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RAPID TESTS IN THE DIAGNOSTICS OF SOME FASTIDIOUS MICROORGANISMS

K. Ivanova, M. Marina

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ABSTRACT

Rapid tests for hydrolysis of DNA, nitrate reduction, phosphatase and urease were developed. We applied the tests for more fastidious and biochemically inactive bacteria like *Campylobacter* /242 strains/, *Helicobacter* /55 strains and 42 biopsies/, and for *Bilophila wadsworthia* /10 strains/ - an anaerobic microorganism recently isolated. Reading the results was for 30 minutes. We compared the rapid tests with conventional ones and they proved to be as exact as them. Rapid tests also exceed conventional by the rapidity of reading the results and easiness to perform.

Key words: Campylobacter, Helicobacter, rapid test

INTRODUCTION

The interest in the microaerophilic spiral-to-curve-shaped bacteria belonging to the species *Campylobacter* and *Helicobacter* has been greatly increased in the recent years. *Campylobacter jejuni* infection is widespread and this microorganism can be isolated in 4 to 30% from the fecal specimens of the patients with acute diarrheal diseases /1/.

Another important pathogen - *Helicobacter pylori* is the causative agent of gastritis in humans. Chronic infection with this organism contributes to formation of gastric and duodenal ulcers and possibly - of gastric carcinoma /8/.

Bilophila wadsworthia /BW/ - an anaerobic microorganism, recently isolated /in 1989/ which participates in severe anaerobic infections /3, 9/.

These microorganisms are biochemically inactive and very fastidious in their growth. That is why there are some difficulties in using the conventional biochemical methods.

The purpose of this study was to develop rapid tests that can be used in the identification and differentiation of *C.jejuni, H.pylori* and *B.wadsworthia*. These tests include: DNA hydrolysis, nitrate reduction, alkaline phosphatase hydrolysis and urease.

MATERIALS AND METHODS

	Bacterial Strains:
Campylobacter sp.	242 strains, 23 of them were referent and were obtained from H.Lior - Canada
C.jejuni	198 strains
C.coli	26 strains
C.lari	*2* strains
Helicobacter pylori	55 strains
duodenal biopsies	42
Bilophila wadsworthia	10 strains

The strains were incubated 24 h before testing - for *Campylobacter* and 48 h - for *H.pylori* and *B.wadsworthia*.

Rapid tests:

The chemical components of the rapid tests are presented on table 1.

Table 1. Ingredients for the rapid tests

Rapid tests / Ingredients	DNA-se	Nitrate reduction	Urease	Alkaline phosphatase
K_2HPO_4		5g		
KH_2PO_4		2g	2g	
Yeast extract		1g	0,5 g	
KNO_3		2g		
Urea			20 g	
Phenol rot			0,012 g	
DNA	2g			
NPPST*				0,4 g
tris buffer pH	100ml			
Distilled water		100ml	100ml	100ml

* NPPST p-nitrophenyl phosphate di-sodiumtetrahydrate

Referent strains *Campylobacter* as well as strains from *Enterobacteriaceae, A.calcoaceticum, A.anitratus* and *S.aureus* have been used as positive and negative controls.

The method of comparision of two alternative distributions with the exact criterion of Fisher was used to determine the sensitivity of the rapid tests when comparing with conventional ones.

RESULTS

The comparison of the rapid tests with the conventional ones is presented on table 2.

All the strains *H.pylori* were positive for DNA-hydrolysis and the strains *Campylobacter* - positive or negative according their biotype.

The results of the rapid tests were read after 30 min., while those of the conventional - after 24 or 48 h. A clear zone was not obtained around the bacterial growth of most of the strains *H.pylori* and some strains *Campylobacter*. That is why those strains needed incubation of 48 h or repeated performances of the tests.

Statistically significant differences were not observed between the methods using the exact criterion of Fisher /$p>0,5$/.

A full coincidence of the results /$p>0,5$/ and 100% sensitivity of the rapid test was observed when comparing between rapid test for nitrate reduction with the conventional ones.

Urease tests were applied directly for 42 duodenal biopsies, 30 of which were urease positive with rapid test for 30 min. From the same biopsies were isolated 30 strains *H.pylori* /100% sensitivity/.

With the conventional test only 26 biopsies were urease positive immediately with the rapid test while with the conventional ones - within 4 h.

Reading the results for the rapid urease test for *B.wadsworthia* was after 30 min., and for conventional - in 48 h.

The test alkaline phosphatase hydrolysis was applied for all strains *H.pylori* /n=55/ and all of them turned positive very quickly in 5 to 30 min., in contrast of the strains *Campylobacter* that remained negative for the same time.

Cultivation of the bacteria for rapid tests was in aerobic conditions while for the conventional - in microaerophilic for *H.pylori* and *Campylobacter* or anaerobic - for *B.wadsworthia*.

CONCLUSION

The rapid tests exceed the conventional by the rapidity of reading the results - 30 min., not within 24 or 48 h. A full coincidence of the results with the conventional tests is observed - 100% sensitivity. Conventional method for urease reaction has shown lower sensitivity /88%/ applied directly for duodenal biopsies.

The rapid tests are easier to perform - it is not necessary to work in sterile conditions because of the heavy not reproducing bacterial suspension. Incubation - in air, not in microaerophilic or anaerobic conditions.

ABBREVIATIONS USED IN THIS PAPER: NONE

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Table 2. Comparison of rapid with conventional tests

Tests	DNA-se positive		Nitrate reduction negative		Nitrate reduction positive		Urease negative		Urease positive		Alkaline phosphatase negative		Sensitivity positive	Sensitivity negative	Sensitivity %
	R	C	R	C	R	C	R	C	R	C	R	C			
Strains /N/															
C.jejuni n=198	84	84	67	67	198	198	0	0	0	0	10	10	0	15	100%
C.coli	24	24	18	18	42	42	0	0	0	0	5	5	-	-	100%
C.lari n=2	1	1	1	1	2	2	0	0	-	-	-	-	-	-	100%
H. pylori n=55	55	55	0	0	0	0	15	15	55	55	0	0	55	0	100%
biopsies n=42	-	-	-	-	-	-	-	-	30	26	12	16	-	-	88%
B. wadsworthia n=10	-	-	-	-	10	10	0	0	10	10	0	0	-	-	100%

R - rapid test
C - conventional test

It is known that the strains *H.pylori* and *B.wadsworthia* do not grow or grow very poorly in liquid media. That is why our rapid tests are better for testing their biochemical activity. Except for species identification rapid tests can be used also for subspecies differentiation such as urease, alkaline phosphatase and nitrate reduction to differentiate *C.jejuni* from *H.pylori.*

Using the fact that one of the basic ways to accelerate the enzyme reaction is the increase of the temperature /in our cases at 56°C/ we achieve acceleration of the process of DNA hydrolysis and prove the enzymes urease and alkaline phosphatase in microorganisms as *Campylobacter, H.pylori* and *B.wadsworthia.*

Rapid tests	Solution	Inoculum	Incubation	Reading the result	Conventional tests
Hydrolysis of DNA	The compound of DNA /Difco/ is dissolved in tris buffer /pH 8,6/ for 30 min at 100°C and poured into test tubes in 0,5 ml	A very heavy inoculum prepared from the strains C.jejunii and H.pylori with an even distribution in all the medium	30 min at 56°C in water bath	Positive-the medium becomes liquified Negative - the medium remains solid	The rapid test was compared with conventional of Lior 151 and Hanninen /4/
Nitrate reductin	0,05 ml from the solution /see table 1/ was diluted with 0,45 ml tap water	A very heavy inoculum pared from the strains C.jejuni, H.pylori and BW	30 min at 37°C	Positive - intensive red Negative - no change after addition of 1 drop 5% anaphtil amine and 5N acetic acid	The rapid test /7/ was compared of color was compared with Ullmann /10/ and Sumanenn /9/
Urea test	0,05 ml from the solution /see table 1/ was diluted with 0,45 ml tap water	A very heavy inoculum prepared from the strains C.jejuni, H.pylori and BW Grinded mass from duodenal biopsy specimens	30 min at 56°C in water bath	Positive - change of color From yellow to red Negative - no change of color	The rapid test 171 was compared with Marshal /6/ and Sumanenn /9/
Alkaline phospatase /2/	0,03 ml from the solution /see table 1/ is poured into the tubes	A very heavy inoculum prepared from the strains C.jejuni and H.pylori	30 min at 56°C in water bath	Positive - change of color in yellow at 5 to 30 min. Negative - no change of color	

REFERENCES

1. Amin N. *Campylobacter* caused diarrhea. Why is at risk? Postgrad Med. 1984, 5,89-97.

2. Cowan, S. Fermentation: Biochemical micromethods for bacteriology. J.Clin.Microbiol. 1953, 8, 391 - 396.

3. Finegold S.M., P.Summanen, S.Hunt Gerardo and E.J. Baron. Clinical importance of *Bilophila wadsworthia.* EurJ.Clin.Microbiol.Infect.Dis., 1992, 11, 1058-1063.

4. Hanninen, M.L. Rapid method for detection of DNA-se of *Campylobacter,*.J.Clin.Microbiol. 1989, 9, 2118-2119.

5. Lior, H., A.Patel. Improve toluidine - blue DNA agar. J.Clin.Microbiol. 1987, 25, 2030.

6. Marshall, B., J.R.Warren, G.J.Francis et al. Rapid urease test in the management of *C.pylori* associated gastritis. Am.J.Gastroenterol. 1987, 82, 200-210.

7. Sakazaki, R., S.Natioca, and F.Ishii. Rapid biochemical and amino-acid decarboxylation tests with preserving solutions for the recognition of enteric bacteria. The Bulletin of the Nippon Veterinary and Zootechnical College, 1959, 18, 18-27.

8. Shibata T., I.Imoto, E.Gabazza. Detection of *H.pylori* in biopsy of patients with gastric carcinoma. Biomedicine&Pharmacotherapy, 1997, 1, 22 -28.

9. Summannen, P. et al. Wadsworth Anaerobic Bacteriology Manual. Fifth Edition, Star Publ. Company, 1993.

10. Ullmann, U. Methods in *Campylobacter?,*. In Methods in Microbiology, 1979, vol.13,Chap.X, 435-451

DETECTION OF EAF PLASMID IN ENTEROPATHOGENIC ESCHERICHIA COLI (EPEC) ISOLATES BY PCR

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SUMMARY

The aim of this study was to demonstrate the EAF plasmid in *Escherichia coli* diarrheagenic isolates, which is responsible for the localized adherence (LA) and the full virulence of EPEC. A total of 53 clinical isolates of EPEC were investigated by PCR using specific primers and amplicons visualized by capillary electrophoresis. Five out of 53 (9.4%) of EPEC strains were pEAF positive. Conclusion: PCR technique is reliable method for detection of pEAF in EPEC isolates. More isolates from different EPEC serotypes should be tested to find out the true prevalence of the plasmid among them. The first investigation for pEAF among EPEC in Bulgaria is presented.

Key words: pEAF, EPEC, PCR

INTRODUCTION

Diarrheal illness caused by *E. coli* is a major public health problem worldwide, particularly among infants younger than 5 years and when foodborne outbreak is appeared. Diarrheagenic *E. coli* have been broadly divided into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), entroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

Detection of diarrheagenic *E. coli* has long relied on the determination of serogroups characteristic for some pathotypes of these bacteria. For instance, serogroups O44, O55, O111, O119 are typical for EPEC, serogroups O6, O25, O78, O148 for ETEC, serogroups O28, O112, O124, O144 for EIEC, serogroups O26, O113, O117, O157 for EHEC (1). *E. coli* of specific serogroups can be associated reproducibly with certain clinical syndromes, but it is not in general the serologic antigens themselves that confer virulence and serogrouping is rarely sufficient to reliably identify a strain as diarrheagenic (1). Rather, the serogroups serve as readily identifiable chromosomal markers that correlate with specific virulent clones (1,2). Nowadays, pathotypes of *E. coli* are defined on the basis of virulence properties demonstrated by phenotypic and/or genotypic methods, i.e., by recognition of their specific virulence factors: toxins, adhesion type, host cell surface modifying factors, invasins, secretion systems which export proteins and pilot them to the target host cells.

EPEC are the major cause of infantile diarrhea predominantly in developing countries but are also identified in developed countries (3,4,5). During infection, EPEC strains attach intimately to the intestinal epithelium and efface the absorptive microvilli, initiating a complex signalling cascade that ultimately leads to diarrhea (6). These pathogenic effects manifest as „attaching and effacing" histopathological hallmark, which is conferred by a large chromosomal island called locus of enterocyte effacement (LEE). EPEC of classic serotypes adheres to cultured cells in a localized pattern (1,7). This localized adherence (LA) is conferred by a large EPEC adherence factor (EAF) plasmid, associated with virulence in epidemiological and volunteer challenge studies, which is present in all typical EPEC strains, and absent or altered in atypical EPEC strains (8,9,10,11). The 60-MDa EAF plasmid harbors the bundle-forming pilus (*bfp*) operon, encoding the type IV pili responsible for LA and the formation of microcolonies on host cells (12).

The aim of our study was to demonstrate the EAF plasmid in strains of EPEC serotypes isolated in the country in 2007.

ABBREVIATIONS USED IN THIS PAPER:
enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), entroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC)

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MATERIALS AND METHODS

Bacterial strains. A total of 53 EPEC strains isolated in Regional Inspetorates of Public Health Control and Prevention (RIOKOZ) in 2007, and sent to National Reference Laboratory of Enteric Pathogens for confirmation, were investigated. O serotypes were determined with antisera manufactured by Bulbio-NCIPD, Ltd, Bulgaria. A strain of *E. coli* O127 (Copenhagen, Denmark) served as a positive control for pEAF in the study.

PCR. Isolation of DNA from the strains was performed using PrepMan Ultra Sample Preparation Reagent (PM) (Applied Byosystems). One loopfull from cultures incubated overnight on McConkey agar (Bulbio-NCIPD, Ltd) ($1x10^6$ CFU) was suspended in 100µl PM in 1.5 ml tube. Then samples were homogenized on vortex for 10-30 sec., followed by heating on thermoblock (100^0C) for 10 min. After centrifugation of samples at 13 000 rpm for 3 min., supernatants were used for analysis. The concentration of isolated DNA (ng/µl) was measured by spectrophotometer GeneQuant pro (Amersham Biosciences) at λ=260 nm. The primers we used were: EAF1: 5'-cag ggt aaa aga aag atg ata a-3' and EAF2: 5'-tat ggg gac cat gta tta tca-3' that amplificate ≈400 bp of EAF (13). Parameters of PCR were: ≈20-40 ng DNA, 0.4 µM EAF1/EAF2, 1xPCR buffer, 200 µM dNTP, 2 mM $MgCl_2$, 4% dimethylsulfoxid, 1U AmpliTaq GoldTM (Applied Biosystems) in 25 µl volume. DNA amplification was performed on QB-96 apparatus (Quanta, Biotech Ltd). Conditions of the reaction were: initial denaturation 95^0C for 6 min., 35 cicles denaturation-95^0C for 20 sec., hybridization-55^0C for 30 sec., and elongation-72^0C for 30 sec; the terminal elongation was at 72^0C for 5 min. Amplicons were analysed by capillary electrophoresis using eGene HDA-GT12 apparatus with method M 500.

RESULTS AND DISCUSSION

Serotyping. Serotypes of EPEC strains were determined as follows: O20 (n=2), O26 (n=2), O44 (n=6), O55 (n=9), O86 (n=2), O111 (n=6), O117 (n=1), O119 (n=6), O125 (n=1), O126 (n=4), O127 (n=6), O128 (n=7), and O146 (n=1).

PCR. Five out of 53 (9.4%) EPEC strains were positive for EAF plasmid (Fig. 1, lanes 5 to 9). Of the 9 *E. coli* O55 EAF positive proved to be 2 strains (22.2%). Of course, their number was low and we need much more strains tested in order to claim that percentage of EAF positive *E. coli* O55 is over 20.

In this study we have demonstrated the EAF plasmid in only 9.4% of the EPEC strains. We think the reason for the low number of EAF positive EPEC in the investigation might be due to: Firstly, it is well known that not all of the EPEC isolates harbor the plasmid. Recent epidemiological evidence indicates an increasing prevalence of atypical EPEC both in developed countries (3,5,14) and in developing countries (15,16). Many atypical EPEC isolates lack the entire EAF plasmid (17); Secondly, part of the strains may have lost the plasmid after

multiple procedures of subculture; Thirdly, primers used probably originate from more variable part of the plasmid.
In conclusion, the PCR technique is a reliable method for detection of the pEAF in EPEC isolates; in Bulgarian isolates the plasmid was detected most frequently in *E. coli* O55, but more isolates from this serotype and other EPEC serotypes should be tested; here, we have presented the first investigation in Bulgaria demonstrating pEAF in clinical isolates of EPEC.

REFERENCES
1. Nataro, J., J.B. Kaper. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 1998; 11 (1): 142-201.
2. Whittam, T.S., M.L. Wolfe, I.K. Wachsmuth, F. Orskov, R.A. Wilson. Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. Infect. Immun. 1993; 61: 1619-1629.
3. Afset, J.E., L. Bevanger, P. Romunstad, K. Bergh. Association of atypical enteropathogenic *Escherichia coli* with prolonged diarrhea. J. Med. Microbiol. 2004; 53: 1137-1144.
4. Kaper, J.B., J.P. Nataro, H.L.T. Mobley. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2004; 2: 123-140.

