs, the number of genital Chlamydia trachomatis infections reported annually has remained high and has continued to rise. In 2009, 23 of the 30 EU/EEA countries reported more than 343 000 cases of genital chlamydia (1,2,3). Although C. trachomatis infection in Bulgaria is a notifiable disease, Bulgaria does not report data and is not included in the report.

Antibiotic treatment is obligatory. If not treated in an early stage or if treated incorrectly, the infection can lead to severe complications, such as pelvic inflammatory disease (PID), ectopic pregnancy, tubal infertility, uretritis, chronic prostatitis, infertility in men and/or erectile disfunction. However, 50-80% of infected men and women are asymptomatic. This high number of unrecognized infected individuals provide the reservoir for spreading the infection to other men and women via sexual transmission (1,2).

The goal of the present study was to analyze the trends of Chlamydia trachomatis identified in our laboratory for a four year period, 2006-2009 among symptomatic patients in the capital city of Sofia, Bulgaria. The population of Sofia is estimated to be 1.247.000 (2008).

MATERIALS AND METHODS

Patients and methods

A total of 512 patient samples from 294 men and 218 women were examined between years 2006 - 2009 by the laboratory of molecular microbiology at NCIPD. Our laboratory developed an in-house PCR method. Primers were selected to detect the Outer Membrane Protein gene omp1 gene. Selected primers were CTRA1-ctgcttacggccgacatatgcaggatg and CTRA2-aagcttgctcgagaccatttaactcca. Amplifications were performed at primer annealing temperature at 65 0C., 1.5M MgCl2, 5% of DMSO in the PCR reaction mix and 35 cycles of DNA amplification. DNA extracted form 30-50 ml of first void urine or cervical swabs were analyzed for men and women respectively. Samples were collected from sexually active 20-50 years old men and women.

RESULTS

During the four year period 512 samples were tested. Positive by PCR were 19 patients of which 12 men and 7 women. Negative by PCR were 494 patients of which 282 men and 211 women. Table 1 presents summary of the results.

The prevalence of C. trachomatis among the symptomatic patients was 4,08% in men and 3,21% in women. The general prevalence of C. tracohomatis infection among the population in the capital city of Sofia of symptomatic patients is estimated to be 3,71 %.

The male-to-female ratio for the four years period is 12:7 meaning that almost 43% more cases were reported in men than in women. The overall rate in men is in the opposite situation as compared to Western countries (1,2), suggesting that the reservoir of the infection are the men.

DISCUSSION

The STDs are major global problem. The chlamydial infections are among the most frequently found and important STDs. According to the World Health Organization (WHO), approximately 89 million people are newly infected with C. tra-

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Table 1. PCR analysis of 512 samples analyzed for C. trachomatis.

	PCR	2006		2007		2008		2009	
		N: patients		N: patients		N: patients		N: patients	
men	pos.	52	2	100	3	65	3	77	4
	neg.		50		97		62		73
women	pos.	48	3	85	2	47	1	38	1
	neg.		45		83		46		37
		Total 100		Total 185		Total 112		Total 115	

chomatis infections annually worldwide (3). Chlamydiae may cause asymptomatic infection which creates the potential for underrecognized transmission with significant implications for both individual and population health. Several laboratory methods are used for the diagnosis of C. trachomatis. These include cytological tests for the detection of intracytoplasmic inclusion bodies, cell culture, Enzyme-linked immunosorbent assay (ELISA), Direct immuno- fluorescence (DIF) and DNA amplification via PCR. Survey performed by ECDC in 2009 reports, that for Chlamydia diagnosis the nucleic acid amplification technique is the reference method in use in the European region (2). No seasonal trends could be observed for the reported C. trachomatis infections through 2006-2009. The data presented here do not cover the situation in the country and must be interpreted with caution because the proportion of the Chlamydia cases that are actually diagnosed and reported is likely to differ from the regions across the country.

The estimated prevalence of the Chlamydia infection among the urban population in Sofia could not be deduced from our results. Official data for the prevalence of the Chlamydia infection in Bulgaria have not been published yet.

Our results demonstrate slight prevalence of the Chlamydia infection among men, thus to be suspected as reservoir of the infection. Diagnoses from certain countries, including Bulgaria cannot be included in trend analyses, as they do not have comprehensive surveillance for STI.

REFERENCES

1. European Centre for Disease Prevention and Control. Sexually transmitted infections in Europe, 1990–2009. Stockholm: ECDC; 2011, http://ecdc.europa.eu/en/publications/Publications/110526_SUR_STI_in_Europe_1990-2009.pdf

2. European Centre for Disease Prevention and Control. Chlamydia control in Europe. 2009. doi 10.2900/11364

3. WHO report, http://www.who.int/vaccine_research/diseases/soa_std/en/index1.html

EVALUATION OF A NEW MOLECULAR TEST FOR THE IDENTIFICATION OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATES

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SUMMARY:
Mycobacterium tuberculosis is a clonal organism, once a single nucleotide polymorphism (SNP) is introduced into the genome it will be passed on to the next generation. Drug resistance in M. tuberculosis strains is to a great extent caused by the presence of SNPs in genes conferring resistance to tuberculostatics. SNPs can be used as discriminative markers for drug resistance as well as to generate robust phylogenetic trees. SNP based species identification is also possible. A new molecular method was recently developed for the analysis of drug resistance and strain typing of M. tuberculosis strains, based on the detection of informative genetic markers such as deletions or single nucleotide polymorphisms (SNPs).
The method evaluated in this study, multiplex ligation-dependent probe amplification (MLPA) [Schouten, 2002] contains three essential steps, a hybridization step followed by a ligation step and an amplification step. The final read-out is performed on the MAGPIX device using the Luminex xTAG bead assay. The multiplexing capacity of the assay allows the simultaneous analysis of up to 47 different molecular markers within one sample and therefore provides complex information from a single assay. 13 markers provided information about resistance to first- and second line drugs based on the detection of specific mutations in the bacterial genome. We have analysed DNA isolated from bacterial culture by MLPA and compared the results to the current molecular method for the detection of drug resistance in TB strains, the MTBDRplus and MTBDRsl assays (HAIN Lifesciences).

Key words: Mycobacterium tuberculosis, drug resistance

INTRODUCTION

Tuberculosis (TB), caused by Mycobacterium tuberculosis (MTB), is one of the most devastating bacterial diseases. Current identification of drug resistance in MTB and proper strain identification is time consuming as it requires combinations of several techniques such as microscopy, bacterial culture and molecular methods. This delays the change from empirical to accurate personalized treatment and limits prevention of transmission.

A new molecular method was recently developed for the analysis of drug resistance and strain typing of MTB strains [Bergval, 2008], based on the detection of informative genetic markers such as deletions or single nucleotide polymorphisms (SNPs). MTB is a clonal organism, once a SNP is introduced into the genome it will be passed on to the next generation. Drug resistance in TB strains is to a great extent caused by the presence of SNPs in genes conferring resistance to specific antibiotics. Therefore SNPs can be used as discriminative markers for drug resistance as well as to generate robust phylogenetic trees. SNP based species identification is also possible.

The method evaluated in this study, multiplex ligation-dependent probe amplification (MLPA) [Schouten, 2002] (Fig. 1) contains three essential steps, a hybridization step followed by a ligation step and an amplification step. The final read-out is performed on the MAGPIX device using the Luminex xTAG bead assay. The multiplexing capacity of the assay allows the simultaneous analysis of up to 47 different molecular markers within one sample and therefore provides complex information from a single assay after only a short period of time and with easy hands-on performance. In this study we have established the MLPA in the laboratory. We have analysed DNA isolated from bacterial culture by MLPA and compared the results to the current molecular method for the detection of drug resistance in TB strains, the MTBDRplus and MTBDRsl assays (HAIN Lifesciences), routinely performed in the TB reference laboratory in Sofia [Bachiyska 2010].

OBJECTIVE

The purpose of this study was to assess the feasibility of the recently developed MLPA assay in a setting which is endemic for multidrug-resistant TB (MDR–TB) and where the information gained by the assay is thought to be of added value. Therefore we analysed a selection of MDR-strains with the recently developed MLPA assay at the National Institute of Infectious and Parasitic Diseases in Sofia.

The demonstration study performed at the National Institute of Infectious and Parasitic Diseases in Sofia is part of a joint project between The Netherlands, France, Georgia and Bulgaria. The leading partner is the Royal Tropical Institute in Amsterdam, The Netherlands.

METHODS

MLPA was performed on nine selected culture isolates from patients with MDR-TB collected between 2009 and 2011 in Bulgaria. The clinical isolates have been defined as MDR-TB based on phenotypic drug resistance testing (DST) using the nitrate-reductase assay [Panaiotov and Kantardjiev, 2002] and Bactec 460TB or Bactec MIGIT 960 system for first line and second line drugs. DNA was extracted from MGIT cultures using buffers containing cetyltrimethylammonium bromide (CTAB). For comparison between the MLPA and MTBDRsl/MTBDRplus, the isolates were analysed by the two reverse hybridisation assays MTBDRplus detecting resistance to first line drugs and MTBDRsl detecting resistance to second line drugs [Hillemann, 2007; Kiet, 2010]. The principle of the MLPA assay was previously described [Bergval, 2008] and is briefly outlined in Figure 1. The MLPA assay consists of a hybridisation, ligation and amplification step followed by analysis of the amplicons using a bead-based assay on the MAGPIX device (Luminex Corporation). In addition to the nine samples, a negative control, a contamination control and an assay control are analysed as extra samples. No DNA template or probe mix is added to the negative control thereby sensing for contamination with PCR products. The contamination control contains DNA from a species unrelated to mycobacteria e.g. Staphylococcus aureus (S. aureus).