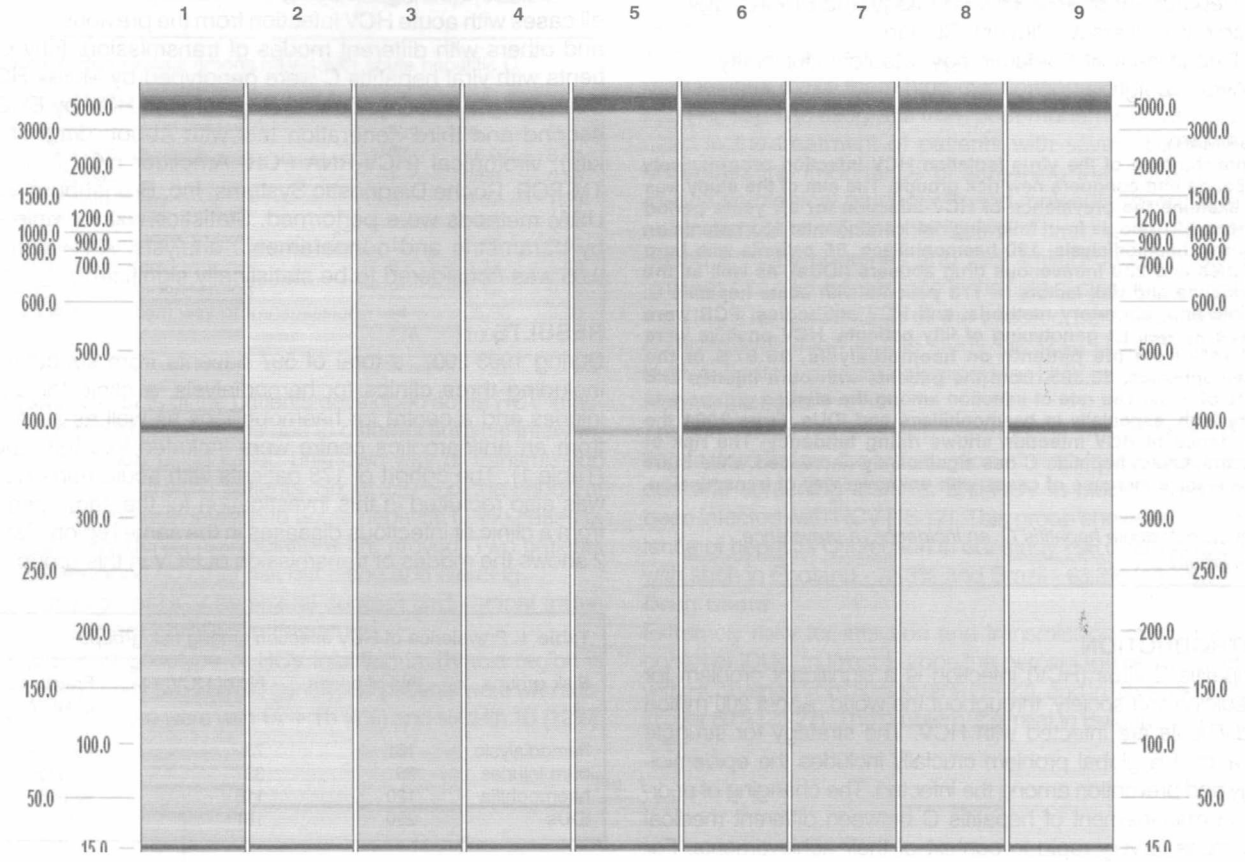

Fig.1. PCR for detection of pEAF in EPEC isolates.

1. *E. coli* O127 positive control (Denmark); 2. negative control (ddH_2O); 3. *E. coli* O127 (№ 159); 4. *E. coli* O111 (№ 361); 5. *E. coli* O127 (№ 172); 6. *E. coli* O128 (№ 219); 7. *E. coli* O55 (№ 221); 8. *E. coli* O119 (№ 284); 9. *E. coli* O55 (№ 374)

5. Nguyen, R.N., L.S. Taylor, M. Tauschek, R. Robins-Browne. Atypical enteropathogenic *Escherichia coli* infection and prolonged diarrhea in children. Emerg. Infect. Dis. 2006; 12: 597-603.
6. Clarke, S.C., R.D. Haigh, P.P.E. Freestone, P.H. Williams. Virulence of enteropathogenic *Escherichia coli*, a global pathogen. Clin. Microbiol. Rev. 2003; 16: 365-378.
7. Cravioto, A., R.J. Gross, S.M. Scotland, B. Rowe. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. 1979; 3: 95-99.
8. Bieber, D., S. Ramer, C.-Y. Wu, W. Murray, T. Tobe, R. Fernandez, G. Schoolnik. Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. Science 1998; 280: 2114-2118.
9. Levine, M.M., J.P. Nataro, H. Karch, M.M. Baldini, J.B. Kaper, R.E. Black, M.L. Clements, A.D. O'Brien. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding and enteroadhesiveness factor. J. Infect. Dis. 1985; 152: 550-559.
10. Nunes, E.B., H.O. Saridakis, K. Irino, J.S. Pelayo. Genotypic and phenotypic characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from children with and without diarrhea in Londrina. Brazil. J. Med. Microbiol. 2003; 52: 499-504.
11. Sunabe, T., Y. Honma. Relationship between O-serogroup and presence of pathogenic factor genes in *Escherichia coli*. Microbiol. Immunol. 1998; 42: 845-849.
12. Mьller, D., P. Hagedorn, S. Brast, G. Heusipp, M. Bielaszewska, A.W. Friedrich, H. Karch, M.A. Schmidt. Rapid identification and differentiation of clinical isolates of enteropathogenic *Escherichia coli* (EPEC), atypical EPEC, and shiga toxin-producing *Escherichia coli* by a one-step multiplex PCR method. J. Clin. Microbiol. 2006; 44 (7): 2626-2629.
13. Franke, J., H. Franke, H. Schmidt, A. Schwarzkopf, L.H. Wieler, G. Baljer, L. Beutin, H. Karch. Nucleotide sequence analysis of enteropathogenic *Escherichia coli* (EPEC) adherence factor probe and development of PCR for rapid detection of EPEC harboring virulence plasmids. J. Clin. Microbiol. 1994; 32: 2460-2463.
14. Trabulsi, L.R., R. Keller, T.A.T. Gomes. Typical and atypical enteropathogenic *Escherichia coli*. Emerg. Infect. Dis. 2002; 8: 508-513.
15. Galane, P.M., M. Le Roux. Molecular epidemiology of *Escherichia coli* isolated from young South African children with diarrheal disease. J. Health Popul. Nutr. 2001; 19: 31-38.
16. Toma, C., Y. Lu, N. Higa, N. Nakasone, I. Chinen, A. Baschkier, M. Rivas, M. Iwanaga. Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. J. Clin. Microbiol. 2003; 41: 2669-2671.
17. Nwaneshindu, A.I., T. Mucci, D.J. Pickard, I.N. Okeke.A second large plasmid encodes conjugative transfer and antimicrobial resistance in O119:H2 and some typical O111 enteropathogenic *Escherichia* strains. J. Bacteriol. 2007; 189 (16): 6074-6079.

INCIDENCE AND PREVALENCE OF HCV INFECTION IN THE THRACIA REGION IN BULGARIA FOR 15 YEARS PERIOD

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SUMMARY

From the time of the virus isolation HCV infection progressively expands and conquers new risk groups. The aim of the study was to examine the prevalence of HCV infection for 15 years period among 597 people from following risk contingents: 161 patents on chronic haemodialysis, 120 haemophiliacs, 86 patients with burn injuries and 230 intravenous drug abusers (IDUs), as well as the incidence and risk factors in 178 patients with acute hepatitis C. Clinic and laboratory methods, anti HCV antibodies, PCR were made as well as genotyping of fifty patients. HCV positive were 45.34% from the patients on haemodialysis, 96.67% of the haemophiliacs, 33.38% from the patients with burn injuries and 70% of IDUs. The rate of infection among the studied groups was very high, especially in haemophiliacs and IDUs. From 2001 the incidence of HCV infection shows rising tendency. The risk of posttrasfusion hepatitis C has significantly decreased while there is a relative increase of cases with unknown way of transmission.

Key words: acute hepatitis C, an incidence, a prevalence, risk groups

INTRODUCTION

Hepatitis C virus (HCV) infection is a significant problem for medicine and society, throughout the world. About 200 million individuals are infected with HCV. The strategy for struggle against this global problem crucially includes the epidemiology and prevention among the infected. The changing of priority in management of hepatitis C between different medical sciences is very rapid in context of their achievements. For example, as acute hepatitis C progresses to chronic infection in 43%-86% of patients [1] and because there is clear evidence that antiviral therapy during the acute phase of HCV infection significantly reduces evolution to chronic illness [2, 3], the identification of patients with newly acquired hepatitis C is useful to the prevention of chronic hepatitis [4]. The precise characterization of the risk groups is necessary in the light of the continuous alteration of their proportion and especially the relative increase of the group with no reported identified risk [5].

The epidemiology of acute hepatitis C has changed during the past decades, and the incidence of new infection has decreased in most developed countries. The introduction of more accurate and sensitive screening tests has led to the control of hepatitis C associated with blood transfusion and administration of blood derivatives [4].

ABBREVIATIONS USED IN THIS PAPER:
Hepatitis C virus (HCV)

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METHODS

A long term trial for 15 years period was completed in six Bulgarian health care centres throughout the country. Four risk groups were determined on purpose as followed: 161 patients on chronic hemodialysis, 120 haemophiliacs, 86 patients with moderate and heavy forms of burn injuries and 230 intravenous drug users. A total of 178 patients with acute hepatitis C treated from 1993 to 2007 in the clinic of infectious diseases in Plovdiv were also enrolled in this investigation. Each patient provided written, informed consent before entering the study. The last cohort comprised all cases with acute HCV infection from the previous groups and others with different modes of transmission. Fifty patients with viral hepatitis C were genotyped by Murex HCV Serotyping 1-6 Assay. The serological (anti HCV by ELISA second and third generation test with Abbott diagnostic kits), virological (HCV-RNA PCR- Amplicor HCV Monitor TM PCR, Roche Diagnostic Systems, Inc. Branchburg, NJ, USA) methods were performed. Statistics was completed by parametric and nonparametric analysis. A P-value of 0.05 was considered to be statistically significant.

RESULTS

During 1993-2007, a total of 597 patients from six centres including three clinics for hemodialysis, a clinic for burn injuries and a centre for haemophiliacs as well as a group from an antinarcotics centre were included into the study (Table 1). The cohort of 178 patients with acute hepatitis C was also recruited in this investigation for the same period from a clinic of infectious diseases in the same region. Table 2 shows the modes of transmission of HCV in this group.

Table 1. Prevalence of HCV infection among risk groups

Risk groups	No of cases	No of HCV(+)	Prevalence of HCV
hemodialysis	161	73	45.34%
burn injuries	86	33	38.37%
haemophilia	120	116	96.67%
IDUs	230	161	70%

The HCV positive in patients on hemodialysis were 73 (32 women and 41 men). The age distribution in this group was median and (range) 47.90 (18-73) years old. Thirty of them were on dialysis twice and 43 trice weekly with duration of dialysis median and (range) 4.67 (1-10) years.

Twenty two from 33 HCV positive patients with average and heavy forms of burns were men and 11 were women. Each of them received more than one blood or plasma transfusions.

One hundred and sixteen patients with haemophilia type A aged between three and 73 years were HCV positive and one of them was female.

The last cohort of intravenous drug users belongs to the population of community required hepatitis C. A correlation between the duration, the number of application and the increased risk for infection was established. One hundred and forty or 60.87% of these cases had duration of injection drug use for one or two years. One third of all had one to three applications daily while the rest injected once or twice weekly. Almost all of them had acquired either tattoo or piercing. They reported sharing of earrings and drug injection paraphernalia among IDUs which increased the risk for transmission of different HCV genotypes.

Incidence of acute HCV

From 2001 to 2004 the incidence of HCV infection showed rising tendency for both Thracia region and the whole country. The intensive indices ranged from 0.5‰ in 1997 to 1.9‰ in 2004 (Figure1).

The common characteristic of both parameters is the low intensity typical for blood born epidemics. The period between years 2000 and 2004 can be determined as epidemic for both the whole country and Thracia region.

Modes of transmission of acute HCV infection

The evolution of epidemic characteristics of acute HCV infection reveals that until year 2000 there is predominance of cases infected after blood or blood products transfusion (surgical procedures, burns, haemophiliacs, healthcare workers, chronic hemodialysis). After this period the cohort of intravenous drug users took on the leadership (Table 2).

Table 2. Risk groups among cases with acute hepatitis C infection

Risk groups	No	%	S_p
Intravenous drug users	82	46.07	3.74
Haemophiliacs	1	0.56	0.56
Patients with burns	6	3.37	1.35
Patients on dialysis	3	1.69	0.96
Patients with multiple blood transfusions	14	7.87	2.02
Healthcare workers	6	3.37	1.35
Patients with Tattoo/Piercing	2	1.12	0.79
Such with unknown way of transmission	64	35.96	3,60
Total	178	100	-

Fourteen or 7.87% of the patients developed acute HCV infection after blood transfusions due to different surgical interventions The group of so called unknown way of transmission consists of 64 (35.96%) patients. Six or 3.37% of the cohort of healthcare workers developed acute hepatitis C. No cases of infection after dental manipulations are proved. Two patients (1.12%) reported no other risk but tattoo and piercing.

Transmission of HCV by sexual contact and vertical transmission are not enrolled in this study.

The prevalent genotype of HCV infection in Thracia region is genotype 1. Forty two from 50 genotyped patients were with subtype 1a (84%); two were with 1a + 1b (4%) and six with 1b (12%).

hygienic standards may influence the risk of HCV infection. This may explain differences found between dialysis centres in one country. However, the differences among centres indicate that local factors could play a role in causing HCV infection [6, 7]. The fight against HCV in dialysis centres is still going on but the percentage of the infected is not low. The level of 45.34% HCV positive patients in the three dialysis centres in Thracia region is relatively high with low rate of the acute form. HCV infection is common among patients with end-stage renal disease. This should be taken in mind according to possible kidney transplantation [8-11].

Burn patients

The multiple plasma and blood transfusions given to severe burn injuries make them high-risk contingent for HCV infection [12, 13]. The allografts from post-mortem skin are widely used in the treatment of patients with severe burns. This underlines the importance of screening that involves both routine serological procedures and molecular microbiological investigation. The latter has not been uniformly introduced in many countries and very limited data is available to assess its cost-benefit ratio in the field of skin donor screening [14]. The percentage of the infected from this cohort is comparatively high in this study (38.37%). This makes the burn patients a distinct risk group for HCV infection.

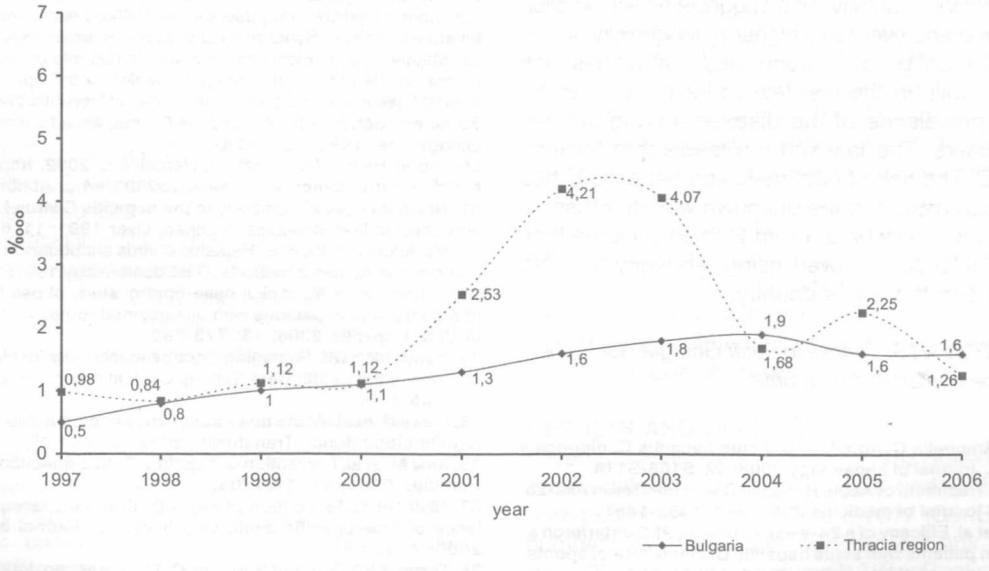
Haemophiliacs

The majority of patients with haemophilia who received clotting factors before the introduction of virus-inactivation methods and screening tests for anti-HCV in blood banks have been infected with HCV [15-17]. This group shows high prevalence of hepatitis C infection in our study - 96.67%, compared with such in England - 76.3% and Brazil - 63.3% [16, 18].

Drug users

Extremely risky for infection and transmission of HCV is the cohort of IDUs. In West Europe the percentage of the infected with HCV varies from 30% to 98% [19-22], and in USA and Japan is over 60% [23-27]. The problem is similar in Bulgaria where the

Fig. 1.



DISCUSSION

Haemodialysis patients

It is well known that haemodialysis patients are at high risk for hepatitis C infection. However, there is a wide range in prevalence rates in different regions of the world, ranging from 1% in the UK to more than 90% in Eastern Europe [6]. To a certain extent, this may reflect the different prevalence of HCV-infected individuals among the general population in these countries. However, the dialysis process itself and the level of

rate of the infected is 70%. The spread of hepatitis C is not untypical for the more experienced IDUs and new cases are reported even after 25 years of intravenous drug exposure, which should include this population in the prevention programmes. New trials indicate that that the focus should be not only on exchange of needles and syringes but also on the shared use of drug injection paraphernalia [28]. The reduction of the infection needs early preventions strategies including more information about the risk of HCV transmission among IDUs [28].

There is an increase of the incidence of the acute viral hepatitis C (AVHC) since 2001 in the clinic of infectious diseases in Plovdiv, Bulgaria. It is due to the growing number of the infected IDUs, which are 46.07% of all cases with AVHC included in this trial. They are followed by the group of patients with unknown way of transmission of HCV - 64 cases or 35.96%, which is not unusual for several other reports[29, 30]. In Japan it reaches 35%, in USA - 22% and in France 20%-40% [31-33]. A possible way of transmission in these cases could be by microlesions when another source of the infection is not found. The anamnestical data are not always reliable and the use of intravenous drugs, sexual contacts, etc. are concealed. Unconfirmed iatrogenic infection is not excluded especially after instrumental examinations and manipulations [34, 35].

In this study the patients infected with AVHC after blood and blood products transfusions are rather low in number. Their rate is 7.87% of all treated with this diagnosis and they were hospitalized at the early ninetieths or before the era of serological testing [34, 36]. Though this percentage is not very high it is necessary to improve the screening methods and better tests like NAT to be used for its elimination.

Healthcare workers with AVHC were 3.37% of all patients. No cases of infection after dental manipulations are proved though there is a sufficient evidence for this way of transmission in the available literature [37]. Extremely rare only in 2 cases (1.12%) tattoo and piercing are incriminated without any other probable risk for HCV infection. Transmission of HCV by sexual contact and vertical transmission are not enrolled in this study, which is in agreement with the data in the literature that these are exceptional ways of HCV infection [38, 39].