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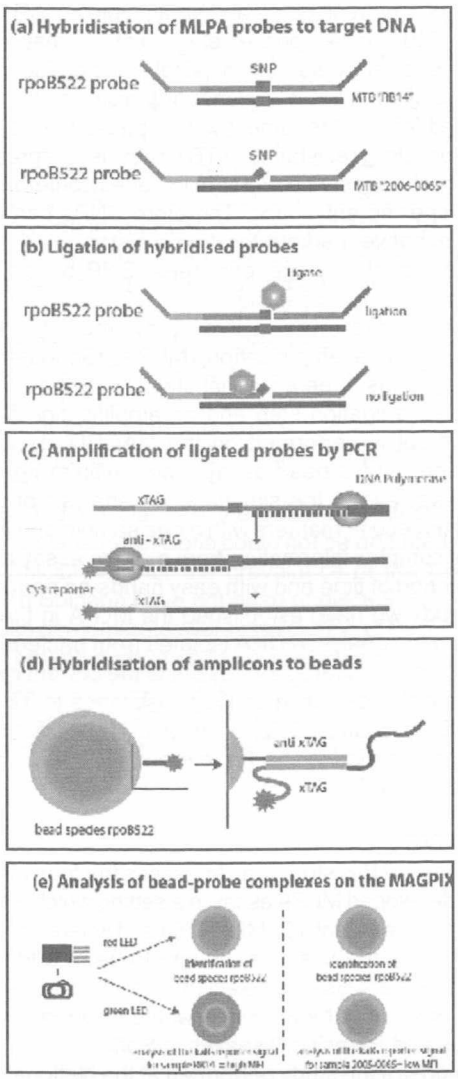

Fig. 1. Overview of the Multiplex Ligation-dependent Probe Amplification (MLPA) assay.
(a) Sequence-specific probes hybridise to the target DNA over night. Each probe consists of a target-specific sequence, xTAG, forward and reverse primer sequences. The rpoB522 probes perfectly match the sequence of the strain RB14 but not strain 2005-0065, indicating that the SNP is only present in strain RB14. (b) Only probes that are 100% complementary to their target sequence hybridise and are ligated by a highly specific ligase. (c) Ligated probes are amplified in a PCR reaction. The reverse primer binds first and amplifies the probes before the labelled forward primer can bind to its complementary sequence generating a probe labelled with a reporter dye. (d) Amplified probes hybridise to their specific bead. Each bead species has a unique xTAG sequence that is complementary to the probe xTAG and is present in multiple copies on the bead surface. (e) Analysis of the bead-probe complexes takes place on the MAGPIX device. A red light emitting diode (LED) and a CCD camera identify first the individual bead species before green LEDs excite the reporter molecules on the probes. The signal is translated into Median Fluorescence Intensity (MFI). The MFI is proportional to the amount of probes bound to the bead.

a MLPA can detect resistance to INH, RIF, STR, EMB, FLQ, AMK, KM, CAP
b MTBDRplus and MTBDRsl detect resistance to INH, RIF, EMB, FLQ, AMK, KM, CAP
c Drug Susceptibility Test (DST) was performed for INH, RIF, STR, EMB, FLQ, AMK, KAN and CAP
INH, isoniazid; FLQ, fluoroquinolones; EMB, ethambutol; RIF, rifampicin; AMK, amikacin; KM, kanamycin; CAP, capreomycin.

Contamination with MTB DNA will be detected with this control. The assay control contains DNA from a MTB strain that has been previously analysed by MLPA and quality controls the assay.
For this study, the results of 13 informative markers for drug resistance were analysed. These markers represent the most prevalent mutations revealed from literature conferring resistance to rifampicin, isoniazid, ethambutol, streptomycin, amikacin, kanamycin, capreomycin and fluoroquinolones. An additional marker detects an MTB-specific sequence within the 16S rRNA locus. Only strains that are positive for this marker are further analysed. Markers for drug resistance included in the assay are listed in Table 1.

Table 1. Drug resistance markers included in the MLPA assay

FLD	Ethambutol	embB-306 (MLPA probes detect wild type MLPA sequence)
	Isoniazid	katG-S315T (high level INH) , inhA-C(-)15T (low level INH)
	Rifampicin	rpoB-V176F, rpoB-S522L, rpoB-H526D, rpoB-H526Y, rpoB-S531L
	Streptomycin	rpsl-43 (MLPA marker detects wt sequence)
SLD	Aminoglycosides Macrocyclic peptides	rrs-1401 (AMK/KAN/CAP) (MLPA probes detect wild type sequence) rrs-1402 (nucleotide change C to G) (CAP)
	Fluoroquinolones	gyrA-A90V, gyrA-D94G

RESULTS

The MLPA technique contains a hybridisation, ligation and amplification step. The successful generation of amplicons can be checked by agarose gel electrophoresis prior to analysis. Products of about 150 bp were amplified in all nine analysed strains (Fig. 2, lane 4,6-13). No products are present in the negative control and products of smaller size are present in the contamination control, which is possibly indicative of unspecific amplification products (Fig. 2, lane 1-3). Following the amplification step, the amplicons are analysed using the bead-based assay. All nine strains analysed were positive for the 16S rRNA marker indicating a genetic background of MTB (data not shown).

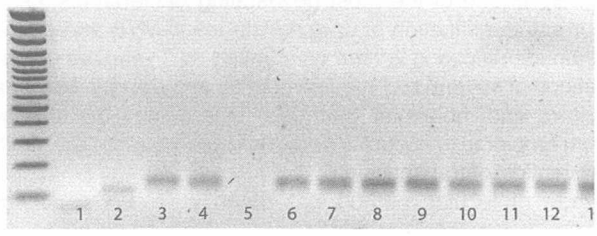

Fig. 2. PCR products obtained after the PCR step of the MLPA assay.
Probes that could bind to their target sequence were amplified and are visible as a PCR product of about 150 nt. Lane 1, negative control. Lane 2, control with S .aureus DNA. Lane 3, assay control with DNA from a well characterised MTB strain. Lanes 4,6-13, samples. Lane 5, empty. 100bp ladder.

The MLPA was positive for markers indicating resistance to rifampicin and isoniazid for all nine strains analysed (Table 2). This finding is consistent with the results from the MTBDRplus assay and the DST.
DST for second line drugs and MLPA was performed for all nine strains but only three strains (32-10, 91-10, 96-10) were investigated for second line drugs using MTBDRsl.
The five strains that were streptomycin resistant on the basis of DST (52-20, 72-10, 90-10, 91-10, 101-10) were determined as streptomycin sensitive by MLPA. A single marker is included in the MLPA assay for the detection of streptomycin resistance. It is possible that the mutation conferring resistance to streptomycin in the five strains is different to the one that can be detected with the marker included in the MLPA assay. Resistance to streptomycin cannot be detected by the MTBDRsl since no specific markers are included.
Phenotypic resistance to ethambutol was detected in five strains (32-10, 9-10, 91-10, 96-10, 101-10). Mutations in the conferring resistance to ethambutol were detected in all three strains analysed by MTBDRsl and in five strains analysed by MLPA (32-10, 52-10, 72-10, 91-10, 101-10). The results of two strains (32-10, 96-10) were consistent between MLPA, MTBDRsl and DST. Mutations conferring resistance to ethambutol were also detected by MLPA in two strains that were phenotypically ethambutol susceptible (52-10, 72-10). Performance of DST for ethambutol is difficult and might in some cases lead to false negative results. Further, mutations indicating resistance to ethambutol could not be detected by MLPA in one phenotypically ethambutol resistant strain that was also positive for the marker indicating ethambutol resistance by MTBDRsl. It is likely that the mutation conferring resistance to ethambutol in the clinical isolates differs from the one which can be detected by the marker included in the MLPA assay.
Four strains (32-10, 52-10, 91-10, 96-10) showed resistance to fluoroquinolones on the basis of DST. Resistance to fluoroquinolones was detected by the MTBDRsl assay in all three analysed strains (32-10, 91-10, 96-10) whereas the MLPA detected resistance to fluoroquinolones in three strains (32-10, 52-10, 96-10), two of them consistent with the strains analysed with the MTBDRsl assay. Although the two most prevalent markers for the detection of fluoroquinolone resistance are included in the MLPA assay it is possible that other mutations conferring resistance to fluoroquinolones are present which could not be detected by MLPA but with the MTBDRsl assay.
Resistance to amikacin/kanamycin/capreomycin was identified in one strain, 91-10, by all three methods.

CONCLUSION

We set out to asses the performance of a new molecular method, MLPA, by comparing the results obtained by MLPA in the national TB reference lab in Sofia, Bulgaria to previously performed DST and MTBDRsl/plus. MLPA allows the detection of molecular markers that confer resistance to antimycobacterial drugs rifampicin, isoniazid, ethambutol, streptomycin, amikacin, kanamycin, capreomycin and fluoroquinolones. Results were obtained after a turn around time of 1 ½ days using DNA isolated from liquid cultures.
In the present study, 13 markers provided information about resistance to first- and second line drugs based on the detection of specific mutations in the bacterial genome. The MLPA assay identified mutations conferring resistance to rifampicin and isoniazid for all isolates and thereby confirmed the MDR-TB status of the clinical isolates.
For the second line drugs discrepancies were identified between MLPA and the MTBDRsl assay. The current MLPA assay is lacking some probes that are present in the MTBDRsl assay. Therefore mutations present in the bacterial genomes could only be detected by MTBDRsl. In case of the non-detected mutation in the embB306 locus, it was previously reported that not all possible codon changes can be detected by the MLPA embB probe [Bergval, 2008]. The strains that were sensitive for specific antibiotics but by interpretation of the MLPA results were resistant, it is most likely that DST led to false negatives due to the difficult performance of DST for drugs such as ethambutol [Da Silva, 2011].
Compared to the MTBDRsl/plus that requires two individual tests to obtain the same results, MLPA revealed the drug resistance in a single assay.
In addition to a shorter turn-around time that currently available methods can offer to obtain the same information, MLPA enables simultaneous detection of 13 markers per sample in a single assay which can be extended to 50 markers per sample. Therefore, with a single assay more information, useful for appropriate treatment or control measures, can be obtained than is currently feasible. Another benefit of MLPA over for instance reverse hybridisation assays such as the MTBDRplus and MTBDRsl is that the choice of genetic markers to be included is completely flexible and can be

Table 2. Comparison of identified drug resistance of phenotypically MDR TB revealed by different molecular methods.