The prevalent genotype of HCV infection in Thracia region is genotype 1 [40] and it is predominant for the whole country as well as in Europe and United States.

The genotyped patients were from the group of these with blood or blood products transfusion which confirms the data that this genotype is more common for the parental way of infection. [41]. Genotype 1b is characteristic for the elder patients and is eventually associated with advanced liver damage, higher viral load and histological activity. This suggests earlier infection with this genotype and eventually higher pathogenicity [42].

In conclusion the cohort of hemophiliacs is at highest risk for hepatitis C while in the last few years there is an increase of the prevalence of the disease among intravenous drug abusers. The last win the leadership for incidence of AVHC. The risk of posttrasfusion hepatitis C has significantly decreased and the unknown way of transmission is in the focus of debate. From 2001 to 2004 the incidence of HCV infection showed rising tendency for both Thracia region and the whole country.

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REFERENCES

1. Mondelli MU, Antonella C, Agostino C. Acute hepatitis C: diagnosis and management. Journal of hepatology 2005; 42: S108-S114.
2. Jaeckel E, et al. Treatment of Acute Hepatitis C with Interferon Alfa-2b. The New England journal of medicine 2001; 345: 1452-1457.
3. Santantonio T, et al. Efficacy of a 24-week course of PEG-interferon a-2b monotherapy in patients with acute hepatitis C after failure of spontaneous clearance. Journal of Hepatology 2005; 42 329-333.
4. Santantonio T, et al. Risk factors and outcome among a large patient cohort with community-acquired acute hepatitis C in Italy. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 2006; 43: 1154-1159.
5. Gungabissoon U, Balogun MA, Ramsay M. Hepatitis C virus: laboratory surveillance in England and Wales, 1992-2004. Epidemiology and infection 2007 135: 541-548.
6. Hinrichsen H, et al. Prevalence and risk factors of hepatitis C virus infection in haemodialysis patients: a multicentre study in 2796 patients. Gut 2002; 51: 429-433.
7. Schneeberger PM, et al. Hepatitis C Virus Infections in Dialysis Centers in The Netherlands: a National Survey by Serological and Molecular Methods. Journal of clinical microbiology 1998; 36: 1711-1715.
8. Abbott KC, et al. Hepatitis C and Renal Transplantation in the Era of Modern Immunosuppression. Journal of the American Society of Nephrology 2003; 14: 2908-2918.
9. Murthy BV, et al. Predictors of GBV-C infection among patients referred for renal transplantation. Kidney international 1998; 53: 1769-1774.
10. Natov S, et al. Serologic and virologic profiles of hepatitis C infection in renal transplant candidates. New England Organ Bank Hepatitis C Study Group. American journal of kidney diseases: the official journal of the National Kidney Foundation 1998; 31: 920-927.
11. Pereira BJ, et al. Effect of hepatitis C infection and renal transplantation on survival in end-stage renal disease1. Kidney international 1998; 53: 1374-1381.
12. Miyoshi K, et al. Hepatic disorder in burn patients. Burns, including thermal injury 1985; 12: 49-53.
13. Coursaget P, et al. Incidence of hepatitis C virus infection in burn patients: detection of anti-C100, anti-C33c and anti-Core antibodies Biomedicine & pharmacotherapy 1991; 45: 445-449.
14. Pianigiani, et al. Prevalence of skin allograft discards as a result of serological and molecular microbiological screening in a regional skin bank in Italy. Burns 2006; 32: 348-351.
15. Baptista-Gonzalez H. Hepatitis C virus infection in patients with hemophilia. Revista de gastroenterologia de Mexico 2002; 67: S75-79.
16. Barbosa A, et al. Prevalence of hepatitis C Virus infection among hemophiliacs in Central Brazil. Memorias do Instituto Oswaldo Cruz 2002; 97: 643-644.
17. Blanchette VS, et al. Hepatitis C infection in children with hemophilia A and B. Blood 1991; 78: 285-289.
18. Brettler DB, et al. Prevalence of hepatitis C virus antibody in a cohort of hemophilia patients [see comments]. Blood 1990; 76: 254-256.
19. Coppola N, et al. Acute hepatitis B and C virus coinfection: a virological and clinical study of 3 cases. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 2003; 36: 528-532.
20. Esposito N, Rossi C. A nested-epidemic model for the spread of hepatitis C among injecting drug users. Mathematical Biosciences 2004; 188: 29-45.
21. Mathei C, Buntinx F, Damme PV. Seroprevalence of hepatitis C markers among intravenous drug users in western European countries: a systematic review. Journal of Viral Hepatitis 2002; 9: 157-173.
22. Proust B, et al. Two Successive Hepatitis C Virus Infections in an Intravenous Drug User. Journal of clinical microbiology 2000; 38: 3125-3127.
23. Villano SA, et al. Incidence and risk factors for hepatitis C among injection drug users in Baltimore, Maryland. Journal of clinical microbiology 1997; 35: 3274-3277.
24. Thorpe LE, et al. Risk of Hepatitis C Virus Infection among Young Adult Injection Drug Users Who Share Injection Equipment. American journal of epidemiology 2002; 155: 645-653.
25. Katayama K, Tanaka J, Yoshizawa H. Past trends in hepatitis C virus infection and route of transmission in Japan. Rinsho byori. The Japanese journal of clinical pathology 2001; 49: 741-746.
26. Evans JL, et al. Gender differences in sexual and injection risk behavior among active young injection drug users in San Francisco (the UFO study). Journal of Urban Health 2003; 80: 137-146.
27. Alter M, Moyer L. The importance of preventing hepatitis C virus infection among injection drug users in the United States. Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology 1998; 18: S6-10.
28. Mathei C, et al. Evidence for a substantial role of sharing of injecting paraphernalia other than syringes/needles to the spread of hepatitis C among injecting drug users. Journal of Viral Hepatitis 2006; 13: 560-570.
29. Lemon SM, Brown EA. Hepatitis C virus. 4th edn. New York: Churchill Livingstone, 1995, pp. 1474-1486.
30. World Health Organization. Hepatitis C 2002, http://www.who.int/csr/disease/hepatitis/whocdscsrlyo2003/en/print.html.
31. Nishioka K, et al. Antibody to the hepatitis C virus in acute hepatitis and chronic liver diseases in Japan. Liver 1991; 11: 65-70.
32. McHutchison JG, et al. Hepatitis C virus antibodies in acute icteric and chronic non-A, non-B hepatitis. Gastroenterology 1991; 101: 1117-1119.
33. Karmochkine M, et al. A case-control study of risk factors for hepatitis C infection in patients with unexplained routes of infection. Journal of Viral Hepatitis 2006; 13: 775-782.
34. Henderson DK. Managing Occupational Risks for Hepatitis C Transmission in the Health Care Setting. Clinical microbiology reviews 2003; 16: 546-568.
35. Larke B, et al. Acute nosocomial HCV infection detected by NAT of a regular blood donor. Transfusion 2002; 42: 759-65.
36. Kew M, et al. Prevention of hepatitis C virus infection. Journal of Viral Hepatitis 2004; 11: 198-205.
37. Mele, et al. Prevention of hepatitis C in Italy: lessons from surveillance of type-specific acute viral hepatitis. Journal of Viral Hepatitis 2000; 7: 30-35.
38. Terrault NA. Sex and Hepatitis C. The American Journal of Gastroenterology 2005; 100: 825-826.
39. Winston DH, Winston DC Managment of hepatitis C by the primary care provider: monitoring guidelines. A monthly newsletter of the hepatitis C support project 2006; 9: 9.
40. Boykinova O, Novakov S. Prevailing subtype in patients with acute hepatitis C virus infection. Clinical Microbiology and Infection 2000; Vol 6, Suppl. 1: 222.
41. Boykinova O, et al. A prospective three year study on patients after acute viral hepatitis C infection. Clinical application of immunology 2002; 1: 106-109.
42. Svirtlih N, et al. Hepatitis C virus genotypes in Serbia and Montenegro: the prevalence and clinical significance. World journal of gastroenterology: WJG 2007; 13: 355-360.

EMERGENCE OF QNR GENES AMONG EXTENDED-SPECTRUM BETA-LACTAMASE-PRODUCING ENTEROBACTERIAL ISOLATES IN A CANCER HOSPITAL IN BULGARIA

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SUMMARY

Objectives: To evaluate the presence of *qnr* genes among enterobacterial isolates carrying extended-spectrum beta-lactamases (ESBLs). Methods: Screening for the *qnrA*, *qnrB* and *qnrS* genes was carried out by PCR amplification with specific primers in 163 non-duplicate, clinically relevant ESBL-producing enterobacterial isolates collected between 2000 and 2005. ESBLs from all *qnr*-positive isolates were identified by PCR amplification and DNA sequencing. Pulsed-field gel electrophoresis was used to investigate the clonality of *qnr*-carrying isolates. Results: Seven *Citrobacter freundii* isolates, each from a different individual, carried *qnrB*. Among them, 4 had *qnrB17*, 2 had *qnrB10* and 1 had *qnrB12*. None of the isolates carried *qnrA* and *qnrS*. Among the *qnrB17*-carrying isolates, 3 possessed bla_{TEM-3} alone and 1had both $bla_{CTX-M-15}$ and bla_{OXA-1}. The isolates with *qnrB10* possessed bla_{TEM-3}. The *qnrB12*-carrying isolate had $bla_{CTX-M-15}$, bla_{OXA-1} and bla_{TEM-1}. Conclusions: The prevalence of *qnr* among enterobacterial clinical isolates carrying ESBLs between 2000 and 2005 was low (4.3%). Only *qnrB* was found and only in *C. freundii* isolates dating since 2002. *qnrA* and *qnrS* were not detected.

Key words: quinolones, resistance, enterobacteria, ESBLs

Until recently, quinolone resistance was believed to arise solely from chromosomal mutations in genes encoding target enzymes or affecting drug accumulation (1). In 1998, the first plasmid-mediated quinolone resistance (Qnr) was reported, in a *Klebsiella pneumoniae* isolate (2). Qnr proteins belong to the pentapeptide repeat family and are able to bind to DNA gyrase and topoisomerase IV, protecting them from the inhibitory activity of quinolones (3). The first Qnr detected, a protein of 218 amino acids, was named QnrA. Since then, two more Qnr determinants have been described, QnrB and QnrS, which share 40% and 59% amino acid identity, respectively, with QnrA. (4).

Qnr has been detected in several members of the *Enterobacteriaceae* family, mainly in *K. pneumoniae*, *Escherichia coli* and *Enterobacter* spp., in different countries (3). Plasmids harbouring *qnr* may also carry extended-spectrum beta-lactamase (ESBL) genes (3-5). The aim of this study was to evaluate the presence of *qnr* genes among enterobacterial isolates carrying ESBLs in a Bulgarian hospital.

ABBREVIATIONS USED IN THIS PAPER:

ESBL - extended-spectrum beta-lactamase; CLSI - Clinical and Laboratory Standards Institute; PCR - polymerase chain reaction; PFGE - pulsed-field gel electrophoresis; NAL - nalidixic acid; CIP - ciprofloxacin; Ak - amikacin; Cm - chloramphenicol; Gm - gentamicin; Km - kanamycin; Nt -netilmicin; Su - sulfonamides; Tc - tetracycline; Tm - tobramycin, Tp - trimethoprim.

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MATERIALS AND METHODS

From January 2000 to December 2005, 163 non-duplicate clinically relevant ESBL-producing enterobacterial isolates obtained at a 252-bed oncology hospital in Sofia were analyzed for the presence of *qnr* genes. These included 94 *E. coli*, 40 *K. pneumoniae*, 10 *Citrobacter freundii*, 7 *Enterobacter cloacae*, 6 *Serratia marcescens*, 2 *Klebsiella oxytoca*, and 1 each of *Enterobacter aerogenes*, *Morganella morganii*, *Providencia rettgeri* and *Escherichia hermannii*. Bacterial identification was carried out using Vitek 2 (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO).

Susceptibility to antimicrobials was determined by disc diffusion, following the Clinical and Laboratory Standards Institute (CLSI) recommendations. Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and at least one of the following antibiotics: cefotaxime, ceftazidime, aztreonam or cefepime, and confirmed by the Etest ESBL strip (AB Biodisk, Solna, Sweden). Nalidixic acid and ciprofloxacin MICs were determined by Vitek 2 (Vitek AMS; bioMerieux Vitek Systems Inc). Susceptibility interpretations were defined according to CLSI-2007 breakpoints. *E. coli* ATCC 25922 was used as an antibiotic-susceptible control.

PCR screening for *qnr* genes was performed on boiled cell lysate of clinical isolates with the Expand High Fidelity PCR system (Roche Diagnostics, Penzberg, Germany). The *qnrA*, *qnrB* and *qnrS* genes were amplified using specific primers and reaction conditions, as previously described (6). All positive results were confirmed by direct sequencing of both strands of amplicons. ESBLs of all *qnr*-positive isolates were identified by PCR amplification and DNA sequencing using primers specific for bla_{VEB-1}, bla_{OXA-1}, bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, as previously described (7, 8). Amplification of DNA was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Amplicons were purified with a QIAquick PCR purification kit (QIAGEN, K.K., Tokyo, Japan) and sequenced with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence analyses and comparison with known sequences were performed with the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

To assess the genetic relatedness of *qnr*-carrying isolates, genomic DNA was isolated and digested with *Spe*I (Bio-Rad, Hercules, CA). Pulsed-field gel electrophoresis (PFGE) was performed with the CHEF Mapper System (Bio-Rad) with the following run parameters: pulses ranging from 5.3 to 34.9 s at 6 V/cm for 19.5 h. A 48.5-kb lambda ladder (Bio-Rad) was used as the DNA size marker. Isolates were considered to be genetically related if their DNA macrorestriction patterns differed by fewer than seven bands (9).

RESULTS AND DISCUSSION

Seven *C. freundii* strains out of the 163 isolates studied, each from a different individual, carried *qnrB* (4.3%). Among the *qnrB*-positive isolates, 4 had *qnrB17* (GenBank accession number AM919399), 2 had *qnrB10* (GenBank accession number DQ631414) and 1 had *qnrB12* (GenBank accession number EU273755). No isolate carried *qnrA* or *qnrS* gene (Table). Among the total isolates studied, 20.9% and 24.5% were susceptible to nalidixic acid and ciprofloxacin, respectively. All *qnr*-positive isolates were nalidixic acid-resistant (MIC, >32 mg/L). For ciprofloxacin, four of the seven *qnr*-positive isolates (57.1%) were susceptible (MIC, 1 mg/L) according to the CLSI. Among the *qnrB*-carrying isolates, 5 possessed bla_{TEM-3} alone, 1had both $bla_{CTX-M-15}$ and bla_{OXA-1}, and 1had $bla_{CTX-M-15}$, bla_{OXA-1} and bla_{TEM-1}. No bla_{SHV} and bla_{VEB-1} were found in any of the *qnr*-positive isolates.

Table 1. Characteristics of *qnrB*-positive *Citrobacter freundii* isolates

Isolates	Date of isolation	Specimen	*qnr gene*	MIC (mg/L)[a]		ESBL	Other resistances [b]	PFGE pattern
				NAL	CIP			
CF 416	04/02/2002	urine	*qnrB10*	>=32	>=4	TEM-3	Ak Cm Gm Km Nt Su Tc Tm Tp	I
CF 612	12/02/2002	urine	*qnrB10*	>=32	>=4	TEM-3	Ak Cm Gm Km Nt Su Tc Tm Tp	I
CF 687	15/02/2002	urine	*qnrB17*	>=32	1	TEM-3	Ak Cm Gm Km Nt Su Tm	II
CF 1250	19/03/2002	urine	*qnrB17*	>=32	1	TEM-3	Ak Cm Gm Km Nt Su Tm	II
CF 1319	22/03/2002	urine	*qnrB17*	>=32	1	TEM-3	Ak Cm Gm Km Nt Su Tm	II
CF 1659	08/04/2005	urine	*qnrB12*	>=32	>=4	CTX-M-15	Cm Gm Km Nt Su Tc Tm Tp	III
CF 3821	29/09/2005	urine	*qnrB17*	>=32	1	CTX-M-15	Gm Km Tc Tm	IV

[a] NAL, nalidixic acid; CIP, ciprofloxacin
[b] Resistance abbreviations: Ak, amikacin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nt, netilmicin; Su, sulfonamides; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim.

PFGE analysis of *Spe*I-digested DNA of the *qnr*-positive isolates showed that the seven *C. freundii* isolates had four different PFGE patterns (Figure and Table). The two *qnrB10*-carrying isolates which showed the same PFGE profile had been obtained from different patients who shared the same surgery ward room. Likewise, three of the *qnrB17*-carrying isolates had the same PFGE profile. These isolates had been obtained from different patients who stayed in the same surgery ward. The remaining two isolates (no. 1659 and no. 3821) showed a different pattern, suggesting a unique clonal background.

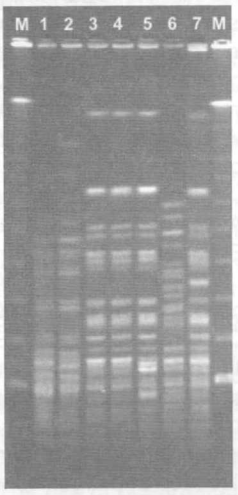

Fig. 1. Pulsed-field electrophoresis gel of DNA fragments generated with *Spe*I from genomic DNA of *qnrB*-positive *C. freundii* isolates. Lanes M 48.5 kb lambda ladder; line 1, isolate CF 416; line 2, isolate CF 612; line 3, isolate CF 687; line 4, isolate CF 1250; line 5, isolate CF 1319; line 6, isolate CF 1659; line 7 isolate CF 3821.

In this work, all *qnr*-positive isolates were resistant to nalidixic acid. Regarding ciprofloxacin, 57% of the isolates were susceptible, but showed decreased susceptibility compared with the antibiotic susceptible control strain. These data agree with previous reports showing that the presence of *qnr* does not necessarily lead to MICs above CLSI breakpoints for resistance to ciprofloxacin (2). The basis for concern regarding the low-level resistance conferred by this mechanism remains the increment this causes in the selection window to high-level quinolone resistance (3).

Our results differ from those of a recent French study, which showed that in a collection of 186 ESBL-producing bacteria obtained from 2002 to 2005 in a hospital in Paris, 2.2% and 1.6% of the isolates carried *qnrA1* and *qnrS1* (qnrB was not evaluated), respectively (5). However, our results are similar to those of a recent report from a Kuwaiti hospital, where 4.7% of 64 ESBL-producing enterobacteria obtained from 2002 to 2004 carried *qnrB* but none carried *qnrA* or *qnrS* (10).

CONCLUSIONS

Our study showed a low prevalence of QnrB determinants among ESBL-producing enterobacterial isolates and the lack of detection of QnrA and QnrS determinants in that collection from Bulgaria. Further studies in more heterogeneous bacterial populations are necessary to comprehensively assess the prevalence of Qnr and its clinical significance.

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REFERENCES

1. Jacoby, G. A. 2005. Mechanisms of resistance to quinolones. Clin. Infect. Dis. 4:S120-126.
2. Martinez-Martinez, L., A. Pascual and G. A. Jacoby. 1998. Quinolone resistance from a transferable plasmid. Lancet 351:797-799.
3. Robicsek, A., G. A. Jacoby and D. C. Hooper. 2006. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. 6:629-640.
4. Nordmann, P. and L. Poirel. 2005. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. J. Antimicrob. Chemother. 56:463-469.
5. Poirel, L., C. Leviandier and P. Nordmann. 2006. Prevalence and genetic analysis of plasmid-mediated quinolone resistance determinants QnrA and QnrS in *Enterobacteriaceae* isolates from a French university hospital. Antimicrob. Agents Chemother. 50:3992-3997.
6. Robicsek, A., J. Strahilevitz, D. F. Sahm, G. A. Jacoby, and D. C. Hooper. 2006. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob. Agents Chemother. 50:2872-2874.
7. Lee, S., Y. Park, M. Kim, H. K. Lee, K. Han, C. S. Kang and M. W. Kang. 2005. Prevalence of Ambler class A and D b-lactamases among clinical isolates of *Pseudomonas aeruginosa* in Korea. J. Antimicrob. Chemother. 56:122-127.
8. Saladin, M., V. T. Cao, T. Lambert, J. L. Donay, J. L. Herrmann, Z. Ould-Hocine, C. Verdet, F. Delisle, A. Philippon and G. Arlet. 2002. Diversity of CTX-M b-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiol. Lett. 209:161-168.
9. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33:2233-2239.
10. Cattoir, V., L. Poirel, V. Rotimi, C-J. Soussy and P. Nordmann. 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. 60:394-397.

PESTICIDES AND IMMUNITY

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SUMMARY

Most pesticides used today are acutely toxic to humans. Pesticides cause poisonings and deaths every year. Acute pesticide poisonings frequently involve organophosphate pesticides, or sometimes their close relatives, the n-methyl carbamates. Symptoms of organophosphate or carbamate poisoning include blurred vision, salivation, diarrhea, nausea, vomiting, wheezing, and sometimes seizures, coma, and death.

Many pesticides damage the immune system. The most immediately noticeable immune reaction to pesticide exposure is an increase in allergic reactivity, often including multiple chemical hypersensitivity. People whose immunity is suppressed by pesticides may also be unable to fight off viral infections or may experience a reactivation of one or more of the herpes viruses. Immunological studies reveal that pesticide-exposure can cause a decrease in the number of B and Tcells.

Lots of clinical, epidemiological and experimental studies show reductions or disruptions in cell-mediated, humoral and non-specific immunity.

Key words: pesticides, human health effects, immunotoxi-city, immunosuppression, autoimmunity

The US Environmental Protection Agency (EPA) defines a pesticide as „any substance or mixture of substances intended for preventing, destroying, repelling, or lessening the damage of any pest" (49).

A pesticide may be a chemical substance, biological agent (such as a virus or bacteria), antimicrobial, disinfectant or device used against pests including insects, plant pathogens, weeds, mollusks, birds, mammals, fish, nematodes (roundworms) and microbes that compete with humans for food, destroy property, spread or are a vector for disease or are a nuisance. Many pesticides are poisonous to humans.

Since before 2500 BC, humans have used pesticides to prevent damage to their crops. The first known pesticide was elemental sulfur dusting used in Sumeria about 4,500 years ago. By the 15th century, toxic chemicals such as arsenic, mercury and lead were being applied to crops to kill pests. In the 17th century, nicotine sulfate was extracted from tobacco leaves for use as an insecticide. The 19th century saw the introduction of two more natural pesticides, pyrethrum which is derived from chrysanthemums, and rotenone which is derived from the roots of tropical vegetables (36).

In 1939, Paul Mьller discovered that DDT was a very effective insecticide. It quickly became the most widely-used pesticide in the world. However, in the 1960s, it was discovered that DDT was preventing many fish-eating birds from reproducing which was a huge threat to biodiversity. Rachel Carson wrote the best-selling book *Silent Spring* about biological magnification. DDT is now banned in at least 86 countries, but it is still used in some developing nations to prevent malaria and other tropical diseases by killing mosquitoes and other disease-carrying insects (29).

ABBREVIATIONS USED IN THIS PAPER: None

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Pesticides are specifically formulated to be toxic to living organisms, and as such, are usually hazardous to humans. Most pesticides used today are acutely toxic to humans. Pesticides cause poisonings and deaths every year (28). Pesticides increased risk of leukemia and cancers (lung, brain, testicular, lymphoma), increase in spontaneous abortions, greater genetic damage, decreased fertilit, liver and pancreatic damage, neuropathy, disturbances to immune systems (asthma/ allergies), increases in stillbirths, decreased sperm counts (44).

The main risks for children are cancer: leukemia and brain cancer, asthma and allergies, polyneuritis with numbness and pain in lower limbs, altered neurological functioning and long-lasting neuro-behavioral impairments, birth defects, neurotoxicity, gangrene (tissue death) of the extremities. Children whose homes and gardens are treated with pesticides have 6.5 times greater risk of leukemia than children living in untreated environments (13, 17, 30, 44).

Acute pesticide poisonings frequently involve organophosphate pesticides, or sometimes their close relatives, the n-methyl carbamates. Symptoms of organophosphate or carbamate poisoning include blurred vision, salivation, diarrhea, nausea, vomiting, wheezing, and sometimes seizures, coma, and death. Mild to moderate pesticide poisoning mimics gastroenteritis, bronchitis, or intrinsic asthma, and even astute clinicians may not link these symptoms to pesticides (41).

Occupational pesticide poisonings are required to be reported in California, and there are approximately 1,500 reported cases per year (301, 401). Efforts to extrapolate to national occupational pesticide poisonings result in estimates of anywhere between 10,000 and 40,000 physician-diagnosed pesticide illnesses and injuries annually among agricultural workers (4).

Chronic effects of pesticide exposure may include adverse effects on neurological function, cancer, reproductive harm, reduced growth and development, and birth defects (12, 36).

Hundreds of millions of farmworkers, farm households, and consumers are probably exposed to dangerous levels of pesticides. Most of these people live in the developing world and the countries of the former Soviet Union. In many cases, they are using chemicals with known acute and chronic toxicity and applying them without adequate safeguards.

Pesticide production is a $30 billion industry, and 62 percent of sales is in the United States,Western Europe and Japan. But pesticides are applied intensively in parts of the developing world and use there is growing more rapidly. The acute toxicity and possible cancer risks of many pesticides have led to bans or use restrictions in the U.S. and Europe. Many of those pesticides are still being produced and used in the developing world and in the former Soviet Union (45).

Hundreds of millions of people are significantly exposed to pesticides every day, either directly in farm and garden use or in residues in water, air and food. Direct bodily measurements of exposure show that even children in rural areas and infants breastfeeding from exposed mothers can receive substantial doses (7, 35, 39).

As much as 85 to 90 percent of pesticides applied to agriculture never reach their targets, but instead disperse into the air, soil and water, and into the bodies of nearby animals and people (38). Farmers and rural residents are immediately exposed, but faraway consumers of agricultural products and fish are also at risk (48).

Pesticides that accumulate up the foodchain are still used in many countries. Measurements of the blood, fat and breast milk of people far from agricultural areas often show significant pesticide residues and enzyme depletions caused by certain pesticides. Far in the Arctic, Canadian Inuits carry as high a concentration of these compounds in their fat as polar bears and beluga whales do (2, 42).

The category pesticides includes insecticides, herbicides and fungicides. Since „cide" means to kill, it is readily apparent that these agents are lethal to various forms of life - insect, plants and fungi. That this lethality may also extend to humans is becoming increasingly apparent especially in the area of damage to the *immune system*. Symptoms of a pesticide-weakened immune system include: skin rashes, nausea, fatigue, depression, leukemia, frequent infections and fever. The scientific evidence suggesting that many pesticides damage the immune system is impressive. Animal studies have found that pesticides alter the immune system's normal structure, disturb immune responses, and reduce animals' resistance to antigens and infectious agents. There is convincing direct and indirect evidence that these findings carry over to human populations exposed to pesticides (45).
The most immediately noticeable immune reaction to pesticide exposure is an increase in *allergic reactivity* often including multiple chemical hypersensitivity. People whose immunity is suppressed by pesticides may also be unable to fight off viral infections or may experience a reactivation of one or more of the herpes viruses. Immunological studies reveal that pesticide-exposure can cause a decrease in the number of B and Tcells. The ratio of T-4 to T-8 helper cells is often reversed similar to the immune abnormalities found in AIDS patients (26).
Lots of studies using accepted scientific methods have shown that many pesticides alter the immune system in experimental animals and make them more susceptible to disease. Pesticides have been found to reduce the numbers of white blood cells and disease-fighting lymphocytes and impair their ability to respond and kill bacteria and viruses. They also alter the development of the thymus and spleen, key immune organs (19).
Studies have documented these effects for many pesticides used in this country and worldwide: organochlorines (e.g., DDT, lindane, chlordane), organophosphates (e.g., malathion, parathion, diazinon), carbamates (e.g., aldicarb, carbofuran, carbaryl) and others (2,4-D, atrazine, captan). Most pesticides now on the market have never been adequately tested in the laboratory for immunotoxic effects (45).
Few studies have assessed the impact of pesticides on the human immune system, partly because such research is extremely difficult to design and implement. Although most people are exposed to chronic low concentrations rather than the high doses used in laboratory tests, no basis exists for assuming that humans are exempt from risk.
Epidemiological studies from Canada and the former Soviet Union find that children and adults exposed to pesticides suffer similar immune system alterations and higher rates of infectious disease. The risks of pesticide-induced immunosuppression are known to be greatest to infants and children, the aged, people malnourished or chronically ill (1, 8, 50).
The mortality rate from the common infectious diseases, already by far the biggest killer in developing nations, may be driven in part by pesticide exposure.
Communicable diseases cause nearly half of all deaths in the developing world, compared to less than 10 percent in industrialized nations (8, 50).
Chronic malnutrition, inadequate health services, and poor sanitation are already serious threats to health. Any additional impairment of natural immune defenses can kill.
In developing nations, controls over pesticide marketing, manufacturing, and use are typically weak. Safety precautions are widely ignored. Symptoms of overexposure such as respiratory trouble, gastric upset, rashes, dizziness, „nerves" and weakness are usually disregarded or misdiagnosed. Doctors in the field found pesticide overexposure three times as common as farmers themselves reported (14).
Isolated Canadian Inuits who eat mainly fish and marine mammals heavily contaminated with accumulated pesticides show pronounced immune system deficiencies, particularly among breast-fed infants and children. Meningitis and inner ear infections occur at rates 30 times that of American children. Many Inuit babies can't be vaccinated, since they don't produce an antibody response (16, 43).
Tuberculosis has grown dramatically in Central Europe and the former Soviet Union where chemical pollution is widespread (8, 50).
Tuberculosis outbreaks are common among immunosuppressed groups.
Cotton workers in Uzbekistan had impaired cell-mediated immunity and suffered higher rates of respiratory, kidney and intestinal infection than residents of other areas using fewer pesticides (18, 22).
Children are particularly susceptible to the effects of pesticides on their immune systems. In the agricultural districts of central Moldova, where pesticides have been used heavily, 80 percent of healthy children had suppressed immunity. Children from these areas were three times more likely to have infectious diseases of the digestive tract, and two to five times more likely to have infectious diseases of the respiratory tract. Workers in pesticide factories and on farms in the area exhibited elevated rates of infectious diseases of the digestive, urinary, respiratory, and female genital tracts (3, 27, 37).
Indian factory workers chronically exposed to pesticides showed a 66 percent drop in blood lymphocyte levels, but tested normal after three months' vacation (20).
A recent plague that killed dolphins in the Mediterranean, the North Sea and the North Atlantic turned out to be common viruses to which the animals were normally resistant. Blood samples from dolphins off the Florida coast showed the mammals had high levels of pesticide residues, major viral infections and weak immune systems (25, 46).
Seal pups captured off the relatively unpolluted Northwest coast of Scotland were fed uncontaminated fish for one year. Half were then fed herring from the polluted Baltic sea. These seals developed high bodily concentrations of pesticides and other organochlorine chemicals and immune system responses a third as strong as in a control group (6). The herring was purchased from local markets and had been intended for human consumption.
Pesticides may increase susceptibility to certain cancers by weakening the immune system's surveillance against cancer cells. Autopsies on dead whales from the highly contaminated St. Lawrence Seaway found high tissue concentrations of certain pesticides and PCBs, as well as severe bacterial infections and frequent tumors (5).
Farmers generally have lower risks of cancer than other men, but have a higher risk for the kinds of cancers found in immune-deficient patients (those with AIDS and those taking immuno-suppressive drugs): Hodgkin's disease, melanoma, multiple myeloma, leukemia (all cancers of the immune system) and cancers of the lip, stomach and prostate. Farmers most heavily exposed to pesticides have the highest relative rates (11, 33, 38, 51).
Some pesticides are linked conclusively to the production of autoimmune antibodies, attacks of the body upon itself (10, 32, 34, 47).
Russian research in the cotton, tobacco and vegetable-growing regions of Samarkand found that people exposed to organo-chloride and organo-phosphate pesticides harbored autoimmune antibodies. Factory workers elsewhere in Russia showed similar symptoms (40).

CONCLUSION

Clinical and epidemiological studies of humans who are occupationally or accidentally exposed to pesticides provide direct evidence that normal immune system structure and functions are thereby altered. In general, these findings are consistent with the experimental evidence, showing reductions or disruptions in cell-mediated, humoral and non-specific immunity.