	Molecular test			Phenotypic test
Sample	MLPA[a]	MTBDRplus[b]	MTBDRsl[b]	DST[c]
30-10	INH, RIF	INH, RIF	ND	INH, RIF
32-10	INH, RIF, EMB, FLQ	INH, RIF	EMB, FLQ	INH, RIF, EMB, FLQ
52-10	INH, RIF, EMB, FLQ	INH, RIF	ND	INH, RIF, STR, FLQ
72-10	INH, RIF, EMB	ND	ND	INH, RIF, STR
90-10	INH, RIF	ND	ND	INH, RIF, STR, EMB
91-10	INH, RIF, EMB, AMK/KM	INH, RIF	EMB, FLQ, AMK/ KAN, CAP	INH,RIF, STR, EMB, FLQ,AMK,KAN,CAP
96-10	INH, RIF, FLQ	INH, RIF	EMB, FLQ	INH, RIF, EMB, FLQ
97-10	INH, RIF	ND	ND	INH, RIF
101-10	INH, RIF, EMB	ND	ND	INH, RIF, STR, EMB

made dependent on the information required. Inclusion of additional drug resistance markers is of interest for diagnostic purposes whereas addition of genotypic markers would provide information on the species level. Country-specific marker panels could contain markers for the most prevalent genotypes circulating in e.g. Bulgaria, Eastern Europe or Asia presuming that indicative markers are available from the literature or can be identified by genome sequencing of representative strains. Another variation of the MLPA could be the addition of markers that identify the most clinically relevant non-tuberculous mycobacteria (NTM) [Ngan, 2011].
In summary, the MLPA is the only molecular test currently available that reveals complex information from a clinical isolate in only a single assay within 1 ½ days turn around time. The MAGPIX device used for the bead-based read out of the MLPA is a robust and open platform that can be used for DNA assays as well as multiplexed immunological assays. These can be purchased as a kit or may also be partially developed by the researchers themselves.
We feel that the combination of MLPA with Luminex technology is promising and can be of great added value in the fight against (MDR-)TB. Initial results obtained in the reference laboratory in Sofia are promising and lessons learned from this feasibility study are a good starting point to further optimise the method.

ACKNOWLEDGMENTS

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REFERENCES

Bachiyska E, Kantardjiev T, Ivanova A, Atanasova Y, Merdzhanov D. Probl. Inf. Parasit. Dis 2010; 38: 4-6

Bergval IL, Vijzelaar RNCP, Dalla Costa ER, Schuitema ARJ, Oskam L, Kritski AL, Klatser PR, Anthony RM. JCM 2008; 46 (2): 689-699

Da Silva PEA, Palomino, JC. J Antimicrob Chemother 2011; 66: 1417–1430

Hillemann D, Ruesch-Gerdes S, Richter E. JCM 2007; 45 (8): 2635-2640

Kiet VS, Lan NTN, An DD, Dung NH, Hoa DV, van Vinh Chau N, Chinh NT, Farrar J, Caws M. JCM 2010; 48 (8): 2934-2939

Panaiotov S, Kantardjiev T. J Clin Microbiol. 2002;40(10):3881

Schouten JA, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Nucleic Acids Research 2002; 30:e57

MOLECULAR-GENETIC METHODS IN THE DIAGNOSTICS OF DENTAL INFECTIOUS CAUSED BY OBLIGATELY ANAEROBIC BACTERIA

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SUMMARY:
The diagnostics of human dental pathogens is essential for the correct treatment of gingivitis, periodontitis and other diseases of the oral cavity. The most often causative agents are anaerobic bacteria such as Fusobacterium nucleatum, Porphyromonas gingivalis and others. Their cultivation is time and work consuming and expensive as well. In the last 20 years a variety of molecular methods for fast diagnosis and typing appear that allow exact determination on species, strain and biovar level. On the other hand development of the quantitative PCR gives a possibility to estimate the ratio of bacterial cells from the normal flora and pathogenic strains – very important for the correct diagnosis.

Key words: Anaerobes, Molecular-Genetic Methods

The infections of the oral cavity are dangerous not only for the tooth health. Much more serious are their complications for the cardio-vascular system and other organs in the body. Many species of anaerobes are colonizing the human oral cavity. They are part of the normal flora but in some conditions they can cause inflammation processes such as gingivitis, periodontitis, etc. Others are participating in the development of cardiac diseases such as myocardial infarctions (1). Most probably periodontitis and dental procedures are participating in transient bacteraemia (2,3). Mastication can release endotoxins into blood stream in patients with periodontitis (4). Infected root canals can be a source of blood contamination during the therapy (5).

Some authors claim that infection may also play a role in the genesis and development of atherosclerotic processes and their complications, based on detection of serological markers related to some agents such as Chlamydia pneumoniae, Helicobacter pylori and Cytomegalovirus. (12,13). Others, however, suggest that the association between coronary artery disease and periodontal disease (PD) is a more incidental phenomenon, since it may be related only with other cardiovascular risk factors (14,15).

Taylor BA et al. conducted a study in patients with advanced periodontitis requiring full-mouth tooth extraction. Elimination of advanced periodontitis by full-mouth tooth extraction reduces inflammatory and thrombotic markers of cardiovascular risk, supporting the hypothesis that treatment of PD may lower the cardiovascular risk (16).

Periodontitis is the most common persistent bacterial infection in the oral cavity of adults. It causes chronic inflammation in tooth supporting tissues, but it also leads to systemic inflammatory response (17,18) specific immune reactions to periodontitis associated bacterial species (19,20) and unfavorable changes in plasma lipoprotein profile (21) and cholesterol metabolism (22).

One of the most serious problems is the diagnostics of anaerobes - causative agents of anaerobe infections.

Cultivation of the anaerobes is time and work consuming and its discrimination is not very exact on strain and biovar level. Therefore molecular-biological techniques were developed and applied in last two decades in the diagnostics of bacteria including anaerobes. It is complicated to estimate the ratio between different species and strains in a clinical sample, which is necessary for correct treatment.

Subgingival microbial samples were taken and evaluated by means of real-time polymerase chain reaction (RT-PCR) for the total amount of bacteria and the following periodontopathogens: Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Parvimonasmicra (formerly Micromonas micros), Dialister pneumosintes, and Campylobacter rectus (23).

Quantitative PCR (qPCR) has recently been used to quantify microorganisms in such complex communities listed above on one hand and dental plaque biofilms on the other (24). And there is variability in the qPCR protocols being used in different cases. The two variables evaluated were the use of DNA content versus actual cell counts to estimate bacterial numbers in mixed plaque samples (24) and the effectiveness of three different universal primers versus species specific primers in amplifying specific target pathogens in these samples (25). The results lead to the development of a standardized protocol that was shown to be highly reproducible as demonstrated by low coefficients of variation. The results also confirmed that this standardized qPCR protocol can be used as a sensitive method for quantifying specific bacterial species in human plaque samples.

The bacterial content of clinical material can be easily estimated using correctly selected primers for determination and discrimination on different level depending on the special needs of the diagnostics.

A specific 16S rDNA PCR and subsequent hybridization reaction is designed to discriminate between strains of Prevotella intermedia and Prevotella nigrescens. This technique was then used to detect the presence of these two bacterial species in acute oral infection (26). A total of 36 pus samples aspirated from 26 peri-apical abscesses, three root canals, three periodontal abscesses, two cases of refractory periodontitis, one cyst and one haematoma were examined (26). A portion of the pus sample can be directly amplified by PCR and the rest of the specimen can be cultured usind classical microbiological methods. The PCR-based technique gave an identical pattern of detection of P. intermedia or P. nigrescens to that obtained by culturing. The presence of P. intermedia and P. nigrescens in pus can be detected rapidly and specifically by direct PCR amplification of 16S rDNA. In the present days sequencing and software analysis of the genes coding 16S rRNA amplified with universal prokaryotic primers is general taxonomic pattern in accordance with Bergey's manual (27). It is applied in dental microbiology of anaerobes.

Severe early childhood caries (ECC), while strongly associated with Streptococcus mutans can be selectively detected (culture, PCR) that is also associated with a widely diverse microbiota using molecular cloning techniques (28). Isolates were purified and partial sequences for the 16S rRNA gene were obtained from 5608 isolates. Sequence-based analysis of the 16S rRNA isolate libraries from blood and acid agars of severe ECC and caries-free children had >90% population coverage, with greater diversity occurring in the blood isolate library. Isolate sequences were compared with taxon sequences in the Human Oral Microbiome Database (HOMD), and 198 HOMD taxa were identified, including 45

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previously uncultivated taxa, 29 extended HOMD taxa, and 45 potential novel groups.

The major species associated with severe ECC included Streptococcus mutans, Scardovia wiggsiae, Veillonella parvula, Streptococcus cristatus, and Actinomyces gerencseriae. S. wiggsiae was significantly associated with severe ECC children in the presence and absence of S. mutans detection. We conclude that anaerobic culture detected as wide a diversity of species in ECC as that observed using cloning approaches. Culture coupled with 16S rRNA identification identified over 74 isolates for human oral taxa without previously cultivated representatives.

The abundance of anaerobic pathogens in combination with the complications of infections caused by them makes molecular-genetic methods very valuable and irreplaceable for timely diagnostics and treatment of patients with dental problems. These methods are also much more sensible and can estimate the pathogens of much lower level.