REFERENCES

1. Barnett, J.B., and K.E. Rodgers, „Pesticides," in J.H. Dean, M.I. Luster, A.E. Munson, and I. Kimber, eds., Immunotoxicology and Immunopharmacology, Second Edition, (Raven Press, Ltd., New York: 1994), 191-211.
2. Barrie, L.A., D. Gregor, B. Hargrave, R. Lake, D. Muir, R. Shearer, B. Tracey, and T. Bidleman, „Arctic Contaminants: Sources, Occurence and Pathways," The Science of the Total Environment, (1992), 122: 1-74.
3. Birnbaum, L.S., „Workshop on Perinatal Exposure to Dioxin-Like Compounds: V. Immunologic Effects," Environmental Health Perspectives, (March 1995), 103(Supplement 2): 157-159.
4. Blondell J. Epidemiology of pesticide poisonings in the United States, with special reference to occupational cases. Occ Med State of the Art Reviews 1997; 12:209-220. Bulletin of the World Health Organization, (1993), 71(5): 625-632.
5. De Guise, S., D. Martineau, P. Beland, and M. Fournier, „Possible Mechanisms of Action of Environmental Contaminants on St. Lawrence Beluga Whales (Delphinapterus leucas)," Environmental Health Perspectives, (May 1995), 103(Supplement4): 73-77.
6. De Swart, R.L., Impaired Immunity in Seals Exposed to Bioaccumulated Environmental Contaminants, Dissertation for Doctorate, (Erasmus Universiteit Rotterdam, Rotterdam, Netherlands, June 1995).
7. Dinham, B., „Review of the Global Pesticide Market," Interim Report: Presenting the Evidence, (The Pesticides Trust, London: June 1994), 19-29. The Pesticide Hazard: A Global Health and Environmental Audit, (Zed Books for The Pesticides Trust, London: 1993).
8. Dolin, P.J., M.C. Raviglione, and A. Kochi, „A Review of Current Epidemiological Data and Estimation of Future Tuberculosis Incidence and Mortality," (World Health Organization, Geneva: 1993), WHO/TB/93.173.
9. Drew, L.L., „Health of Canada's Aboriginal People," Canadian Journal of Public Health, (March-April 1992), 83(2): 163-164.
10. Druet, P., „Metal-Induced Autoimmunity," Human and Experimental Toxicology, (1995), 14: 120-121
11. Figgs, L.W., M. Dosemeci, and A. Blair, „United States Non-Hodgkin's Lymphoma Surveillance by Occupation 1984-1989: A Twenty-Four State Death Certificate Study," American Journal of Industrial Medicine, (1995), 27: 817-835.
12. Garry V, Schreinemachers D, Harkins M, Griffith J. Pesticide appliers, biocides, and birth defects in rural minnesota. Environ Hlth Persp 1996; 104:394-399.
13. Hammond, M., 1995, Pesticide Bylaws: Why We Need Them and How to Get Them. Consultancy for Alternative Education, Quebec.
14. Jeyaratnam, J., „Acute Pesticide Poisoning: A Major Global Health Problem," World Health Statistics Quarterly, (1990), 43:139-144.
15. Jeyaratnam, J., K.C. Lun, and W.O. Phoon, „Survey of Acute Pesticide Poisoning Among Agricultural Workers in Four Asian Countries," Bulletin of the World Health Organization, (1987), 65(4); 521-527.
16. Julien, G., J.D. Baxter, M. Crago, H.J. Ilecki, and F. Therien, „Chronic Otitis Media and Hearing Deficits Among Native Children of Kuu-jjuaraapik (Northern Quebec): A Pilot Project," Canadian Journal of Public Health, (January/February 1987), 78: 57-61.
17. Journal of the American Medical Association 1989;30:1306. Mayo Clinic;Medical Toxicology 1988;3:350-75. National Poisons Unit, Guy's Hospital, London, England.
18. Kaloyanova, F.P., and M.A. El Batawi, „Organo-chlorine Compounds," Human Toxicology of Pesticides, (CRC Press, Inc., Boca Raton, Florida: 1991a), 59-100.
19. Kendall, M.D., and M.A. Ritter, eds., Thymus Up–date 4: The Thymus in Immunotoxicology, (Har-wood Academic Publishers, London: 1991).
20. Khan, S.A., and S.A. Ali, „Assessment of Certain Hematological Responses of Factory Workers Exposed to Pesticides," Environmental Contamination and Toxicology, (1993), 51: 740-747.
21. Kovalchuk, L.V., and A.N. Cheredayev, „The Actual Problems of Estimation of the Human Immune System at the Modern Stage," Immunologia, (1990), (5): 4-7, (in Russian).
22. Kovtyukh, L.P, „Health Status of the Population of Moldova in Districts with Heavy Pesticide Application, Summary Report, 1990-1993", Report prepared for the World Resources Institute, Program in Economics and Population, (Moldovan Branch of Ecological and Genetic Monitoring, Kishinev: 1995).
23. Kozlyuk, A.S., L.A. Anisimova, and I.G. Shroit, [Immunologic Methods in Hygiene Studies], (Sti-intsa, Kishinev: 1987a), (in Russian).
24. Kozlyuk, A.S., G.V. Kushnir, and I.G. Shroit, „[The Characteristics of the Immune Status of Children in Various Districts of Moldovian SSR]," [Proceedings of the First Republic All-Union Immunological Congress, Volume 2], (Moskow: 1989), 221, (in Russian).
25. Lahvis, G.P., R.S. Wells, D.W. Kuehl, J.L. Stewart, H.L. Rhinehart, and C.S. Via, „Decreased Lymphocyte Responses in Free-Ranging Bottlenose Dolphins (Tursiops truncatus) Are Associated with Increased Concentrations of PCBs and DDT in Peripheral Blood," Environmental Health Perspectives, (May 1995), 103(Supplement 4): 67-72.
26. Legro, William. Under Seige. Organic Gardening. April 1988.
27. Lindstrom, G., K. Hooper, M. Petreas, R. Stephens, and A. Gilman, „Workshop on Peri–natal Exposure to Dioxins-Like Compounds: I. Summary," Environmental Health Perspectives, (March 1995), 103(Supplement 2): 135-142.
28. Litovitz TL, Smilkstein M, Felberg L, 1996 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 1997; 15:447-500.
29. Lobe, J., „WHO urges DDT for Malaria Control Strategies," Sept 16, 2006, Inter Press Service, cited from Commondreams.org;
30. Lowengart, et al., 1987, Journal of National Cancer Institute, 79: 39-46.
31. Maizlish N, Rudolph L, Dervin K. The surveillance of work-related pesticide illness: an application of the Sentinal Event Notification System for Occupational Risks (SENSOR). Am J Pub Hlth 1995; 85:806-811.
32. Majoor, G.D., K. Wiel, H. Wodzig, and P.J.C. Van Breda Vriesman, „Cyclosporin-Induced Autoimmunity," in M.D. Kendall, and M.A. Rit-ter, eds., Thymus Update 4: The Thymus in Immunotoxicology, (Harwood Academic Publishers, Switzerland: 1991), 179-200.
33. Maroni, M., and A. Fait, Health Effects in Man from Long-Term Exposure to Pesticides: A Review of the 1975-1991 Literature, (Elsevier Scientific Pub–lishers Ireland, Ltd.: 1993).
34. McConnachie, P.R., and A.C. Zahalsky, „Immune Alterations in Humans Exposed to the Termiti-cide Technical Chlordane," Archives of Environ–mental Health, (July/August 1992), 47(4): 295-301. „Immunological Consequences of Exposure to Pentachlorophenol," Archives of Environ–mental Health, (July/August 1991), 46(4): 249-253.
35. McDougall, L., L. Magloire, C.J. Hospedales, J.E. Tollefson, M. Ooms, N.C. Singh, and F.M.M. White, „Attitudes and Practices of Pesticide Users in Saint Lucia, West Indies," Bulletin of the Pan American Health Organization, (1993), 27(1): 43-51.
36. Miller, G. Tyler Jr. (2002). Living in the Environment (12th Ed.). Belmont: Wadsworth/Thomson Learning.
37. Moldova, Central Statistical Board, „Statistical Reports, 1983-1992," 02.03076, Number 141, (Kishinev: 1992), (in Russian).
38. Moses, M., E.S. Johnson, W.K. Anger, V.W. Burse, S.W. Horstman, R.J. Jackson, R.G. Lewis, K.T. Maddy, R. McConnell, W.J. Meggs, and S.H. Zahm, „Environmental Equity and Pesticide Exposure," Toxicology and Industrial Health, (1993), 9(5): 913-959.
39. Nieto, O., Morbilidad y Mortalidad por Plaguicidas en Antioquia, 1978-1989, Servicio Seccional de Salud de Antioquia, (Medellin: 1990), (in Spanish).
40. Nikolaev, A.I., L.A. Katsenovich, and S.T. Atabaev, Pestitsidy i Immunitet [Pesticides and Immunity], (Meditsina USSR, Tashkent: 1988), (in Russian).
41. Ordin DL. Editorial: Surveillance for pesticide-related illness-lessons from California. Am J Pub Hlth 1995; 85:762-763.
42. Pearce, F., „Tropical Toxins Ruin Arctic Catch," New Scientist, (July 5,1995), 147(1987): 7.
43. Proulx, J.-F., „Meningitis in Hudson's Bay, Northern Quebec, Canada," Arctic Medical Research, Abstracts of the 7th International Congress on Cir-cumpolar Health, (1987), 45.
44. Rea, William J., 1996, Pesticides. Journal of Nutritional and Environmental Medicine 6, 55-124.
45. Repetto R., SS. Baliga., 1996, Pesticides and the Immune System: The public health risks.
46. Simons, M., „Dead Mediterranean Dolphins Give Nations Pause," The New York Times, (February 2,1992), 10.
47. Thrasher, J.D., R. Madison, and A. Broughton, „Immunologic Abnormalities in Humans Exposed to Chlorpyrifos: Preliminary Observations," Archives of Environmental Health, (March/April 1993), 48:(2): 89-93.
48. Utsch, H., Pesticide Poisoning in a Rice and a Vegetable Growing Area of Sri Lanka, Dissertation for Ph.D., (Universitat Zurich, Zurich: 1991).
49. What is a Pesticide? (US EPA definitions) retrieved June 24, 2006
50. WHO Report on the Tuberculosis Epidemic, 1995, (WHO, Geneva: 1995b), WHO/TB/95.183.
51. Zahm, S.H., D.D. Weisenburger, R.C. Saal, J.B. Vaught, P.A. Babbitt, and A. Blair, „The Role of Agricultural Pesticide Use in the Development of Non-Hodgkin's Lymphoma in Women," Archives of Environmental Health, (September/October 1993), 48(5): 353-358.

CASE REPORTS OF *HELICOBACTER PYLORI* INFECTION ASSOCIATED WITH MULTI-DRUG RESISTANT STRAINS IN CHILDREN

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SUMMARY

Successful eradication of *H. pylori* is curative. However, antibacterial resistance is the major cause for treatment failure. The present study focuses on two cases of *H. pylori* infection in children (0.6% of 357 children evaluated since 1996), associated with strains exhibiting uncommon multi-drug resistance to amoxicillin, macrolides and metronidazole.

Keywords: Helicobacter, resistance, multidrug, clarithro-mycin, metronidazole, amoxicillin, tetracycline, cipro-floxacin, breakpoint susceptibility testing, E test, agar dilution method, children

INTRODUCTION

Helicobacter pylori infection is a chronic and often life-long infection that is strongly associated with gastritis and peptic ulcers and is a risk factor for gastric malignancy (1). *H. pylori* infection is most often intrafamilial and spreads by oral-oral or fecal-oral transmission (2). The prevalence of *H. pylori* infection in children with abdominal complaints ranges from 10% to 85% and is high in developing and some other countries (3-5). In Bulgaria, the infection is common in childhood (6). The children should be a focus of constant attention in the diagnostics and treatment of the infection because of the risk for development of peptic ulcers and gastric malignancy later in life.

Eradication of the infection leads to clinical cure but is successful in only 65-90% of cases, and the antibacterial resistance is the major cause for treatment failure (7). In children, the primary resistance rates to metronidazole and clarithromycin have ranged from 16 to 43% and from 9.5 to 28.3%, respectively, in most European countries, and those of post-treatment resistance are often higher (7-13), (Table 1). Because children are thought to be more frequently treated by macrolides for respiratory tract infections than the adults, *H. pylori* strains from children have often exhibited higher primary resistance to clarithromycin than those from adult patients (14). *H. pylori* resistance to amoxicillin and tetracycline has been reported rarely and only in some countries (15,16). Triple resistance to amoxicillin, metronidazole and clarithromycin is uncommon in *H. pylori*. However, Japanese authors have observed an increase in *H. pylori* resistance rates to amoxicillin from 2000 to 2003 (17). The **aim** of the study was to draw attention to two *H. pylori* positive children whose strains exhibited uncommon multi-drug resistance to amoxicillin, macrolides and metronidazole.

Table 1. *H. pylori* resistance to antibacterials in childhood (7-11,14).

Resistance (%) to	Clarithromycin	Metronidazole
Primary resistance in:		
Europe (adults)	2 - 23 (1.7-23.4)	20-40 (15-41)
Europe (children)	9.5 - 28.3	16 - 43
Northern Europe	3.3	30.4
Southern Europe	32.5	28.7
Eastern Europe	17.5	31.2
Western Europe	18.2	31.6
Secondary Resistance	11.4 - 23 - 67	31.6 - >50%

MATERIAL AND METHODS

Bacterial strains: Totally, 357 *H. pylori* strains isolated from non-treated children with gastroduodenal diseases were evaluated since 1996. A single antral biopsy per child was taken. The specimens were transported in Stuart transport medium (Merck) for <5 h. A smear was prepared from part of the specimen and was used for modified Gram staining with carbol fuchsin. A part of specimen was placed in 10% urea agar medium and the color change was read after incubation at 35°C for 30 min, 3 h and 24 h. The remaining part of specimen was homogenized and inoculated onto Columbia blood agar (Becton Dickinson) with vancomycin 10 mg/L , trimethoprim 5 mg/L , cefsulodin 5 mg/L and amphotericin B 5 mg/L, and/or 10% sheep blood, and 1% IsovitaleX. Both selective and non-selective media were used for primary culture. Plates were incubated microaerophilically for 3 to 10 days. Identification was made by Gram staining of the colonies, lack of aerobic growth on blood agar plates and testing for urease, oxidase and catalase. Strain growth was expressed semi- quantitatively as follows: sparse density - growth into the 1st or 1st and 2nd quadrants; moderate - growth into the 3rd quadrant; and abundant - growth into all quadrants of the plate.

Antibacterial susceptibility: The antibacterial susceptibility of the strains was tested by a breakpoint susceptibility testing (BST). Two drops (about 60 ml) of *H. pylori* suspensions, prepared in Mueller-Hinton broth (NCIPD) to obtain Mc Farland standard 3, were inoculated on Mueller-Hinton blood agar plates (NCIPD) and the following drug concentrations (mg/L): metronidazole 8, 16 and 32, clarithromycin or azithromycin 0.25, 0.5, 1 and 2, clindamycin 2 and 4, amoxicillin 0.5, 1 and 2, ciprofloxacin 1 and tetracycline 4. The incubation was performed microaerophilically at 35°C for 48 to 72 h. If no growth appeared on the plates, the strain was considered as susceptible to the given concentration of the agent.

Minimal inhibitory concentrations (MICs) of amoxicillin, penicillin, clarithromycin and metronidazole against the resistant strains were determined by E test. Mc Farland 3 suspensions in Mueller-Hinton broth were used to flood Mueller-Hinton blood agar plates. E test strips (AB Biodisk) were placed on the plates and they were incubated at 35°C for 48-72 h in microaerophilic conditions. The results were read according to the supplier's recommendations.

The susceptibility of the resistant strains was also tested by a modified disk-diffusion method. *H. pylori* suspensions in Mueller-Hinton broth (3 McFarland standard), were spread

ABBREVIATIONS USED IN THIS PAPER:
BST- breakpoint susceptibility testing, MDDM- modified disk diffusion method, MTZ = metronidazole, CLA = clarithromycin, AMO = amoxicillin, NA- non-available

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onto Mueller-Hinton blood agar plates by sterile cotton swabs. The plates were briefly dried and disks containing the following drugs (MG): ampicillin (10), clarithromycin (15), azithromycin (15), erythromycin (15), clindamycin (2), metronidazole (5), tetracycline (30) and ciprofloxacin (5) were added. The incubation was performed as above. The diameters of inhibitory zones were measured in millimeters. The resistance breakpoints (mg/L) were: metronidazole >8, clarithromycin and azithromycin ≥1, amoxicillin and penicillin >0.5, tetracycline >4 (18,19) clindamycin >2 and ciprofloxacin >1. With the modified disk-diffusion method, strains with inhibitory zone diameters of <16 mm for metronidazole (20,21), ≤22 mm for clarithromycin (Grignon et al., 2002) and azithromycin, ≤17 mm for erythromycin (22), and <30 mm for the other drugs, were considered as resistant.

Statistical analysis: chi-square with or without Yates correction.

RESULTS AND DISCUSSUION

In our previous study, *H. pylori* infection was detected in 61.7% of the untreated Bulgarian children with gastroduodenal diseases (6). The infection was common even in the youngest patients, with about half (51.4%) of the children aged 1-5 years being *H. pylori*-positive. The overall infection incidence in Bulgarian pediatric patients was higher than that (25.6-30.5%) reported in some European countries and China (23-25).

Primary resistance rates to metronidazole were stable and oscillated around 13-16% since 1996. The primary metronidazole resistance rates in Bulgarian children were less common than those usually reported in European pediatric patients (14).

The primary clarithromycin resistance exhibited a slight tendency to increase from 10.4% in 1996-1999 to 18.7% in 2005-2007, (P >0.20) (Fig. 1). The primary clarithromycin resistance rates in Bulgarian children were similar to those usually reported in pediatric patients from Eastern and Western Europe. In several countries, the *H. pylori* resistance has exhibited a strong tendency to increase (i.e., the clarithromycin resistance in children in Belgium and Spain) or has remained still absent or rare such as the resistance to amoxicillin and tetracycline (7, 9, 10).

Amoxicillin resistance in *H. pylori* has been observed in single countries since 1999, (7). In the present study, amoxicillin resistance was low and occasional and present only in 1996-1999 and 2003-2004. In Europe, the resistance to amoxicillin in children was also exceptional (0.6%), (14). Likewise, the alarming triple resistance to amoxicillin, metronidazole and clarithromycin was uncommon (0.6% of 357 strains) and found in only two children over an 11-year period of time.

CASE REPORT NO 1: A 10-year-old girl, living in Sofia with only her grand-mother and in poor socioeconomic and hygienic conditions, presented to the University Pediatric Hospital, Sofia, with symtoms of epigastralgia. The history of epigastralgia had started one year earlier. No data about previous *H. pylori* eradication treatment were available. Endoscopy revealed a chronic superficial gastritis. Examination showed no evidence of other diseases. The rapid urease test in the hospital was positive only after 48 h incubation.

One antral biopsy specimen was sent to the microbiology laboratory for examination. *Helicobacter*- like organisms were seen on the direct Gram stained smear of the specimen. The laboratory rapid urease test was negative after 3 and 24 hour incubation. By culture, abundant *H. pylori* growth was observed after three-day incubation on both selective and non-selective media.

The *H. pylori* strain exhibited a multi-drug resistance to amoxicillin, metronidazole and clarithromycin by the BST and E test. By the E test, the MIC of penicillin was 2 mg/L. The strain was resistant to azithromycin and clindamycin by BST. By the disk diffusion method, the inhibitory diameters around the disks with ampicillin, clarithromycin, azithromycin, erythromycin, clindamycin and metronidazole were narrow, ranging from 6 to 12 mm. However, the strain was susceptible to tetracycline and ciprofloxacin by the BST and modified disk diffusion method (Table 2).

The girl was treated with metronidazole, ciprofloxacin, a proton pump inhibitor (omeprazole) and colloidal bismuth subcitrate for 14 days. Unfortunately, the girl has not presented to the hospital for evaluating the success of the eradication.

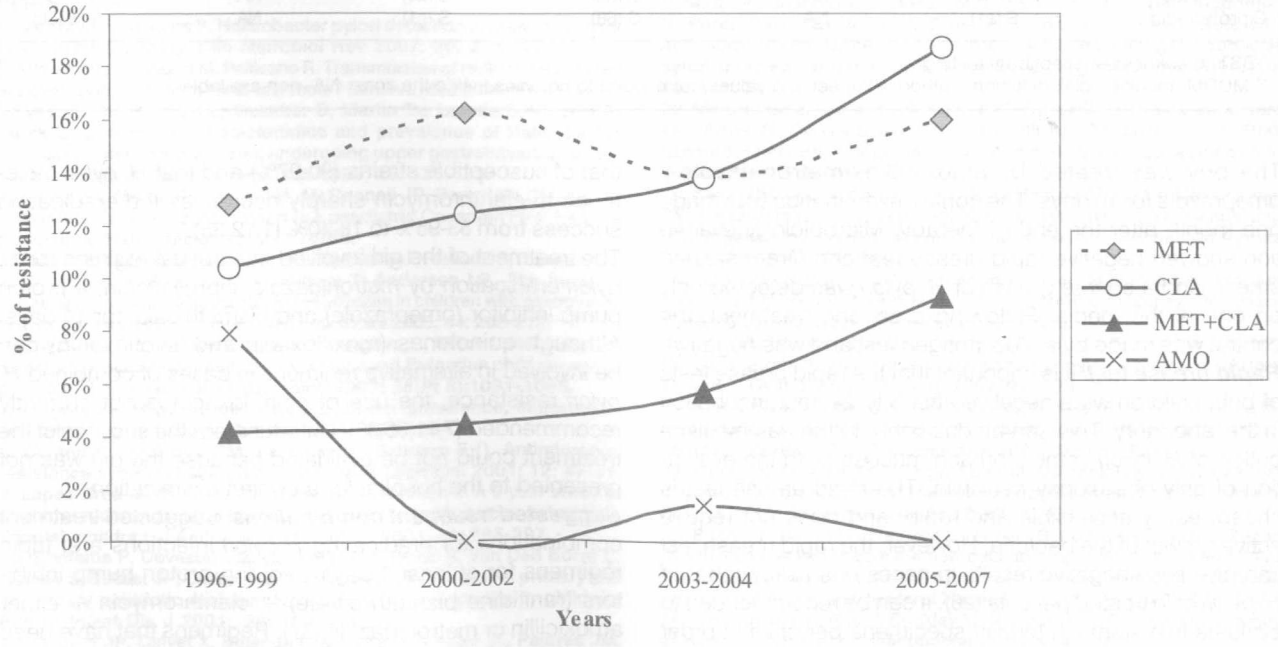

Figure 1. Primary *H. pylori* resistance in untreated Bulgarian children in 1996-2007

MTZ = metronidazole, CLA = clarithromycin, AMO = amoxicillin

CASE REPORT NO 2: A 13-year-old boy, living with his parents in a small village of South-western Bulgaria, was admitted to the University Pediatric Hospital, Sofia, with nausea and epigastralgia, mainly if fasting and at night. The type of his parents' profession was manual. No history of previous *H. pylori* eradication treatment was reported. Examination of the boy showed a coated tongue and epigastralgia. Endoscopy revealed antral nodularity and duodenal ulcer. According to the laboratory results, the blood values, the urinalysis and the erythrocyte sedimentation rate were normal. There was no evidence of other diseases.

One antral biopsy specimen was taken from the boy. No *Helicobacter* like organisms were seen on the direct Gram stained smear of the specimen. In the laboratory, the rapid urease test was negative. By culture, abundant growth of *H. pylori* was detected on the selective plate and moderate growth was observed on the non-selective plate after five-day incubation. The strain was multi-drug resistant to amoxicillin, metronidazole and clarithromycin by the BST and E test, and the MIC of penicillin was 1 mg/L. The strain was resistant to azithromycin and clindamycin by the BST. By the modified disk diffusion method, the inhibitory diameters around the disks with ampicillin, clarithromycin, azithromycin, erythromycin, clindamycin and metronidazole were narrow (from 6 to 11 mm). The strain was susceptible to tetracycline and ciprofloxacin by the BST and modified disk diffusion method (Table 2).

Table 2. Susceptibility patterns of *H. pylori* strains with multi-drug resistance from two children

H. pylori strain	Case No. 1			Case No. 2		
Method for susceptibility testing to	BST* (MIC, mg/L)	E test (mg/L)	MDDM** (mm)	BST (mg/L)	E test (mg/L)	MDDM (mm)
Amoxicillin	R (2)	R* (2)	NA	R (1)	R (1)	NA
Ampicillin	NA	NA	R (10)	NA	NA	R (11)
Penicillin	NA	R* (2)	NA	NA	R (1)	NA
Clarithromycin	R (>2)	R (12)	R (12)	R (>2)	R (48)	R (6)
Azithromycin	R (>2)	NA	R (10)	R (>2)	NA	R (6)
Erythromycin	NA	NA	R (10/6#)	NA	NA	R (6)
Clindamycin	R (>4)	NA	R (15/6#)	R (>4)	NA	R (6)
Metronidazole	R (32)	R (32)	R (6)	R (>32)	R (128)	R (6)
Tetracycline	S** (£4)	NA	S (62)	S (£4)	NA	S (36)
Ciprofloxacin	S (£1)	NA	S (68)	S (£1)	NA	S (38)

* BST- breakpoint susceptibility testing
** MDDM- modified disk diffusion method, #the second values correspond to colonies inside the zone. NA- non-available

The boy was treated by amoxicillin+metronidazole+omeprazole for 10 days. The control examination was made one month after the end of therapy. Microbiology evaluation showed negative rapid urease test and Gram stained smear, and a sparse growth of *H. pylori* was detected only on one of the media. Following a second treatment, the control was made by a stool antigen test and was negative.

Rapid urease test: It is important that the rapid urease tests of both children were negative after 3 to 24 hour incubation in the laboratory. This can be due either to the patchy distribution of *H. pylori* in the stomach mucosa or to the evaluation of only one biopsy per child. The rapid urease test is cheap, easily accessible and rapid, and does not require active growth of the bacteria. However, the rapid urease test can give false negative results in cases of small number of *H. pylori* or in treated patients (26). It can be recommended to evaluate two stomach biopsy specimens per child in order to optimize the diagnostics of *H. pylori* infection.

Possible risk factors for resistance: The risk factors for both *H. pylori* infection and antibacterial resistance in the girl can be associated with living in poor socioeconomic conditions. Because of poor living conditions, the children of Turkish nationality living in Germany are at high risk for *H pylori* infection (27). In the USA, black race adult patients are at high risk for *H pylori* resistance (28). The girl lives only with her grand-mother and, again, this can be related with unfavorable living conditions and care. Manual type of profession of the parents of the boy could be a risk factor for *H. pylori* infection. Higher education may be important for preventing the infection (29).

Multidrug resistant *H. pylori* strains were found in children with different places of living (the capital city and a small village). Multidrug resistance in *H. pylori* is unusual; however, combined resistance to beta-lactams, nitroimidazoles, and other agents has been recently reported (30-32). The triple *H. pylori* resistance was low but present; although according to Kim et al. (33), the amoxicillin resistance has not influenced the success of eradication. However, the detection of H. Pylori strains with amoxicillin MIC of ≥0.032 mg/L could be important because of the reported decreased eradication success in such cases according to several authors (34).

The eradication of the infection in the boy required two consecutive treatment regimens. It may be speculated that the first therapy decreased the number of viable *H. pylori* in the stomach mucosa and, thus, the second regimen eliminated the remaining and partially damaged bacteria. However, it is important to stress that the eradication rate of metronidazole resistant strains is lower (about 75%) than that of susceptible strains (90-97%) and that *H. pylori* resistance to clarithromycin sharply decreases the eradication success from 83-95% to 18-40% (1,12,35).

The treatment of the girl involved an unusual regimen for *H. pylori* eradication by metronidazole, ciprofloxacin, a proton pump inhibitor (omeprazole) and bismuth salts for 14 days. Although quinolones (moxifloxacin and levofloxacin) can be involved in alternative regimens in cases of combined *H. pylori* resistance, the use of ciprofloxacin is not currently recommended (7,11,35,36). Unfortunately, the success of the treatment could not be evaluated because the girl was not presented to the hospital for a control examination.

Suggested treatment combinations: Suggested treatment combinations for eradicating *H pylori* infections are: triple regimens for at least 7 days involving proton pump inhibitors (ranitidine bismuth citrate) + clarithromycin + either amoxicillin or metronidazole (37). Regimens that have been usually >80% efficacious in children have been: a two-week therapy with a nitroimidazole and amoxicillin; a two-week regimen of bismuth, amoxicillin and metronidazole; a one- to two-week regimen of a PPI, clarithromycin and

amoxicillin; and a two-week regimen of a PPI, clarithromycin and metronidazole, (38). In failure of eradication, quadruple regimen can be used in older children (e.g. proton pump inhibitors + bismuth subsalycilate/subcitrate+ metronidazole + tetracycline) with a success of 67-90%, (37).
Possible regimens for third-line therapy are the sequential regimens, (39). A 10-day sequential treatment in children has involved omeprazole+ amoxicillin for 5 days, followed by omeprazole+ clarithromycin+ tinidazole for the following 5 days. *H pylori* eradication has been detected in 97.3% versus 75.7% by triple therapy (omeprazole, amoxicillin, and metronidazole) for 1 week with a good compliance (>95%). Addition of probiotics to the eradication regimens seems appropriate. *In vitro* activity of lactobacilli against *H. pylori* has been detected, although *H. pylori* eradication by lactobacilli has been uncommon (40). At low and increased pH, *Lactobacillus delbrueckii* subsp. *bulgaricus* strains inhibited the growth of 38.9 to 88.9% and 27.8 to 66.7% of *H. pylori* strains, (41). Ingestion of *Lactobacillus casei* can decrease *H. pylori* density in gastric mucosa (42). Moreover, lactobacilli reduce both gastric inflammation and side effects of therapy for *H. pylori* eradication (43).

CONCLUSION

The resistance patterns in *H. pylori* are not constant. In the present study, an alarming triple resistance to amoxicillin, metronidazole and clarithromycin was found in two children. Recently, similar triple resistance has been reported in 6.8% of 44 Chinese children, (44). Although uncommon, the multi-drug resistance could hinder the *H. pylori* eradication in the affected patients and could slowly increase in the future. *H. pylori* eradication associated with multidrug resistant strains or eradication failures strongly require susceptibility testing of the isolates and should be handled on a case-by-case basis by specialists.

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REFERENCES

1. Megraud F, Lehours P. Helicobacter pylori detection and antimicrobial susceptibility testing. Clin Microbiol Rev 2007; 20: 280-322.
2. Mladenova I, Durazzo M, Pellicano R. Transmission of Helicobacter pylori: are there evidences for a fecal-oral route? Minerva Med 2006, 97: 15-18.
3. Mention K, Michaud L, Guimber D, Martin De Lasalle E, Vincent P, Turck D, Gottrand F. Characteristics and prevalence of Helicobacter heilmannii infection in children undergoing upper gastrointestinal endoscopy. J Pediatr Gastroenterol Nutr 1999; 29: 533-539.
4. Sinha SK, Martin B, Sargent M, McConnell JP, Bernstein CN. Age at acquisition of Helicobacter pylori in a paediatric Canadian First Nations population. Helicobacter 2002; 7:76-85.
5. Janulaityte-Gunther D, Kucinskiene R, Kupcinskas L, Pavilonis A, Labanauskas L, Cizauskas A, Schmidt U, Wadstrom T, Andersen LP.. The humoral immuneresponse to Helicobacter pylori infection in children with gastrointestinal symptoms. FEMS Immunol Med Microbiol 2005; 44: 205-212.
6. Boyanova L, Lazarova E, Jelev C, Gergova G, Mitov I. Helicobacter pylori and Helicobacter heilmannii in untreated Bulgarian children over a period of 10 years. J Med Microbiol. 2007; 56(Pt 8):1081-1085.
7. Megraud F. H pylori antibiotic resistance: prevalence, importance, and advances in testing. Gut 2004, 53:1374-1384.
8. Canton R, de Argila CM, de Rafael L, Baquero F (). Antimicrobial resistance in Helicobacter pylori. Rev Med Microbiol 2001; 12: 47-61.
9. Lopez-Brea M, Martinez MJ, Domingo D, Alarcon T. A 9 year study of clarithromycin and metronidazole resistance in Helicobacter pylori from Spanish children. J Antimicrob Chemother 2001; 48: 295-297.
10. Bontems P, Devaster JM, Corvaglia L, Dezsofi A, Van Den Borre C, Goutier S, Butzler JP, Cadranel S. Twelve year observation of primary and secondary antibiotic-resistant Helicobacter pylori strains in children. Pediatr Infect Dis J 2001;, 20: 1033-1038.
11. Gisbert JP, Calvet X, Bujanda L, Marcos S, Gisbert JL, Pajares JM. 'Rescue' therapy with rifabutin after multiple Helicobacter pylori treatment failures. Helicobacter 2003; 8: 90-94.
12. Peitz U, Sulliga M, Wolle K, Leodolter A, Von Arnim U, Kahl S, Stolte M, Borsch G, Labenz J, Malfertheiner P. High rate of post-therapeutic resistance after failure of macrolide-nitroimidazole triple therapy to cure Helicobacter pylori infection: impact of two second-line therapies in a randomized study. Aliment Pharmacol Ther 2002; 16: 315-324.
13. Heep M, Kist M, Strobel S, Beck D, Lehn N. Secondary resistance among 554 isolates of Helicobacter pylori after failure of therapy. Eur J Clin Microbiol Infect Dis 2000; 19: 538-541.
14. Koletzko S, Richy F, Bontems P, Crone J, Kalach N, Monteiro ML, Gottrand F, Celinska-Cedro D, Roma-Giannikou E, Oderda G, Kolacek S, Urruzuno P, Martinez Gomez MJ, Casswall T, Ashorn M, Bodanszky H, Megraud F. Prospective multicentre study on antibiotic resistance of Helicobacter pylori strains obtained from children living in Europe. Gut 55: 2006; 1711-1716.
15. Meyer JM, Silliman NP, Wang W, Siepman NY, Sugg JE, Morris D, Zhang J, Bhattacharyya H, King EC, Hopkins RJ. Risk factors for Helicobacter pylori resistance in the United States: the surveillance of H. pylori antimicrobial resistance partnership (SHARP) study, 1993-1999. Ann Intern Med 2002; 136:13-24.
16. Dore MP, Osato MS, Realdi G, Mura I, Graham DY, Sepulveda AR. Amoxycillin tolerance in Helicobacter pylori. J Antimicrob Chemother 1999; 43: 47-54.
17. Watanabe, K., Tanaka, A., Imase, K., Tokunaga, K., Sugano, H., Kai, A., Ishida, H., Itoh, T., Takahashi, S. Amoxicillin resistance in Helicobacter pylori: studies from Tokyo, Japan from 1985 to 2003. Helicobacter 2005;10, 4 - 11.
18. Megraud, F., Lehn, N., Lind, T., Bayerdurffer E, O'Morain C, Spiller R, Unge P, van Zanten SV, Wrangstadh M, Burman CF. Antimicrobial susceptibility testing of Helicobacter pylori in a large multicenter trial: the MACH 2 study. Antimicrob Agents Chemother 1999; 43: 2747-2752.
19. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. Tenth informational supplement. Approved standard M100-S10 (M7). 2000, NCCLS, Wayne, Pa.
20. McNulty C, Owen R, Tompkins D, Hawtin P, McColl K, Price A, Smith G, Teare L; PHLS Helicobacter Working Group. Helicobacter pylori susceptibility testing by disc diffusion. J Antimicrob Chemother 2002; 49: 601-609.
21. Chaves S, Gadanho M, Tenreiro R, Cabrita J. Assessment of metronidazole susceptibility in Helicobacter pylori: statistical validation and error rate analysis of breakpoints determined by the disk diffusion test. J Clin Microbiol 1999; 37: 1628-1631.
22. Grignon B, Tankovic J, Megraud F, Glupczynski Y, Husson MO, Conroy MC, Emond JP, Loulergue J, Raymond J, Fauchere JL. Validation of diffusion methods for macrolide susceptibility testing of Helicobacter pylori. Microb Drug Resist 2002; 8: 61-66
23. Koletzko, S., Konstantopoulos, N., Bosman, D., Feydt-Schmidt, A., van der Ende, A., Kalach, N., Raymond, J. & Rьssmann, H.. Evaluation of a novel monoclonal enzyme immunoassay for detection of Helicobacter pylori antigen in stool from children. Gut 2003; 52, 804 806.
24. Wong, K.K., Chung, P.H., Lan, L.C., Lin, S.C. & Tam, P.K. Trends in the prevalence of Helicobacter pylori in symptomatic children in the era of eradication. J Pediatr Surg 2005; 40, 1844-1887.
25. Sykora, J., Hejda, V., Varvarovska, J., Stozicky, F., Siala, K. & Schwarz, J. Helicobacter heilmannii gastroduodenal disease and clinical aspects in children with dyspeptic symptoms. Acta Paediatr 2004; 93, 707-709.
26. De Korwin JD. Advantages and limitations of diagnostic methods for H. pylori infection. Gastroenterol Clin Biol 2003; 27(3 Pt 2): 380-390.
27. Rothenbacher D, Inceoglu J, Bode G, Brenner H. Acquisition of Helicobacter pylori infection in a high-risk population occurs within the first 2 years of life. J Pediatr 2000; 136:744-748.
28. Duck WM, Sobel J, Pruckler JM, Song Q, Swerdlow D, Friedman C, Sulka A, Swaminathan B, Taylor T, Hoekstra M, Griffin P, Smoot D, Peek R, Metz DC, Bloom PB, Goldschmidt S, Parsonnet J, Triadafilopoulos G, Perez-Perez GI, Vakil N, Ernst P, Czinn S, Dunne D, Gold BD. Antimicrobial resistance incidence and risk factors among Helicobacter pylori-infected persons, United States. Emerg Infect Dis 2004; 10: 1088-1094.
29. Moreira ED Jr, Santos RS, Nassri VB, Reis AT, Guerra AL, Alcantara AP, Matos JF, Carvalho WA, Moura CG, Silvani CS, Sant'Ana CS. Risk factors for Helicobacter pylori infection in children: is education a main determinant? Epidemiol Infect 2004; 132: 327-335.
30. Kwon DH, Lee M, Kim JJ, Kim JG, El-Zaatari FA, Osato MS, Graham DY. Furazolidone- and -resistant Helicobacter pylori: Prevalence and role of genes involved in metronidazole resistance. Antimicrob Agents Chemother 2001; 45: 306-308.
31. Toracchio S, Marzio L. Primary and secondary antibiotic resistance of Helicobacter pylori strains isolated in central Italy during the years 1998-2002. Dig Liver Dis 2003;35: 541-545.
32. Hu CT, Wu CC, Lin CY, Cheng CC, Su SC, Tseng YH, Lin NT. Resistance rate to antibiotics of Helicobacter pylori isolates in eastern Taiwan. J Gastroenterol Hepatol 2007; 22: 720-723.
33. Kim N, Kim JM, Kim CH, Park YS, Lee DH, Kim JS, Jung HC, Song IS. Institutional difference of antibiotic resistance of Helicobacter pylori strains in Korea. J Clin Gastroenterol 2006; 40: 683-687.
34. Domingo D, Alarcyn T, Vega AE et al. Microbiological factors that influence the eradication of Helicobacter pylori in adults and children. Enferm Infecc Microbiol Clin. 2002; 20 (9):431-434
35. Bazzoli F, Berretti D, De Luca L, Nicolini G, Pozzato P, Fossi S, Zagari M. What can be learnt from the new data about antibiotic resistance? Are there any practical clinical consequences of Helicobacter pylori antibiotic resistance? Eur J Gastroenterol Hepatol 1999; 11 (Suppl 2): S39-S42.
36. Mc Loughlin, R, Racz I, Buckley M, O'Connor HJ, O'Morain C. Therapy of Helicobacter pylori. Helicobacter 2004; 9: 42-48.
37. Malfertheiner P, Megraud F, O' Morain C, Hungin APS, Jones R, Axon A, Graham DY, Tytgat G& The European Helicobacter pylori Study Group (EHPSG). Current concepts in the management of Helicobacter pylori infection-The Maastricht 2-2000 Consensus Report. Aliment Pharmacol Ther; 2002; 16: 167-180.