REFERENCES:

1. Perssona G.R., Ohlssonc O., Petterssond T., Renvertc S. Chronic periodontitis, a significant relationship with acute myocardial infarction. Eur Heart J. 2003; 24(23):2108-15.
2. Herzberg MC, Meyer MW. Effects of oral flora on platelets: possible consequences in cardiovascular disease. J Periodontol 1996; 67:1138–42.
3. Daly CG, Mitchell DH, Highfield JE et al. Bacteremia due to periodontal probing: a clinical and microbiological investigation. J Periodontol 2001; 72:210–4.
4. Quirynen M, Mongardini C, de Soete M et al. The role of chlorhexidine in the one-stage full-mouth disinfection treatment of patients with advanced adult periodontitis. Long-term clinical and microbiological observations. J Clin Periodontol 2000; 27:578–89.
5. Debelian GJ, Olsen I, Tronstad L. Bacteremia in conjunction with endodontic therapy. Dent Traumatol 1995;11:142–9.
6. Muhlestein JB, Anderson JL. Infections serology and atherosclerosis: how bundersome is the risk? Circulation. 2003; 107: 220-2.
7. Smieja M, Gnarpe J, Lonn E, Gnarpe H, Olsson G, Yi O. Multiple infectious and subsequent cardiovascular events in the heart outcomes prevention evaluation (HOPE) study. Circulation. 2003; 107: 251-7.
8. Hujoel PP, Draugsholt M, Spiekerman C. Periodontal disease and coronary heart disease risk. JAMA. 2000; 284: 1406-10.
9. Hung HC, Willett W, Joshipura KJ, Merchant A, Rosner BA, Ascherio A. Oral health and peripheral arterial disease. Circulation. 2003; 107: 1152-7.
10. Taylor BA, Tofler GH, Carey HMR, Morel-Kopp MC, Philcox S, Carter TR. Full-mounth tooth extraction lowers systemic inflammatory and thrombotic markers of cardiovascular risk. J Dent Res. 2006; 85: 74-8.
11. Loos BG, Craandijk J, Hoek FJ, Wertheim-van Dillen PM, van der Velden U. Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. J Periodontol 2000;71:1528-1534.
12. Mattila K, Vesanen M, Valtonen V, et al. Effect of treating periodontitis on C-reactive proteins: A pilot study. BMC Infectious Diseases 2002;2:30-32.
13. Ebersole JL, Taubman MA, Smith DJ, Genco RJ, Frey DE. Human immune responses to oral micro-organisms. I. Association of localized juvenile periodontitis (LJP) with serum antibody responses to Actinobacillus actinomycetemcomitans. Clin Exp Immunol 1982;47:43-52.
14. Pussinen PJ, Vilkuna-Rautiainen T, Alfthan G, Mattila K, Asikainen S. Multiserotype enzyme-linked immunosorbent assay as a diagnostic aid for periodontitis in largescale studies. J Clin Microbiol 2002;40:512-518.
15. Lösche W, Karapetow F, Pohl A, Pohl C, Kocher T. Plasma lipid and blood glucose levels in patients with destructive periodontal disease. J Clin Periodontol 2000; 27: 537-541.
16. Pussinen PJ, Jauhiainen M, Vilkuna-Rautiainen T, et al. Periodontitis decreases the antiatherogenic potency of high density lipoprotein. J Lipid Res 2004;45:139-147.
17. Nonnenmacher C, Stelzel M, Susin C, Sattler AM, Schaefer JR, Maisch B, Mutters R, Flores-de-Jacoby L. Periodontal microbiota in patients with coronary artery disease measured by Real-Time polymerase chain reaction: A case-control study. J Periodontol. 2007 ;78(9):1724-30.
18. Syrjanen J, Peltola J, Valtonen V, Livanainen M, Kaste M, Huttunen JK. Dental infections in association with cerebral infarction in young and middle-aged men. J Intern Med 1989;225:179-184.
19. Mackenzie RS, Millard HD. Interrelated effects of diabetes, arteriosclerosis and calculus on alveolar bone loss. J Am Dent Assoc 1963;66:192-198.
20. Stubbs S, Park SF, Bishop PA, Lewis MA. Direct detection of Prevotella intermedia and P. nigrescens in suppurative oral infection by amplification of 16S rRNA gene. J Med Microbiol. 1999 Nov;48(11):1017-22.
21. Bergey S. Systematic Bacteriology Vol 2 Part A p. 15.
22. Tanner AC, Mathney JM, Kent RL, Chalmers NI, Hughes CV, Loo CY, Pradhan N, Kanasi E, Hwang J, Dahlan MA, Papadopolou E, Dewhirst FE. Cultivable Anaerobic Microbiota of Severe Early Childhood Caries J Clin Microbiol. 2011;49(4):1464-74.

PERIODONTAL ANAEROBIC INFECTIONS AND CARDIOVASCULAR DISEASES

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SUMMARY:
For a long time the anaerobic bacteria and other periodontal pathogens were considered as causative agents of the infections of the teeth and oral cavity. In the past years series of studies of different investigators showed relation between the oral microflora and dental and cardiovascular diseases. Therefore in the present days diagnostics of the oral bacteria (mostly anaerobes) is important both for dentists and cardiologists to make a correct decision for the prophylaxis and treatment of dental and cardiovascular diseases. In this review we discuss some important aspects about the health problems for humans related with the oral anaerobes.
Key words: Periodontal Infections, Cardiovascular Disease, Anaerobes

Cardiovascular diseases are widely spread. The World Health Organization has reported about 16.6 million death cases per year in the world. 7.1 million of them are caused by coronary arterial disease [1]. Periodontal diseases are even more often – in more than half of the children and in almost all the adult population in the world [2] and chronic paradontitis affects 10-15% of the adults [3, 4].

The progress of science and the modern conception about the etiology and pathogenesis of the periodontal diseases directed many investigators to seek systematic influences of the periodontal diseases on cardiovascular and other systems. Investigators in the field of periodontal medicine in the last 20 years are working on the following tasks: influence of the periodontal infection on different processes related with cardiovascular diseases, whether periodontal diseases are risk factor for cardiovascular diseases, which is the role of the inflammation and the immunity, is direct participation of periodontal pathogens possible in the pathogenesis of cardiovascular diseases, are they mutual genetic factors determining this two kind of diseases?

Discovering the correlation between periodontal and cardiovascular diseases will help the clinicians not only to affect a disease but for a full prophylaxis and treatment of the patient.

Pathogenic mechanisms in the appearance and development of periodontal diseases. Risk factors for periodontal diseases. [5]

Periodontitis is considered as an infectious disease in the literature. Significant changes in the understanding of the agents of periodontal diseases took place in the last three decades. About 500 different bacterial species are related with the tooth plaque [6]. The most often identified periodontal pathogens are three microaerophillic species (Aggregatibacter actinomycetemcomitans, Campylobacter rectus and Eikenella corrodens) and seven anaerobes (Porphyromonas gingivalis, T. forsythensis, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, Eubacterium, spirochetes) [6,7].

One of the most significant achievements in the understanding of the etiology of periodontal diseases is the consideration of the tooth plaque as a biofilm (microcolonies and single cells incorporated in high moisturized mainly anionic exopolimeric matrix) [8]. Bacteria achieve different from their planctonic forms properties thanks to the biofilm and their virulence increases [9].

Investigation on different factors of virulence shows that the bacteria can damage tissue or release anti-inflammatory cytokines and other cell mediators of the macroorganism and cause indirect maceration. The bacteria are etiologic factor but not sufficient for periodontal disease – the factors of the reactivity of the organism are important too.

Periodontites are family of diseases with similar general pathogenic mechanisms in the investigated connective tissue and bone destruction [10]. Modern conception is that subgingival biofilm enters apically in gingival sulcus disrupting the connection between the crown of the connective epithelium and the tooth. The connective epithelium is converting in pocket epithelium – periodontal pocket appears and the only barrier between the bacterial biofilm and the connective tissue becomes the thin partial ulcerating pocket epithelium which is transparent for the bacterial products /as lipopolysaccharides – LPS/ and the bacteria reach connective tissue and blood vessels. Some lipopolysaccharides and other factors of virulence of the Gram-negative microorganisms from the subgingival biofilm activate the cells of the connective and sulcular epithelium to proliferate and to secret proinflammatory mediators including IL-8, IL-1a which attract and activate neutrophils. Blood vessels of microcirculation are sore and high conductive. Endothelial cells are activated too and neutrophils and then monocytes and lymphocytes migrate through the vascular wall into extra-vascular space where they form the inflammatory-cell infiltrate. Infiltrated monocytes are activating from lipopolysaccharides and interferon-γ (IFN-γ) for secretion of IL-1β, TFN-a, PGE2 and matrixmetaloproteinases (MMP – big group of enzymes, which destruct the collagen and all the components of the connective tissue extracellular matrix).

The property of some microorganisms to invade the tissues of the periodontium gives them high virulence. Such ability have Porpyromonas gingivalis (Sandros, J., Papadanou, P.N., Nannmark, U. & Dahlen, G. Porphyromonas gingivalis invades human pocket epithelium in vitro. Journal of Periodontal Research, 1994, 29, 62-69)and Actinobacillus actinomycetemcomitans. (Meyer, D. H., Sreenivasan, P.K. & Fives-Taylor, EM. Evidence for invasion of a human oral cell line by Actinobacillus actinomycetemcomitans. Infection and Immunity, 1991, 59, 2719-2726.)

The pathogenesis of periodontitis takes place with participation of cellular and humoral immune response. Infiltrated T- and B-lymphocytes are activated by different bacterial antigens and cytokines and begin to secret interleukins (IL-2, -3, -4, -5, -6, -10) and IFN-γ. T-cell clones are expanding and B-cells differentiate to antibody-producing plasmatic cells. IFN-γ induces and increases the production of prostaglandin E2 (PGE2) and matrixmetaloproteinases (MMP). These molecules mediate destruction of extracellular matrix of the gingiva and periodontal ligament and resorbtion of the alveolar bone.

Expanding periodontal lesion incorporates fibroblasts, endothelial and epithelial cells. That causes increasing of the destruction of the collagen, extra-cellular matrix of the connective tissue and alveolar loss of bone as well.

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General mechanisms in the pathology of periodontitis are affected by both genetic and environmental risk factors that are different on different stages of the disease [10]. The clinical picture is a result of the complex interaction between microbic factor, the common mechanisms of the inflammatory and immune reaction and the risk factors such as genetic factors, diabetes, drugs or diseases causing immune suppression, male sex, age, race/ethnos, bad oral hygiene, stress, tobacco smoking, which may be present or absent in different life stages. These are risk factors for other systematic diseases including cardiovascular [10].

The periodontal lesion is a long-term source of bacterial antigens, Gram-negative bacteria, cytokines and other proinflammatory mediators [11,12].