38. Khurana R, Fischbach L, Chiba N, Veldhuyzen van Zanten S. An update on anti-Helicobacter pylori treatment in children. Can J Gastroenterol; 2005; 19: 441-445.
39. Francavilla R, Lionetti E, Castellaneta SP, Magista AM, Boscarelli G, Piscitelli D, Amoruso A, Di Leo A, Miniello VL, Francavilla A, Cavallo L, Ierardi E. Improved efficacy of 10-Day sequential treatment for Helicobacter pylori eradication in children: a randomized trial. Gastroenterology; 2005; 129: 1414-1419.
40. Hamilton-Miller JM. The role of probiotics in the treatment and prevention of Helicobacter pylori infection. Int J Antimicrob Agents 2003; 22: 360-366.
41. Boyanova L, Stephanova-Kondratenko M, Gergova G, Mitov I. Activity of Lactobacillus delbrueckii subsp. bulgaricus against a widespread and long-lasting infection. First part: Alimentary industry 2005; LIV (№ 10): 18-20; and LIV (№ 11): 21-23.
42. Sgouras D, Maragkoudakis P, Petraki K, Martinez-Gonzalez B, Eriotou E, Michopoulos S, Kalantzopoulos G, Tsakalidou E, Mentis A. In vitro and in vivo inhibition of Helicobacter pylori by Lactobacillus casei strain Shirota. Appl Environ Microbiol 2004, 70: 518-526.
43. Armuzzi A, Cremonini F, Bartolozzi F, Canducci F, Candelli M, Ojetti V, Cammarota G, Anti M, De Lorenzo A, Pola P, Gasbarrini G, Gasbarrini A. The effect of oral administration of Lactobacillus GG on antibiotic-associated gastrointestinal side-effects during Helicobacter pylori eradication therapy. Aliment Pharmacol Ther 2001;15: 163-169.
44. Chen J, Chen FB, Yu JD, Chen XJ, Li ZY, Zhang XP. Prevalence of Helicobacter pylori resistant to clarithromycin, amoxicillin and metronidazole in children. Zhonghua Er Ke Za Zhi 2004; 42: 769-771.

PREVALENCE OF THE FLUOROQUINOLONE-MODIFYING ACETYLTRANSFERASE GENE AAC(6')-IB-CR AMONG EXTENDED-SPECTRUM BETA-LACTAMASE-PRODUCING ENTEROBACTERIAL ISOLATES IN A CANCER HOSPITAL IN BULGARIA

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SUMMARY

Objectives: To determine the prevalence of the fluoroquinolone-modifying aminoglycoside acetyltransferase-encoding gene *aac(6')-Ib-cr* among enterobacterial isolates carrying extended-spectrum beta-lactamases (ESBLs). Methods: One hundred and sixty three non-duplicate, clinically relevant ESBL-producing enterobacterial isolates collected between 2000 and 2005 were screened for *aac(6')-Ib* by PCR. *aac(6')-Ib-cr* was identified by sequencing. PCR amplification and DNA sequencing identified beta-lactamase genes in all *aac(6')-Ib-cr*-positive isolates. Pulsed-field gel electrophoresis (PFGE) was used to investigate the clonality of *aac(6')-Ib-cr*-carrying isolates. Results: Ninety-nine of 163 ESBL-producing isolates (60.7%) were positive for *aac(6')-Ib* including *Escherichia coli* (65/94), *Klebsiella pneumoniae* (21/40), *Citrobacter freundii* (7/10), *Enterobacter cloacae* (1/7), *Klebsiella oxytoca* (1/2) and single isolates of *Enterobacter aerogenes*, *Morganella morganii*, *Providencia rettgeri* and *Escherichia hermannii*. The *aac(6')-Ib-cr* variant was identified in 52 (31.9% of all) isolates including 47 *E. coli*, 2 *C. freundii*, and 1 each of *K. pneumoniae*, *E. aerogenes* and *M. morganii*. Among the *aac(6')-Ib-cr*-positive isolates, 32 had $bla_{CTX-M-15}$, bla_{OXA-1} and bla_{TEM-1}, 16 had $bla_{CTX-M-15}$ and bla_{OXA-1}, and 2 had $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{SHV-12} and bla_{TEM-1}. The remaining 2 isolates had bla_{TEM-1} and ESBL of none of the studied types. PFGE of 47 *E. coli* digests displayed 30 different patterns, suggesting the limited importance of clonal spread of *aac(6')-Ib-cr* gene. Conclusions: The *aac(6')-Ib-cr* variant was present in various species in the family *Enterobacteriaceae* and highly prevalent in *E. coli* isolates (50%). The majority of *aac(6')-Ib-cr*-carrying isolates co-produced CTX-M-15 and OXA-1 beta-lactamases. The spread of multiresistant isolates expressing the aminoglycoside- and fluoroquinolone-modifying enzyme AAC(6')-Ib-cr together with ESBLs is a worrisome development requiring continuous monitoring.

Keywords: quinolones, resistance, enterobacteria, ESBLs

Quinolone resistance is traditionally associated with multiple chromosomal mutations in the genes encoding target enzymes, efflux pumps or porin channels (1). In addition, *qnr* genes responsible for plasmid-mediated quinolone resistance (PMQR) have been found in clinical *Enterobacteriaceae* (2). In 2006, a new mechanism of transferable resistance to ciprofloxacin and norfloxacin has been described: enzymatic inactivation by a variant of the common aminoglycoside acetyltransferase AAC(6')-Ib named AAC(6')-Ib-cr for ciprofloxacin resistance (3). The *cr* variant enzyme reduced modestly the activity of ciprofloxacin by *N*-acetylation of its piperazinyl amine but the presence of the encoding gene, *aac(6')-Ib-cr*, increased substantially the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin (3). Since *aac(6')-Ib-cr* has often been reported to be associated with extended-spectrum beta-lactamase (ESBL) genes (4, 5), the aim of this study was to determine the prevalence of *aac(6')-Ib-cr* variant among ESBL-producing enterobacterial isolates in a Bulgarian hospital.

ABBREVIATIONS USED IN THIS PAPER:
ESBL - extended-spectrum beta-lactamase; PFGE - pulsed-field gel electrophoresis; PMQR - plasmid-mediated quinolone resistance; CLSI - Clinical and Laboratory Standards Institute; PCR - polymerase chain reaction.

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MATERIALS AND METHODS

A total of 163 non-repetitive clinically relevant ESBL-producing enterobacterial isolates were collected at a 252-bed oncology hospital in Sofia between 2000 and 2005. These included 94 *Escherichia coli*, 40 *Klebsiella pneumoniae*, 10 *Citrobacter freundii*, 7 *Enterobacter cloacae*, 6 *Serratia marcescens*, 2 *Klebsiella oxytoca*, and 1 each of *Enterobacter aerogenes*, *Morganella morganii*, *Providencia rettgeri* and *Escherichia hermannii*. Isolates were identified to the species level with Vitek 2 (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO).

Susceptibility to antimicrobials was determined by disc diffusion, following the Clinical and Laboratory Standards Institute (CLSI) recommendations. Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and at least one of the following antibiotics: cefotaxime, ceftazidime, aztreonam or cefepime, and confirmed by the Etest ESBL strip (AB Biodisk, Solna, Sweden). MIC of ciprofloxacin was determined using Etest (AB Biodisk). Susceptibility interpretations were defined according to CLSI-2007 breakpoints. *E. coli* ATCC 25922 was used as an antibiotic-susceptible control.

PCR screening for *aac(6')-Ib* gene was performed on boiled cell lysate of clinical isolates with the Expand High Fidelity PCR system (Roche Diagnostics, Penzberg, Germany) using primers 5'-ACTGAGCATGACCTTGCGATGC-3' and 5'-TTAGGCATCACTGCGTGTTCG-3' to give a 516-bp product. PCR conditions were 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s for 30 cycles. All positive results were further analysed by direct sequencing of amplicons to identified *aac(6')-Ib-cr*. ESBLs of all *aac(6')-Ib-cr*-positive isolates were identified by PCR amplification and DNA sequencing using primers specific for bla_{PER-1}, bla_{VEB-1}, bla_{GES-1}, bla_{OXA-1}, bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes, as previously described (6, 7). Amplification of DNA was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Amplicons were purified with a QIAquick PCR purification kit (QIAGEN, K.K., Tokyo, Japan) and sequenced with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence analyses and comparison with known sequences were performed with the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

To assess the genetic relatedness of *aac(6')-Ib-cr*-carrying isolates, genomic DNA was isolated and digested with *Xba*I (Bio-Rad, Hercules, CA). Pulsed-field gel electrophoresis (PFGE) was performed with the CHEF Mapper System (Bio-Rad) with the following run parameters: pulses ranging from 5.3 to 49.9 s at 6 V/cm for 20.0 h. A 48.5-kb lambda ladder (Bio-Rad) was used as the DNA size marker. Isolates were considered to be genetically related if their DNA macrorestriction patterns differed by fewer than seven bands (8).

RESULTS AND DISCUSSION

Ninety-nine out of the 163 ESBL-producing isolates studied (60.7%) were positive for *aac(6')-Ib* gene. These included *E. coli* (65/94), *K. pneumoniae* (21/40), *C. freundii* (7/10), *E. cloacae* (1/7), *K. oxytoca* (1/2) and single isolates of *E. aerogenes*, *M. morganii*, *P. rettgeri* and *E. hermannii*. The *aac(6')-Ib-cr* variant was identified in 52 (31.9% of all) isolates including 47 *E. coli*, 2 *C. freundii*, and 1 each of *K. pneumoniae*, *E. aerogenes* and *M. morganii* (Table 1).

Table 1. Distribution of *aac(6')-Ib-cr* gene among ESBL-producing *Enterobacteriaceae* isolates

Species	Total isolates n	No. of isolates (%) with: *aac(6')-Ib-cr* gene		Any *aac(6')-Ib* gene	
E. coli	94	47	(50.0)	65	(69.1)
K. pneumoniae	40	1	(2.5)	21	(52.5)
C. freundii	10	2	(20.0)	7	(70.0)
E. aerogenes	1	1		1	
M. morganii	1	1		1	
Others*	17	0		4	(23.5)
Total	163	52	(31.9)	99	(60.7)

* *E. cloacae* (n = 7), *S. marcescens* (n = 6), *K. oxytoca* (n = 2), *P. rettgeri* (n =1), *E. hermannii* (n = 1)

Forty-three of the 52 *aac(6')-Ib-cr*-positive isolates (82.7%) were ciprofloxacin-resistant, with MICs ranging from 12 to >32 mg/L, and nine were susceptible to ciprofloxacin (MICs, 0.047 to 1 mg/L) according to the CLSI. In addition, 78.8% were resistant to gentamicin and 48% to trimethoprim-sulfamethoxazole, respectively.

Among the 52 *aac(6')-Ib-cr*-carrying isolates, 50 possessed both $bla_{CTX-M-15}$ and bla_{OXA-1}. Of these, 2 isolates also carried bla_{SHV-12} and 32 - bla_{TEM-1}. The remaining two isolates had bla_{TEM-1} and ESBL of none of the studied types. (Table 2).

Table 2. Distribution of the *aac(6')-Ib-cr* gene according to beta-lactamases produced by *Enterobacteriaceae* isolates

Species (n)	No. of *aac(6')-Ib-cr*-positive isolates producing: CTX-M-15, OXA-1 TEM-1	CTX-M-15, OXA-1	CTX-M-15, OXA-1, TEM-1 SHV-12	unknown ESBL*
E. coli (47)	30	13	2	2
C. freundii (2)	1	1		
K. pneumoniae (1)	1			
E. aerogenes (1)		1		
M. morganii (1)		1		
Total (52)	32	16	2	2

* unknown ESBL=not of the TEM, SHV, CTX-M, VEB, PER or GES type

The molecular characterization of *aac(6')-Ib-cr*-positive *E. coli* isolates by PFGE showed great genomic diversity among them. The 47 isolates gave 30 major patterns showing that most of these strains were not clonal (Figure).

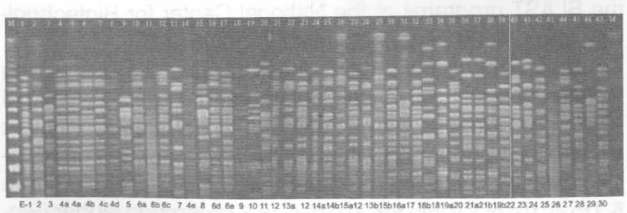

Fig. 1. Pulsed-field electrophoresis gel of *Xba*I-digested genomic DNA for 47 *aac(6')-Ib-cr*-positive *E. coli* isolates. Numbers designating PFGE patterns are shown below the gel. Lanes M, 48.5 kb lambda ladder; lines 1 to 47, *E. coli* isolates

AAC(6')-Ib-cr, a novel PMQR protein, was first described in 2006, but is now recognized to be widely disseminated. This variant of the aminoglycoside acetyltransferase gene *aac(6')-Ib* confers resistance to aminoglycosides (kanamycin, tobramycin, amikacin) and low-level resistance to certain quinolones (ciprofloxacin, norfloxacin). Furthermore, the *aac(6')-Ib-cr* carriage could facilitate the selection of higher level chromosomal resistance mutations when quinolones are used (3). In this work, most of the isolates carrying *aac(6')-Ib-cr* variant were resistant to ciprofloxacin, probably reflecting its ability to promote higher level quinolone resistance mutations.

The present study demonstrated a high prevalence of the *aac(6')-Ib* gene (61%) and its *cr* variant (32%) among 163 ESBL-producing clinical *Enterobacteriaceae*, being highest in *E. coli* (50%). The reported prevalence of *aac(6')-Ib-cr* varies from 14% in ceftazidime-resistant *Enterobacteriaceae* in USA, being highest in *E. coli* (32%), to 51% among multidrug-resistant *E. coli* isolates from Shanghai, China (3, 9). The prevalence of *aac(6')-Ib-cr*, found in this work, was relatively high and similar to that reported from Shanghai.

Of particular interest was that the majority of *aac(6')-Ib-cr*-carrying isolates co-produced CTX-M-15 extended-spectrum brta-lactamase. *E. coli* producing CTX-M-15 associated with *aac(6')-Ib-cr* have been described in several reports from different continents (3-5). It seems that the pandemic spread of CTX-M-15 plasmids and strains is partly responsible for the increase in *aac(6')-Ib-cr*.

CONCLUSIONS

Our study showed that ESBL-producing isolates with *aac(6')-Ib-cr* were present in various species in the family *Enterobacteriaceae* and highly prevalent among *E. coli* isolates in that collection from Bulgaria. The majority of *aac(6')-Ib-cr*-carrying isolates co-produced CTX-M-15 and OXA-1 beta-lactamases. The spread of multiresistant isolates expressing the aminoglycoside- and fluoroquinolone-modifying enzyme AAC(6')-Ib-cr together with ESBLs is a worrisome development requiring continuous monitoring.

ACKNOWLEDGEMENTS: *The authors express their sincere gratitude to the Japan Society for the Promotion of Science for their Grant-in-Aid for Scientific Research, No. 17-05764, to carry out this work.*

REFERENCES

1. Jacoby, G. A. 2005. Mechanisms of resistance to quinolones. Clin. Infect. Dis. 4:S120-126.
2. Martinez-Martinez, L., A. Pascual, and G. A. Jacoby. 1998. Quinolone resistance from a transferable plasmid. Lancet 351:797-799.
3. Robicsek A., J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, K. Bush, and D. C. Hooper. 2006. Fluoroquinolone modifying enzyme: a novel adaptation of a common aminoglycoside acetyltransferase. Nat. Med. 12:83-88.
4. Karisik, E., M.J Ellington, R. Pike, R.E. Warren, D.M. Livermore, and N. Woodford. 2006. Molecular characterization of plasmids encoding CTX-M-15 b-lactamases from *Escherichia coli* strains in the United Kingdom. J. Antimicrob. Chemother. 58:665-668.
5. Machado, E., T.M. Coque, R. Cantyn, F .Baquero, J.C. Sousa, L. Peixe, and Portuguese Resistance Study Group. 2006. Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing *Enterobacteriaceae* strains containing the *aac(6')-Ib-cr* gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme. Antimicrob. Agents Chemother. 50:3220-3221.
6. Lee, S., Y. Park, M. Kim, H. K. Lee, K. Han, C. S. Kang and M. W. Kang. 2005. Prevalence of Ambler class A and D b-lactamases among clinical isolates of *Pseudomonas aeruginosa* in Korea. J. Antimicrob. Chemother. 56:122-127.
7. Saladin, M., V. T. Cao, T. Lambert, J. L. Donay, J. L. Herrmann, Z. Ould-Hocine, C. Verdet, F. Delisle, A. Philippon and G. Arlet. 2002. Diversity of CTX-M b-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiol. Lett. 209:161-168.
8. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33:2233-2239.
9. Park C. H., A. Robicsek, G. A. Jacoby, D. Sahm and D. C. Hooper. 2006. Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. 50:3953-3955.

ACANTHAMOEBA CULBERTSONI PROMOTES GROWTH AND SURVIVAL OF HELICOBACTER PYLORI

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ABSTRACT

On the basis of free-living *Acanthamoeba culbertsoni* strain investigation was made of the invasion, growth, and survival of reference strain *H. pylori* CCUG 17874 and 3 clinical isolates of *H. pylori*. The interaction of amoebae and bacteria has been studied by cultivating them in proteose yeast glucose (PYG) medium and proteose peptone horse serum (PPHS} medium in microaerophilic conditions and aerobic atmosphere. The survival of *H. pylori* in amoebae has been demonstrated by routine methods of its cultivation. The growth of *H. pylori* and its survival in media containing amoebae was found to be considerably longer for a period of 14 days (period of observation) when compared with the bacterial cultures which did not contain amoeba cells. The latter did not survive even an overnight incubation. By immunofluorescent staining of samples from mixed cultures using *H. pylori* antisera it was shown that on the fourth day amoeba cells contained vacuoles filled up with fluorescein stained bacteria and the bacterial number rose up to 8 -12 day. In conclusion: The presence of *H. pylori* in amoeba cells could be interpreted as a mechanism of survival in the environment.

Key words: Acanthamoeba culbertsoni, H. pylori, survival

INTRODUCTION

A growing body of evidence in the literature has documented the intracellular survival of bacteria in free-living protozoans (8,9,11). Bacterial pathogens that multiply and survive in amoebae include: *Legionella* spp., mycobacteria, *Vibrio cholerae, E. coli* 0157, *Helicobacter* spp, *Campylobacter* (1, 2, 3, 6, 12, 13).