The periodontal diseases are often accompanied by transient bacteraemia – invasion of many microorganisms and their products [including lipopolysaccharides (LPS)] in blood circulation where they can activate leucocytes, thrombocytes or endothelial cells. Even good oral hygiene - brushing of the teeth every day and their treatment with thread of healthy patients results to bacteraemia. Medical procedures like "scaling and root planning" in patients with plaque-induced periodontal diseases cause bacteraemia inducing humoral immune response [13].

Page's conception about pathogenic mechanisms of periodontal diseases is explaining their relation with some common diseases and conditions: cardiovascular diseases, diabetes, abnormal births of babies with low weight, osteoporosis [14]. There is a hypothesis about the etiological role of periodontitis for the development of these systematic diseases and conditions [14].

Pathologic processes significant for cardiovascular diseases [15].

Atherosclerosis is a local increasing the thickness of the arterial intima and media on the places of disturbance of the blood flow where its separation from the main stream is possible. In those conditions are possible infiltration and retention of plasmatic proteins (especially low density lipoproteins -LDL), plasmatic cholesterol and fibrinogen.

Monocytes' adhesion in human atheromatous lesion is the critical first stage of the atherosclerosis mediated by adhevise molecules (intercellular adhesive molecule – 1 (ICAM – 1), endothelial leukocyte adhesion molecule – 1, (ELAM-1), vascular cell adhesion molecule – 1, (VCAM-1) which are regulated by the endothelium expressed under lipopolysacchadide and proinflammatory cytokines and prostaglandines activity [16].

Oxidation of the lipids of arterial wall and conversation of fibrinogen to fibrin and fibrin destruction products (FDP) are important factor for further atherogenesis: proliferation of smooth-muscle cells of arterial wall and accumulation of lipids in these cells and in blood monocytes, which are converting to the so-called "foam cells". Monocytes in the lesion are transformed to macrophages, causing further destruction with the help of enzymes [17]. The described processes show the fundamental role of the inflammation in the mediation of all stages of arteriosclerosis and suggest that the infection including the periodontal one, may be a risk factor in the pathogenesis of artheriosclerosis such as tobacco smoking, hypertension, hyperlipidemia and oxidative stress.

Periodontal data related to the atheroscerosis suggest that that can be observed simultaneously as periodontal-atherosclerotical syndrome (PAS) [18]. Generalized periodontitis correlate more often with PAS than with localized light periodontitis which is compatible with the greater microbial load, increasing the cardiovascular risk [18].

Arterial thrombosis follows the rupture of the atheromatous plaque and thrombocytes and blood coagulation are activated by coagulation factor VII. The thromb of the thrombocytes and fibrin may bung partially or fully the artery which can cause ischemic symptoms with different clinical expression. Thrombenobolism is blocking of the artery and is a conventional reason for a stoke or ischemia of a limb.

Connection between periodontal infection and pathogenesis of cardiovascular diseases.

The mechanisms of interaction between such complicated and multifactor diseases as periodontal and cardiovascular are still unclear, but there are several main hypotheses: direct incorporation of bacteria from periodontal infection in the processes of atherogenesis and thrombosis [19], participation of inflammatory mediators of the macroorganism which is a result of the periodontal disease in the processes of atherogenesis and thrombosis [16,20], the presence of common independent risk factors for the periorodontal diseases and atherosclerosis, such as age, male sex, tobacco smoking, alcohol drinking, plasmatic fibrinogen, leukocytes number, diabetes [16,20],co-interaction of the factors listed bellow.

Scientific proofs of the systematic activity of the periodontal infection.

The periodontal and cardiovascular diseases and their interaction have some specificity. Plaque-induced periodontal diseases have chronic progression and this makes them long-term source of both bacteria and their products in the blood circulation and long-term acting mediators for protection of the macroorganism.

The importance of the microorganisms that cause infections, including periodontal is a direct infection of the vascular endothelium. Investigations show that in 97 patients with non-lethal miocardic infarction is much more probable to find periodontal P. gingivalis and B. forsythus in periodontal lesions in comparison to 233 control patients without heart diseases [21]. Investigations of human lesions got from endarterectomy with help of 16S rDNA find in high percentage of the cases periodontopatogens like B. forsythus, P. gingivalis, A. actinomycetemcomitans, and P. intermedia together with human cytomegalovirus and C. pneumonia [22, 23]. These different oral bacteria are found in some arteries in patients with Buerger's disease as well [23].

In other investigations the presence in atheromatous plaques of DNA of periodontal bacteria observed in subgingival tooth plaque of the same patients is not confirmed [24]. The participation of periodontopathogens in the formation of the tooth plaque is unproved. Further investigations must be followed to prove or cancel this theory.

Porphyromonas gingivalis invasion is not in gingival epithelial cells only, estimated in vitro, but in cell cultures from endothelium of coronary and carotid arteries established by electron-microscopy investigation and gives reason to think about its direct participation in the process of atherogenesis [25,26,27] while the production of endothelium-converting enzyme, a vasoconstrictor activating the endothelium, plays role in the mechanisms of hypertension [28]. The participation of Porphyromonas gingivalis and its LSP in the pathogenesis of atherosclerosis is confirmed by its ability to bind LDL with macrophages and to form "foam cells" on a culture study [29]. Some periodontal pathogens like P. gingivalis demonstrate an ability to interact with endothelial surface and to induce proliferation of smooth-muscle cells. They can cause damage of the vasomotor functions of the endothelial cells and atherosclerosis [30,31,32].

Lipopolysaccharides (LPS) in the blood stream – products of Gram – negative microorganisms bind lipopolysaccharide-binding protein (LBP) which is a plasmatic transport protein [33]. If bound to CD14 receptors of endothelial, epithelial or smooth-muscle cells they form LSP-LPB-CD14 complexes that can bind to blood vessel on the turbulent places and create atheromatous plaque. If LPS-LPB binds to membrane CD14 receptors of monocytes, macrophages, neutrophyles

cell activation is caused [34]. Activated cells release cytokines (IL-1, IL-6, TNF-a). [35]. The leading role of inflammatory factor IL-1 in atherosclerosis induced by bacteria and high lipid diet is confirmed by experimental investigation. After ablation of IL-1R1 of mice ApoE+/- (apolipoprotein E heterozygote model) and loading of Porphyromonas gingivalis and lipids progression of atheromatous plaque is reduced [35].

Activated cells (monocytes, macrophages, lymphocytes) can influence expression of adhesive molecules of endothelial cells [36]. They can inhibit lipoproteinlipase and to cause lipemia [37]. Mitogenesis and fibrinogen production can be stimulated [52]. Then leukocytes infiltrate to subintima and smooth-muscle cells proliferate in the lesion [16]. The increase of fibrinogen and leukocytes are related with the cardiovascular disease and periodontitis [16].

There is a theory based on epidemiological investigations that haemostatic indicators are included in potential biological mechanisms and with the help of them the risk factors – tobacco smoking, hyperlipidemia and infections including oral infections can cause vessel changes. We have not seen published direct proves about the influence of periodontal infection on these indicators.

Immune stage of organism's reaction can be observed in patients with stroke or history of cardio-vascular disease who are seropositive for IgA against Porphyromonas gingivalis in comparison with control group or health non-seropositive [38]. The combined antibody response to P. gingivalis and A. actinomycetemcomitans is related to coronary disease [39]. High levels of antibodies against periodontopathogens in the serum indicate that significance of periodontal diseases in the progression of cardiovascular diseases.

In patients with periodontal diseases is found out a production of antibodies against bacterial heat-shock proteins of Bacteroides forsythus and Porphyromonas gingivalis [40,41]. Heat shock proteins (HPSs) are produced in response to different external influences. Because HSPs of the heart and the vessels have similar antigen properties like the bacterial HSPs (of Bacteroides forsythus and of Porphyromonas gingivalis) antibodies cross-react with HSPs of the heart and the vessels [41,42]. There is a hypothesis that atherosclerosis is an autoimmune disease caused by immune reaction (cell and humoral) against HSP-60 expressed by endothelial cells damaged by classic risk factors of atherogenesis like hypertensitivity, high level of chlolesterol, effects of tobacco smoking, etc. [43,44]. There are also contradictory data: IgG against human HSP60, IgA and IgG against bacterial Hsp65 are on the same level like in investigated patients with periodontitis and healthy persons and the levels of IgA anti-Hsp60 antibodies are statistically lower in patients with periodontitis. Those data show deficiency in the possibility for a humoral immune response to Hsp60/65 in patients with chronic periodontitis and this probably supports the development of a chronic inflammation.

The data considered above offer an ethiologic explanation of the epidemiologically estimated relation between periodontal and cardiovascular diseases. The investigation of inflammatory and immunological biomarkers connected with clinical results would give new proofs about the relation between the systematic disease and the chronic oral infection.

The influence of the active thrombocytes for the development of atheromatous lesion is also discussed in the literature. Thrombocytes are part of the haemostatic process which is critical for the formation of the atheromatous plaque. These cells can regulate the release of haemokines from the monocytes in the inflammatory lesion [45,46]. The thrombocytes are an object of effect of different microorganisms including those from the bacterial biofilm [19]. It is suggested that circulating microorganisms Streptococcus sanguis inducing the aggregation of thrombocytes from rabbit and man in vitro can cause coronary thrombosis and symptoms of a myocardial infarction (MI). These data correlate with the thrombogenic mechanism explaining the additional risk of periodontitis for a myocardial infarction [47].

Genetic polymorphism as a risk factor in the pathogenesis of periodontal and cardiovascular diseases.

Genetic factors influence the inflammatory and immune response of the organism in general. It is clear today that genetic factors are very important for the predisposition to periodontitis and the determination of the progression and the severity of this disease.

There are no proofs of common genetic factors causing periodontal and cardiovascular diseases. The polymorphism of IL-1 and TNF-a gene families could have a significant importance for both diseases if consider the inflammatory compound in their pathogenesis. Specific IL-1 – gene polymorphism is risk factor related with an increased risk for periodontitis and cardiovascular diseases, independent from other risk factors [48].

Periodontal infection as a risk factor for cardiovascular diseases.

Cardiovascular diseases are often a reason for death – about 29% of the total world mortality [6]. Cardiovascular diseases include hypertension, coronary disease (myocardial infarction and angina pectoris), peripheral arterial disease, stroke and atherosclerosis as a reason for all cardiovascular diseases. Epidemiological investigations discover many risk factors related with the frequency of cardiovascular diseases. Some of them cannot be influenced (sex, age) but others are object of influence – hypertension, dyslipidemia, tobacco smoking, diabetes, physical inactivity or obesity [49]. These factors are important for the determination of risk persons, but they are not responsible for expression of all cardiovascular diseases. Epidemiological investigations give a basis to tell that chronic infections are an independent risk factor for cardiovascular diseases [50,34,51]. Periodontal diseases are a controllable risk factor for cardiovascular diseases [52, 20, 53] and there are not enough proofs for a relation between them [20]. In 9760 persons investigated by De Stefano et al. (1993) [37] is found approximatly three times higher risk for a coronary disease of the heart of persons with periodontal disease compared with the controls. Epidemiological investigations show that severe periodontal diseases are related with 25-90% higher risk for cardiovascular diseases after a decrease of the influence of the other risk factors [52].

Experimental and clinical researches present data about the role of infection and inflammation in the development of atherosclerosis [56,57]. Malita et al. find a statistically significant relation between the periodontal disease's progression and the stage of atherosclerosis during x-ray investigation and diagnostic coronary angiography in males with coronary heart diseases [58].

Beck et al. (1996) [52] investigate 1147 healthy persons, 207 of whom develop heart diseases in the next 18 years. X-ray investigations of alveolar bone loss are used to estimate the stage of periodontitis. The results show significant relation between bone loss and cardiovascular disease, lethal heart incident or stroke. Later Beck et al. (2001) [59] provide first proofs that periodontitis is related with subclinical atherosclerosis. Data analysis of 6017 persons showed that heavy periodontitis are related with increased thickness of carotid artery's wall in the region of intima media.

Investigations suggest that clinical properties and laboratory indicators of chronic infection accompanied by periodontal diseases may be related to cardiovascular diseases. But there are other opinions. Investigation of Mattila et al., 2000 [60] describes dental pathology in patients with different cardiovascular diseases. It includes adults and seniors and

no significant differences in the presence of periodontal diseases between patients with and without cardiovascular diseases was observed. These results are in contrast to former investigations of the same authors who suggest that a probable reason is the high age of the observed persons. The diagnostics and exact typing of anaerobic pathogens is very important for the determination of the connection between dental and cardiovascular diseases, and their diagnostics and treatment.

REFERENCES:

1. Southerland, J., et al. Commonality in chronic inflammatory diseases: periodontitis, diabetes, and coronary artery disease. Periodontology 2000, 40, 2006, 130-143.
2. Dzhemileva, T. Diseases of periodontium. 1999, Acer, Sofia, Bulgaria
3. Papapanou, P. Periodontal diseases: epidemiology. Ann Periodontol, 1996, 1, 1, 1-36.
4. Papapanou, P. Epidemiology of periodontal diseases: an update. J Int Acad Periodontol, 1999, 1, 4, 110-6.
5. Page, R. The pathobiology of periodontal diseases may affect systemic diseases: inversion of paradigm. Ann Periodontol, 1998, 3, 1, 108-120.
6. Amar, S., X. Han. The impact of periodontal infection on systemic diseases. Med Sci Monit 2003, 9, RA291-299.
7. Marina, M., Bolyarova, T., Ivanova, K., Dzhemileva, T. Calcium antagonists-induced gingival overgrowth – examination of subgingival microflora. ANAEROBE 2000, International Congress of the Federation of Anaerobe Societies, July 10-12, 2000, Manchester, England, Abstr., p.147, poster VIIb – P8.
8. Costerton, J. Overview of microbial biofilms. J Ind Microbiol, 1995, 15, 3, 137-40.
9. Tatakis, D., P. Kumar. Etiology and patogenesis of periodontal diseases. Dent Clin N Am, 2005, 49, 3, 491-516.
10. Page, R., Beck, J. Risk assessment for periodontal diseases. Int Dent J, 1997, 47, 61-67.
11. Leivadaros, E., et al. A pilot study into measurements of markers of atherosclerosis in periodontitis. J Periodontol, 2005, 76, 121-128.
12. Prabhu, A., et al. Detection of local and systemic citokines in adult periodontitis. J Periodontol, 1996, 67, 515-522.
13. Chen, H. Humoral immune responses to Porphyromonas gingivalis before and following therapy in rapidly progressive periodontitis patients. J Periodontol, 1991, 62, 12, 781-791
14. Kim, J., S. Amar. Periodontal disease and systemic conditions: a bidirectional relationship. Odontology, 2006, 94, 10-21.
15. Lowe, G. Etiopathogenesis of cardiovascular disease: haemostasis, trombosis, and vascular medicine. Ann Periodontol, 1998, 3, 1, 121-126.
16. Kinane, D. Periodontal diseases contributions to cardiovascular disease: an overview of potential mechanisms. Ann Periodontol, 1998, 3, 1, 142-150.
17. DeStefano, F., R. Anda, H. Kahn, et al. Dental disease and risk of coronary heart disease and mortality. Br Med J, 1993, 13, 306, 688–691.
18. Offenbacher, St., et al. Periodontitis – atherosclerosis syndrome: an expanded model of pathogenesis. J Periodont Res, 1999, 34, 346-352.
19. Helzberg, M., M. Mayer. Dental plaque, platelets, and cardiovascular diseases. Ann Periodontol, 1998, 3, 1, 151-160.
20. Beck, J., et al. Periodontitis: a risk factor for coronary heart disease? Ann Periodontol, 1998, 3, 1, 127-141.
21. Genco, R., et al. Periodontal microflora related to the risk for myocardial infarction: A case control study. J Dent Res, 1999, 78, (Spec. Issue), 457, (Abstr. 2811).
22. Haraszthy, V., et al. Identification of periodontal pathogens in atheromatous plaques. J Periodont, 2000, 71, 10, 1554-60.
23. Zambon, J., et al. Identification of periodontal pathogens in atheromatous plaques. J Dent Res, 1997, 76, (Spec. Issue), 408, Abstr. 3159.
24. Cairo, F., et al. Periodontal pathogens in atheromatous plaques. A controled clinical and laboratory trial. J Periodontal Res, 2004, 39, 6, 442-446.
25. Deshpande, R., M. Khan, C. Genco. Invasion of aortic and heart endothelial cells by Porphyromonas gingivalis. Infect Immun, 1998, 66, 5337-5343.
26. Deshpande, R., M. Khan, C. Genco. Invasion of aortic and heart endothelial cells by Porphyromonas gingivalis. Infect Immun, 1998, 66, 5337-5343.
27. Progulske-Fox, A., et al. Porphyromonas gingivalis virulence factors and invasion of cell of the cardiovascular system. J Periodontol Res., 1999, 34, 12, 393-399.
28. Awano, S., et al. Sequencing, expression and biochemical characterization of the Porphyromonas gingivalis pep O gene encoding a protein homologous to human endothelin-converting enzime. FEBS Lett, 1999, 460, 139-144.
29. Qi, M., H. Miyakawa, H. Kuramitsu. Porphyromonas gingivalis induces murine macrophage foam cell formation. Microb Pathog, 2003, 35, 6, 259-267.
30. Chun, Y., et al. Biological foundation for periodontitis as a potential risk factor for atherosclerosis. J Periodontal Res, 2005, 40, 87-95.
31. Harris, T., L. Ferrucci, R. Tracy, et al. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. Am J Med, 1999, 106, 506–512.
32. Schachinger, V., et al. Prognostic Impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation, 2000, 101, 1899-1906.
33. Tobias, P., J. Gegner, R. Tapping, et al. Lipopolysacharide-dependent cellular activation. J Periodont Res, 1997, 32, 99-103
34. Geerts, S., et al. Further evidence of the association between periodontal conditions and coronary artery disease. J Periodontol, 2004, 75, 1274-1280.
35. Chi, H., et al. Interleukin-1 receptor signaling mediates atherosclerosis associated with bacterial exposure and/or a high-fat diet in a murine apolipoprotein E heterozygote model: Pharmacotherapeutic implications. Circulation, 2004, 110, 1678-1685.
36. Gamble, J., Y. Khew-Goodall, M. Vadas. Transforming growth factor-beta inhibits E-selectin expression on human endothelial cells. J Immunol, 1993, 150, 10, 4494-503.
37. Beutler, B., A. Cerami. Recombinant interleukin 1 suppresses lipoprotein lipase activity in 3T3-L1 cells. J Immunol, 1985, 135, 6, 3969-3971.
38. Pussinen, P., et al. Antibodies to periodontal pathogens and stroke risk. Stroke, 2004, 35, 2020-2023.
39. Pussinen, P., et al. Antibodies to periodontal pathogens are associated with coronary heart disease. Arterioscler Tromb Vasc Biol, 2003, 23, 1250-1254.
40. Sojar, H., I. Glurich, R. Genco. Heat shock protein 60-like molecule from Bacteroides forsythus and Porphyromonas gingivalis: Molecular mimicry. J Dent Res, 1998, 77, (Spec. Issue), 666, Abstr. 275.
41. Maeda, H., et al. Heat shock protein 60 (Gro EL) from Porphyromonas gingivalis: Molecular cloning and sequence analysis of its gene and purification of the recombinant protein. FEMS Microbiol Lett, 1994, 119, 129-136
42. Tabeta, K., K. Yamazaki, H. Hotokezaka et al. Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. Clin Exp Immunol, 2000, 120, 285–293.
43. Wick, G. Atherosclerosis – an autoimmune disease due to an immune reaction against heat-shock protein 60. Herz, 2000, 25, 2, 87-90.
44. Wick, G., et al. Atherosclerosis as an autoimmune disease: an update. Trends Immunol, 2001, 22, 12, 665-669.
45. Weyrich, A., et al. Activated platelets signal chemokine synthesis by human monocytes. J Clin Invest, 1996, 97, 6, 1525-34.
46. Weyrich, A., S. Lindemann, G. Zimmerman. The evolving role of platelets in inflammation. J Thromb Haemost, 2003, 1, 9,1897-905.
47. Helzberg, M., M. Mayer. Effects of oral flora on platelets: possible consequences in cardiovascular disease. J Periodontol, 1996, 67, 10 Suppl., 1138-1142.
48. Kornman, K., et al. Interleukin-1 genotypes and the association between periodontitis and cardiovascular disease. J Periodont Res, 1999, 34, 6, 353-357.
49. Solberg, L., J. Strong. Risk factors and atherosclerotic lesions. A review of autopsy studies. Arteriosclerosis, 1983, 3, 3, 187-98.
50. Kim, J., S. Amar. Periodontal disease and systemic conditions: a bidirectional relationship. Odontology, 2006, 94, 10-21.
51. Beck, J., St. Offenbacher. Systemic effects of periodontitis: epidemiology of periodontal disease and cardiovascular disease. J Periodontol, 2005, 76, 11, Suppl, 2089-100
52. Beck, J., R. Garcia, G. Heiss, et al. Periodontal disease and cardiovascular disease. J Periodontol, 1996, 67, (10 Suppl), 1123-1137.
53. Mattila, K., M. Nieminen, V. Valtonen et al. Association between dental health and acute myocardial infarction. Br Med J, 1989, 25, 298, 779–78
54. DeStefano, F., R. Anda, H. Kahn, et al. Dental disease and risk of coronary heart disease and mortality. Br Med J, 1993, 13, 306, 688–691.
55. Beck, J., R. Garcia, G. Heiss, et al. Periodontal disease and cardiovascular disease. J Periodontol, 1996, 67, (10 Suppl), 1123-1137.
56. Libby, P. Inflammation in atherosclerosis. Nature, 2002, 19-26, 420, 6917, 868-74.
57. Libby, P., P. Ridker, A. Maseri. Inflammation and atherosclerosis. Circulation, 2002, 5, 105, 9, 1135-43.
58. Mattila, K., et al. Dental infections and coronary atherosclerosis. Atherosclerosis, 1993, 103, 205-210.
59. Beck, J. et St. Offenbacher. The association between periodontal diseases and cardiovascular diseases: A state-of-the science review. Ann Periodontol, 2001, 6, 1, 9-15.
60. Mattila, K., et al. Age, dental infections, and coronary heart disease. J Dent Res, 2000, 79, 2, 756-760.

TESTING OF THE CONTROL STRAIN ESCHERICHIA COLI O 104 USING HYBRID MATERIALS BASED ON SILVER NANOPARTICLES

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SUMMARY:
The Minimal Bactericidal Concentration and the zone of inhibition of two types of hybrid materials containing silver nanoparticles have been determined. These results could increase the possibilities of their usage for diagnostic, prevention and treatment of Enterohemorragic Escherichia coli infections.

Key words: E.coli O104, hybrid materials, silver nanoparticles

INTRODUCTION:

The first small outbreak of acute gastroenteritis caused by E.coli O104:H21 in Helena, Montana 1994 [1] and the outbreak of Shiga toxin-producing E.coli O104 (STEC O104:H4) infections in Germany and France in 2011 [2] stimulated huge investigations on the methods for identification of rarely isolated serotypes E.coli [3-5]. Sero-typing is confirmed in the practice, easy done and quick method for orientation about the agent that causes the infection. The EU supports the quick identification of new incidence of HUS and STEC infections that could be a part of the existed outbreak in Germany or of the cluster in France since they could also be potential alarm of new cluster of the infection. This kind of information should be immediately shared with the organizations, which are accountable for the public health to be in the possibility to take epidemic and microbiological discoveries and to alarm the persons responsible for the safety in the Food Agencies. In addition it should be increased the capacity of the national reference laboratories in view on the fact that the confirming of the serotype is from critical importance. According to EU case definition for diarrhea and haemolytic uremic syndrome (HUS) caused by epidemic strain shiga toxin 2 producing E.coli (STEC) O104:H4 the isolation of a STEC strain of confirmed epidemic case is the isolation of a STEC strain of serogroup O 104 and fulfilling epidemiological criteria for a probable case. Therefore, the routine producing of the agglutinating antiserum E.coli O104 could be a great help in such situation. It is well known the wide application of silver ions in different areas such as food industry, medicine and other [6]. However, the silver nanoparticles and especially these, which are stabilized [6-13] showed better properties in comparison to the silver ions [7-9] and it is possible to apply them for the diagnostics of outbreaks by an Enterohemorrhagic Esherichia coli. The main aim of this study is to test the biochemical and antimicrobial properties of the control strain E.coli O 104 Kopenhagen and to determine the Minimal Bactericidal Concentration (MBC) of silver nanoparticles (AgNps) stabilized via polyvinyl alcohol (PVA/AgNps).

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MATERIALS AND METHODS:

A control strain E.coli O 104 Kopenhagen (lyophilized on 20.09.1974) as a part from the lab bacterial-strain-collection ("Bul Bio -NCIPD"), Polyvinyl alcohol (PVA) (Sigma–Aldrich; 87–88% hydrolyzed, Mw = 13,000–23,000 mol-1); HNO3 (Riedel de Haln, standard solution 2 mol/L); silver nitrate (Acros Organics); tetraethyl orthosilicate (TEOS) (Fluka) were used as received without further purification. Nutrition media in disposable petri dishes and Staff set ("Bul Bio-NCIPD") are used for the biochemical identification. Antimicrobial disks, produced by "Bul Bio-NCIPD" and "BBL", disks impregnated with PVA/AgNps/TEOS hybrid material with initial concentration of silver nitrate 3.7 mg/ml were used [14]. TEM images clearly demonstrate the formation of spherical silver nanoparticles, homogenously distributed in PVA/TEOS matrix obtained by annealing the films 100° C for 60 min. The average particle size increased slightly from 5.0 ± 0.7 nm to 6 ± 1.8 nm when the concentration of the AgNO3 in the initial mixture is increased [14]. Hybrid material PVA/AgNps, synthesized by thermal reduction of AgNO3 at 100 °C in the presence of polyvinylalcohol (PVA) as a stabilizing agent with established by ICP-OES concentration of silver: 156.902 mg/L. Conventional microbiological methods for testing the biochemical properties, which are performed with the aid of software program PIB were used [15]. The testing of the antimicrobial sensitivity was performed according to the Disk Diffusion Method [14, 16-17]. The MBC of the control E.coli O104 strain with the hybrid material was performed using twofold macro-dilutions [18].

RESULTS AND DISCUSSION:

1. Culture and biochemical characteristic of the strain E.coli O 104 Kopenhagen.

In the present study, the biochemical and antimicrobial properties of the control strain E.coli O 104 Kopenhagen lyophilized in 20.09.1974 was studied. Initially to refresh the culture Soya Casein Digest Broth was used and after this inoculation the strain was cultivated using three different types of solid nutrient media as Mac Conkey Agar (Figure 1a), Sheep Blood Agar (Figure 1b) and Common agar. Using Mac Conkey Agar on the surface were observed typically lactose-positive species and intermediate-large colonies. Using Sheep Blood Agar a growth without haemolysis on the surface was observed. The culture on the Common Agar was used for the biochemical identification.

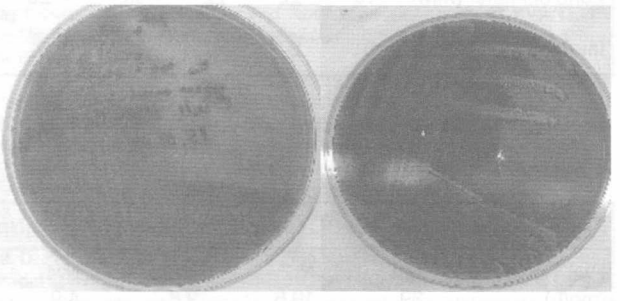

Figure 1: Culture on: a) Mac Conkey agar, b) Sheep Blood Agar

To confirm the species, staff set for identification of isolates belonging to the family Enterobacteriaceae („Bul Bio – NCIPD") was used. After 24-hours incubation at 37°C the biochemical reactions were determined and then the results were processed with the software program PIB "Probalistic Identification of Bacteria" [15] (Table 1).
According to the software program PIB the identification threshold should have score of ID ≥ 0.95 which mean that the identification of the bacterial strain is performed correctly. In our case the score was 0.99742 and this result confirms that the isolate is E.coli.

2. Testing the antimicrobial sensitivity of the control strain E.coli O104 with conventionally used for prevention and treatment antimicrobials and with two hybrid materials containing silver nanoparticles.
The control strain E.coli O104 was tested for sensitivity using Disk Diffusion Method (DDM) of CLSI with the antimicrobial disks: ampicillin (A), ampicillin-sulbactam (A/S), amoxicillin-clavulanic acid (AmC), aztreonam (Azt), imipenem (I), gentamicin (G), ciprofloxacin (Cp), cefuroxime (Cx), cefamandole (Cm), cefotaxime (Ct), ceftazidime (Cz), ceftriaxone (Cft), cefepime (Ce) and disks impregnated with PVA/AgNps/TEOS hybrid material with initial concentration of silver nitrate 3.7 mg/ml. The zones of the antimicrobial disks were accounted according to the norms indicated in CLSI M100-S17 (Table 2, Figure 2). It was established that the control strain E.coli O104 is susceptible to all tested antimicrobials. The detected zone of inhibition using impregnated with PVA/AgNps/TEOS disk was in the limits of susceptibility, which was established by earlier testing with clinical, resistant to antimicrobials strains, isolated from human [19]. (Insert Table2, Figure2)
To determine the Minimal Bactericidal Concentration (MBC) of PVA/AgNps with silver concentration of 157 mg/L against the strain E.coli O104 (Table 3, Figure 3), a macro-dilutions method was used [18]. The bacterial suspension was standardized to 0.5 MF with densitometer, which is equivalent to 1-2x108 CFU E.coli [16].

Table 1. Biochemical identification of the control strain as Escherichia coli

tests	results	tests	results
Motility 37°C	+	Indole	+
Yellow pigment	-	Inositol	-
Red pigment	-	Lactose	+
Mac Conkey	+	Maltose	+
Catalase	+	Mannitol	+
Arginine dihydrolase	-	Raffinose	-
Lysine decarboxylase	+	Rhamnose	+
Ornithine decarboxylase	-	Salicin	+
Urease	-	Sorbitol	+
H2S	-	Sucrose	-
Simmons citrate	-	Trehalose	+
VP 25°C	-	Xylose	+

Table 2. Inhibition zone in mm at testing the sensitivity of the control strain E. coli O104 toward disks impregnated with antimicrobial materials and disks impregnated with PVA/AgNps/TEOS using DDM.

E.coli O104	A mm	A/S mm	AmC mm	Azt mm	I mm	G mm	Cp mm	Cx mm	Cm mm	Ct mm	Cz mm	Cft mm	Ce mm	PVA/ AgNps /TEOS mm
Zone diameter (mm)	19	22	22	30	25	22	31	24	30	36	31	30	34	11
Interpretation (CLSI)	S	S	S	S	S	S	S	S	S	S	S	S	S	

Table 3. Determination of MBC of the hybrid material PVA/AgNps with E.coli O104

PVA/AgNps (mg/L)	№1 39	№2 19.6	№3 9.8	№4 4.9	№5 2.5	№6 1.2	№7 0.6	№8 0.3	№9 0.15	№10 0.08
E.coli O104 (Cfu)	0	0	0	0	0	0	0	1	≥100	Confluent growth

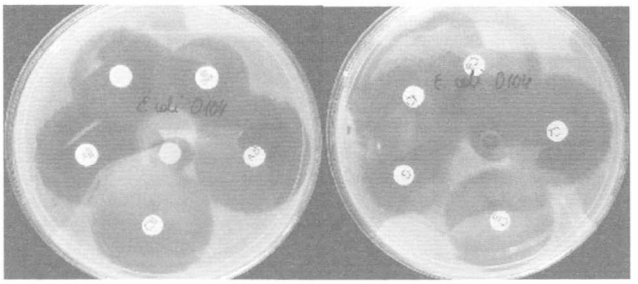

Figure 2: DDM for testing the sensitivity of the control strain E. coli O104 with different antimicrobial disks; in the middle of the petry dishes are placed disks impregnated with PVA/AgNps/TEOS hybrid material.

The MBC of the hybrid material PVA/AgNps against the strain E.coli O104 was 0.3±0,1 mg/L. This value of MBC corresponds to our previous results [20], where the used strain was E.coli ATCC 25922 and hybrid material was in the form of silver loaded micelles on the basis of poly(vinyl alcohol)-b-polyacrilonitrile (PVOH-b-PAN). In those case the determined MBC was 0.36 μg/ml.

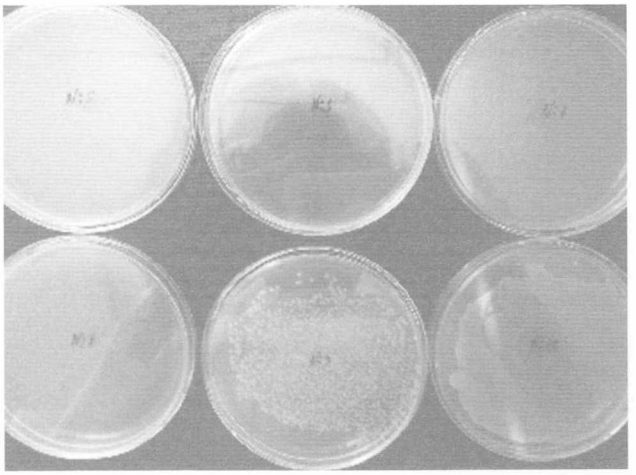

Figure 3: MBC of PVA/AgNps tested with E.coli O104

List of the used abbreviations:

A- ampicillin
A/S - ampicillin-sulbactam
AmC - amoxicillin-clavulanic acid
Azt - aztreonam
AgNps - silver nanoparticles
AgNO3 - silver nitrate
CLSI - Clinical and Laboratory Standards Institute
Ce - cefepime
Cft - ceftriaxone
Cm - cefamandole
Cp - ciprofloxacin
Ct - cefotaxime
Cx - cefuroxime
DDM - Disk Diffusion Method
EU - European Union
E.coli – Escherichia coli
G - gentamicin
HNO3 - Nitric Acid
HUS- Hemolytic-uremic syndrome
I - imipenem
ID - identification value
ICP-OES - Inductively Coupled Plasma Spectroscopy
MBC - Minimal Bactericidal Concentration
PVA - polyvinyl alcohol
PVOH-b-PAN - poly(vinyl alcohol)-b-polyacrilonitrile
PIB - Probabilistic Identification of Bacteria
STEC - Shiga toxin-producing E.coli
TEOS - tetraethyl orthosilicate
TEM - Transmission electron microscopy

The obtained value of MBC was much lower than the cited in the literature value as cytotoxic when using silver nanoparticles. The silver nanoparticles showed a cytotoxic effect only at concentration higher than 30 mg/L [10].

CONCLUSION:

The performed microbiological tests confirmed the species of the tested control strain E.coli O104 with ID 0.99742. It was sensible to all tested antimicrobials as well as to the disk impregnated with PVA/AgNps/TEOS. The MBC of 0.3±0,1 mg/L was determined as this concentration is under the limit for cytotoxic concentration of silver nanoparticles. These results are optimistic for an application of such kind of hybrid materials for producing of in vitro diagnostic medical devices, prevention and treatment in case of Enterohemorrhagic E.coli infections.

1. Outbreak of Acute Gastroenteritis Attributable to Escherichia coli Serotype O104:H21 --
Helena, Montana, 1994, MMWR, July 14, 1995 / 44(27);501-503 available from http://www.cdc.gov/mmwr/preview/mmwrhtml/00038146.htm
2. Investigation Update: Outbreak of Shiga toxin-producing E. coli O104 (STEC O104:H4)
Infections Associated with Travel to Germany Updated July 8, 2011 (FINAL Update), available from http://www.cdc.gov/ecoli/2011/ecolio104/
3. M. A. Chattaway, T. Dallman, I.N. Okeke, J. Wain (2011) Enteroaggregative E. coli O104 from an outbreak of HUS in Germany 2011, could it happen again?, J Infect Dev Ctries 5(6):425-436.
4. K. A. Bettelheim (2003), Non-O157 Verotoxin-Producing Escherichia coli: A Problem, Paradox, and Paradigm, by the Society for Experimental Biology and Medicine.
5. Scheutz F, Møller Nielsen E, Frimodt-Møller J, Boisen N, Morabito S, Tozzoli R, Nataro JP, Caprioli A. (2011), Characteristics of the enteroaggregative Shiga toxin/verotoxinproducing Escherichia coli O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011., Euro Surveill. 2011; 16(24).
6. A.Petica, S. Gavriliu, M. Lungu, N. Buruntea, C. Panzaru (2008), Colloidal silver solutions with antimicrobial properties, Materials Science and Engineering B 152 , 22–27.
7. K. M. Hurst (2006) "Characteristics and applications of antibacterial nano-silver", available from http://nanotechnology.my-place.us/nanosilver.pdf
8. SharmaV.K. , Yngard RA, Lin Y. (2009) Silver nanoparticles: Green synthesis and their antimicrobial activities. Advances in Colloid and Interface Science; 145:83-96.
9. M. Rai, A. Yadav, A. Gade, Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances 2009; 27:76-83.
10. Panacek A., Kolar M., Vecerova R., Prucek R., Soukupova J., Krystof V., Hamal P., (...), Kvitek L. Antifungal activity of silver nanoparticles against Candida spp. (2009) Biomaterials, 30 (31), pp. 6333-6340
11. H. Kong and J. Jang (2006) One-step fabrication of silver nanoparticle embedded polymer nanofibers by radical-mediated dispersion polymerization, first published as an Advance Article on the web 13th June 2006 DOI: 10.1039/b605286j.
12. J.-Ch. Olivier (2005) Drug Transport to Brain with Targeted Nanoparticles, The Journal of the American Society for Experimental NeuroTherapeutics, Vol. 2, 108–119, January 2005 © The American Society for Experimental NeuroTherapeutics, Inc..
13. K.-S. Chou, Ch.-Ch. Chen (2007), Fabrication and characterization of silver core and porous silica shell nanocomposite particles, Microporous and Mesoporous Materials 98.
14. R. Bryaskova, D. Pencheva, G.M. Kale, U. Lad, T. Kantardjiev (2010), Synthesis, Characterisation and Antibacterial Activity of PVA/TEOS/Ag-Np Hybrid Thin Films, Journal of Colloid and Interface Science, doi: 10.1016/j.jcis.2010.04.091.
15. T.N.Bryant (1993), Binary, "A compilation of probalistic bacterial identification matrices", Volume 5 p207-210.
16. CLSI M2-A9, Vol.26 No. 1 „Performance Standards for Antimicrobial Disk Susceptibility Tests", Approved Standard-Ninth Edition.
17. CLSI M100-S17 Performance Standards for Antimicrobial Susceptibility Testing, Seventeenth Informational Supplement.
18. CLSI M26-A, Vol. 19 No. 18 "Methods for Determining Bactericidal Activity of Antimicrobial Agents", Approved Guideline, September 1999.
19. D. Pencheva, R. Bryaskova, T. Kantardjiev (2010), Testing the bactericidal activity of PVA/TEOS/Ag-Np hybrid thin films onto clinical strains with proven resistance toward one or more antimicrobial agents, Journal Problems of Infectious and Parasitic Diseases, Vol. 38, N1.
20. R. Bryaskova, D. Pencheva, M. Kyulavska, D. Bozukova, A. Debuigne, C. Detrembleur, Antibacterial activity of poly(vinyl alcohol)-b-poly(acrylonitrile) based micelles loaded with silver nanoparticles, Journal of Colloid and Interface Science (2009), doi:10.1016/j.jcis.2009.12.040.

Figure legends