Helicobacter pylori infection is one of the most common bacterial infections worldwide. It colonizes about 60% of the world population. *H. pylori* causes chronic superficial gastritis in humans, and infection of this organism predisposes the host to the development of peptic ulcer disease and gastric adenocarcinoma (4,7). The mode of transmission of infection is not entirely understood. The reservoir of *H. pylori* is the digestive tracts of humans and some primates. There is some evidence for faecal-oral transmission because *H. pylori* is shed in the faeces after turnover of the gastric mucosa, and has been detected in sewage and water (5,10). Thus the survival of *H. pylori* suggests the involvement of environmental factors.

The objective of our study was to follow up the interaction of *H. pylori* with free-living amoeba with a view to elucidate the mechanism of its survival in the environment.

ABBREVIATIONS USED IN THIS PAPER: NONE

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MATERIALS AND METHODS

AMOEBA. *Acanthamoeba culbertsoni* strain A-I was used. It was treated with antibiotics to become bacteria free culture. The pure amoeba cultures were cultivated in 2% proteose peptone medium with 5% horse serum.

HELICOBACTER PYLORI. Clinical strains of *H. pylori* 315, 347 and 352 isolated in Military Medical Academy, Sofia, and the reference strain *H. pylori* CCUG 17874 were used for co-cultivation with *A. culbertsoni.*

CO-CULTIVATION of amoeba with *H. pylori* was done by cultivating them in proteose yeast glucose (PYG) medium and proteose peptone horse serum (PPHS) medium. 96-h amoeba cultures (10^4 cells/ml densities) were mixed with 72-h cultures of *H. pylori* (10^7 bacterial cells/ml). PYG and PPHS mixed cultures were incubated in microaerophilic conditions (Gas Pak, Campy Pak, BBL) and aerobic atmosphere at 37°C for 14 days (period of observation). Minimal medium not containing amoeba inoculated with *H. pylori* strains served as a control of its survival.

ASSESSEMENT OF BACTERIAL SURVIVAL. The interaction of amoeba and helicobacter, and survival of bacteria were assessed by plating the mixed cultures onto Brucella agar (Difco) supplemented with 7% defibrinated sheep blood (incubation at least 4 days in microaerophilic conditions at 37°C), and by direct immunofluorescence assay.

The samples from mixed cultures were taken at 0 day (immediately after mixing amoebae and helicobacters), and at 1-st, 2-nd, 4-th, 8-th, 12-th, and 14-th days.

Identification of *H. pylori* was done on the basis of its typical colony appearance, the typical morphology of organisms by Gram staining and positive tests for catalase, oxidase and urease.

Immunofluorescent staining. 0.5% formalin was added to suspensions (amoeba/bacteria) for 24 h at 37°C followed by centrifugation and then the sediments were used for preparations. The slides were fixed on flame and stained with fluorescent anti-helicobacter rabbit serum by routine technique. Fluorescence microscope MLD-2 was used for observation of microscopic preparations.

RESULTS

We have found growth of all strains of *H. pylori* on Brucella agar from both experimental and control settings from platings immediately after mixing the two organisms. After 24h incubation no growth of *H. pylori* have been found from the control tubes, and single colonies of *H. pylori* were observed from the experimental tubes PPHS medium, whereas grouth from the PYG medium was seen after 4-th day.

Platings on 2-nd and 4-th day of incubation were negative for *H. pylori* from both experimental and control tubes. At 8-12-th day of incubation of mixed cultures (amoeba and

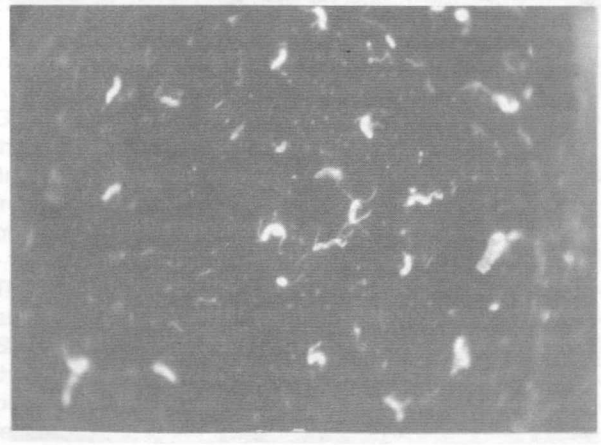

Fig. 1. Non infected amoebae and *H. pylori*

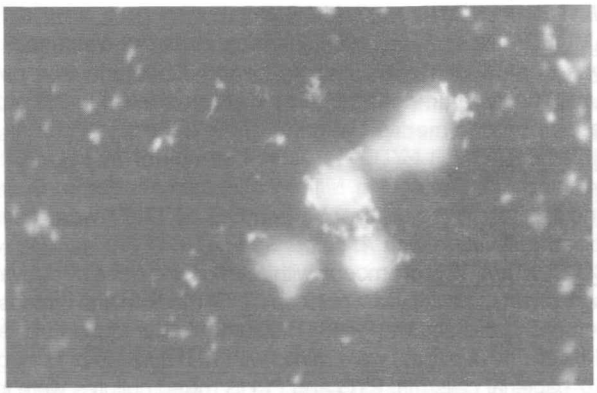

Fig. 2. Adherence of *H. pylori* on the surface of *A. culbertsoni*

helicobacters) there was a heavy growth *of H. pylori* on plates of Brucelfa agar, and no growth from control tubes. We have found that co-cultivation of amoebae and helicobacters in PPHS medium resulted in more heavy growth of *H. pylori* strains. By the immunofluorescent staining after 1-2 days co-cultivation we have observed a lot of bacteria that were out of amoe-

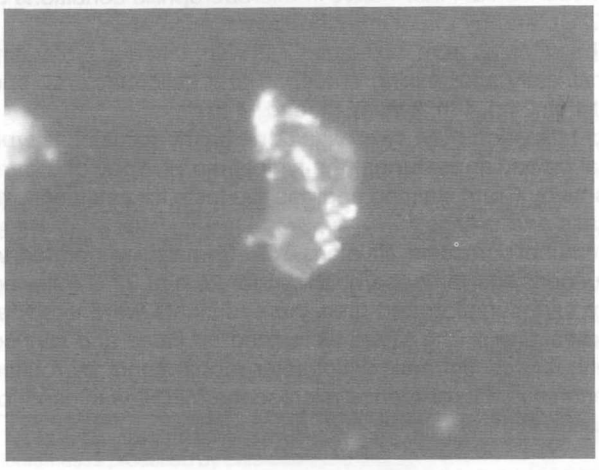

Fig. 3. Invasion of *H. pylori* in amoeba cell

bae (Fig. 1). At 2-4 days of co-cultivation adherence of bacteria onto amoeba surfaces was demonstrated (Fig. 2). At 8-th day we have observed a invasion of helicobacters in amoeba cells (Fig. 3) manifested by single fluorescent vacuoles whose numbers has increased at 12-14-th day (Fig. 4). In this period destructed amoeba cells have also been observed (Fig. 5).

Fig. 4. Fluorescent vacuoles filled up with *H. pylori*

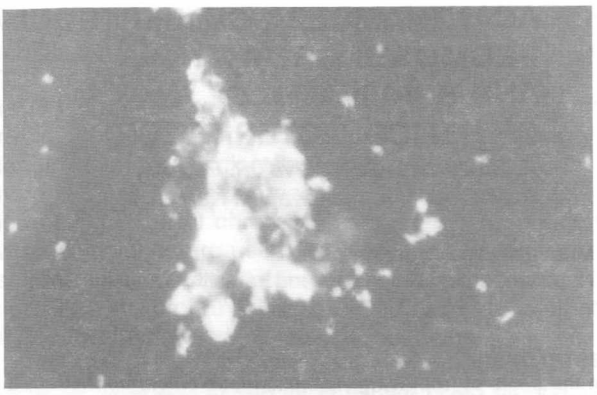

Fig. 5. Destructed amoeba cells

DISCUSSION

The results obtained from us show that the interaction of *H. pylori* with amoeba is a dynamic process in which the initial phase (2-4-th day) is characterized by decreasing number of bacteria to theirs full disappearance or probably transition to non-culturable forms. From 8-th to 12-th day in presence of amoeba we have observed reappearance of *H. pylori* and its growth on Brucella agar.

The data from immunofluorescent assay show that *H. pylori* adhere to amoeba surfaces followed by phagocytosis and presumably bacteria develop in phagosomes and cause destruction of amoeba cells in some cases. More details in this respect could be obtained by electron microscopy studies.

Our results show that *H. pylori* survived in *A. culbertsoni* for 14 days (period of observation). It is well known that some bacterial pathogens multiply and survive in amoebae (1,2,3,6,12,13). In the literature reviewed we have found but one report concerning survival of *H. pylori* in free-living amoebae (13). In this article we report successful co-cultivation of *H. pylori* with *A. culbertsoni.* Our results confirm survival of *H. pylori* within *Acanthamoeba* spp.

In conclusion the ability of *H. pylori* to remain viable in the presence of free-living amoebae could be interpreted as a mechanism of survival in the environment. If this is true it may turn out that free-living amoebae are potential vectors for the spread of *H. pylori.*

REFERENCES

1. Adekambi, T., S.B. Salah, M. Khlif, D. Raoult, M. Drancourt. 2006. Survival of environmental *Mycobacteria* in *Acanthamoeba polyphaga.* Appl Environ. Microbiol. 72:5974-5981.
2. Axelsson-Olsson, D., J. Waldenstrom, T. Broman, B. Olsen, M. Homberg. 2005. Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni.* Appl. Environ. Microbiol. 71:987-992.
3. Barker, J., T..T. Humphrey, M.W.R. Brown. 1999. Survival of *Escherichia coli* O157 in a soil protozoan: implications for disease. FEMS Microbiol. Lett. 173:291-295.
4. Blaser, MJ. 1996. The bacteria behind ulcers. Sci. Am. 274:92-97.
5. Bunn, J.E., W.G. Mackay, J.E. Thomas, D.C. Reid, L.T. Weaver. 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. Lett. Appl. Microbiol. 34:450- 454.
6. Cirillo, J.D.,S.L. Cirillo, L. Yan, L.E. Bermudez, S. Falkow, L.S Tompkins. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila.* Infect. Immun. 4427-4434.
7. Cover, T.L., M.J. Blaser. 1996. *Helicobacter pylori* infection, a paradigm for chronic mucosal inflammation: pathogenesis and implications for eradication and prevention. Adv. Intern. Med. 41:85-117.
8. Greub, G., D. Raoult. 2004. Microorganisms resistant to free living amoebae. Clin. Microbiol. Rev. 17:413-433.
9. Harf, C. 1993. Free living amoebae: interactions with enviromental pathogenic bateria. Endocytobiosis Cell Res. 10:167-183.
10. Lu, Y., T.E. Redlinger, R. Avitia, A. Galindo, K. Goodman. 2002. Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. Appl. Environ. Microbiol. 68:1436-1439.
11. Snelling, W.J., J.E. Moore, J.P. McKenna, D.M. Lecky, J.S. Dooley. 2006. Bacterial-protozoa interactions: an update on the role these phenomena play towards human illness. Microbes Infect. 8:578-587.
12. Thorn, S., D. Warhurst, B.S. Drasar. 1992. Association of *Vibrio cholerae* with fresh water amoebae. J. Med. Microbiol. 36:303-306.
13. Winiecka-Krusnell, J., K. Wreiber, A. von Euler, L. Engstrand, E. Linder. 2002. Free-living amoebae promote growth and survival of *Helicobacter pylori.* Scand. J. Infect.Dis. 34:253-256.

CLONING AND EXPRESSION OF RECOMBINANT BORRELIA BURGDORFERI PROTEINS

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ABSTRACT

Development of ELISA tests with recombinant antigens is a new stage in improving serological diagnosis of Lyme brreliosis, especially in terms of its specificity. Major *B. burgdorferi* proteins that induce immune response OspA and OspC (outer surface proteins) were cloned. *OspA* and *ospC* genes were amplified from genomic DNA of *B. burgdorferi* sensu stricto strain B31 using specific, especially designated for the purpose, primers. Each gene was integrated in the plasmid expression vector pGEX-2T. DNA constructs were used to transform *E. coli* strain DH5 6. Specificity of the obtained proteins was confirmed by specific anti-OspA and anti-OspC monoclonal antibodies in immunoblot.

Key words: Borrelia burgdorferi, Lyme borreliosis, recombinant proteins

INTRODUCTION

Lyme borreliosis is the most common tick-borne infection is USA and Europe. Since clinical manifestations are not specific, Lyme borreliosis is included in differential diagnosis of various other disorders. The only exception is erythema migrans, which is typical for early Lyme disease. Serological confirmation is required for all other clinical presentations of the disease.

Reliable diagnosis is essential to ensure adequate treatment of patients with Lyme borreliosis as well as to exclude this diagnosis in patients with other illnesses. Currently, routine diagnosis of Lyme borreliosis is serological and relies on immunoenzyme (ELISA) and immunoblot tests. First generation ELISA tests, based on whole-cell antigens, show high-rate false positive reactions because of cross-reacting antigens (1,2). Second generation ELISA tests, based on purified or recombinant *B. burgdorferi* antigens, are now introduced. However, still diagnostics of Lyme borreliosis is far from being perfect.

Investigations on some highly specific *B. burgdorferi* antigens gave encouraging results (1,5-11). Unfortunately, immune responses in patients, infected with *B. burgdorferi*, vary significantly and even some key antigens are not always expressed and detected immunologically (1). This causes problems for serological testing by ELISA tests with recombinant antigens. The question now is which antigens in what combination will ensure the best diagnosis of Lyme borreliosis.

OspC is among the major *B. burgdorferi* antigens that elicit early immune response against all three Borrelia species (*B. burgdorferi* sensu strico, *B. garinii* and *B. afzelii*) (5, 6, 8, 9, 12, 13). OspC is responsible for dissemination of borreliae in the tick and establishment of the infection in patients. Boreliae without OspC are not able to infect mice (14). OspC is a virulence factor for *B. burgdorferi*.

ABBREVIATIONS USED IN THIS PAPER: None

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OspC is very polymorphic - 21 major groups have been identified according to its sequence homology. It is assumed, that selective pressure of the immune system is directly responsible for the OspC polymorphism (17,18). Interestingly, only a few OspC groups have been detected in disseminated Lyme borreliosis (A, B, C, D, I, K, N). Over 80% of these infections are due to the OspC groups A and K.

OspA is also a major *B. burgdorferi* antigen but, in contrast to OspC, it is expressed during late Lyme borreliosis. When OspC is expressed, OspA is repressed (early stage) and when OspA is expressed, OspC is repressed (late stage). Amount of antibodies to OspA directly correlates with severity and duration of Lyme arthritis - as higher is antibody level as more severe and with longer duration will be the arthritis (1).

It is clear, that each of *B. burgdorferi* antigens posses specific advantages and disadvantages. That is why, neither one could be used solely for serological diagnosis. Opportunities for improvement of diagnostic specificity and sensitivity are hidden in proper combination of recombinant protein antigens.

Our goal in this study was cloning, expression, purification and proving of major immunodominant *B. burgdorferi* proteins - recombinant OspC and OspA.

MATERIALS AND METHODS

Genome DNA isolation from *Borrelia burgdorferi*

Genome DNA from *B. burgdorferi* was isolated by phenol extraction using a standard procedure (19) or with a DNA extraction kit (Genomic Prep Cells and Tissue DNA Isolation kit; Amersham Biosciences).

Polymerase Chain Reaction (PCR) and *E. coli* DH56 transformation

Amplification of *ospA* gene from *Borrelia burgdorferi* was carried out using primers designed in accordance with the sequence published under the accession number X85739 in the NCBI nucleotide database and were as follows *FospA* ATGAAAAAATATTTATTGGGA and *RospA* TTATTTTAAAGCGTTTTTAATT. The reaction mix contained 20ng genome DNA, 200nM of the forward and reverse primers, 200мM of the four deoxynucleotides, 1.8mM $MgCl_2$ and 1.25 U *Pfu* DNA polymerase (Fermentas).

The amplification program used was as follows: initial DNA denaturation at 95°C for 10 min, 30 cycles of denaturation at 95°C for 1.5 min, primer annealing at 61°C for 2.5 min. and product elongation at 72°C for 2 min as well as final elongation at 72°C for 10 min. The PCR reactions were carried out on Techne Thermal Cycler PHC-1A. The presence of the amplification product was confirmed by analytical electrophoresis on a 0.8% agarose gel run in 0.5x TBE buffer.

Amplification of *ospC* gene from *Borrelia burgdorferi* was carried out using primers designed in accordance with the sequence published under the accession number AF500203 in the NCBI nucleotide database and were as follows *FospC* ATGAAAAAGAATACATTAAGTG and *RospC* AATAATATTGATTTTAATTAAGGG. The reaction mix was identical with those used for *ospA* amplification but instead of 1.8mM $MgCl_2$ 4mM were used. The amplification program used differed from those described for *ospA* only in terms of temperature of primer annealing - 48°C and time for product elongation - 1 min in each cycle.

The obtained by PCR DNA fragment was subjected to 5' and 3' end digestion with the restriction endonucleases *BamH*I and *EcoR*I (10U/мl; Fermentas) and was cloned in the plasmid vector pGEX-2T (Amersham Biosciences). The pGEX-2T vector contains a gene for Glutathione-S-transferase (*GST*) upstream of its multiple cloning site. Successful ligations have led to recombinant *GST-ospA* and *GST-ospC*

fusion genes formation. The resulting DNA constructs were used for transformation in *E. coli* strain DH5б. Ligations of *ospA* and *ospC* in the multiple cloning site of pGEX-2T and the correct orientation of the genes were confirmed by restriction fragment analysis following digestion with *BamHI-PstI* and *XbaI-PstI*, respectively.

Expression and Western blotting of the recombinant *GST-OspA* and *GST-OspC* fusion proteins

Expression of *ospA* and *ospC* in transformed *E. coli* DH5б cells was induced with 1mM IPTG (isopropyl-в-D-thiogalactopyranoside). The transformed cells were cultivated in LB medium at 37°C. Cultivation was continued to an OD_{600} = 0.7 - 0.9 and after addition of 1mM IPTG was extended for a further two hours. The cultures were centrifuged and the cells were lysed by sonication after addition of 5mM PMSF (phenyl-methyl-sulfonyl-fluoride). The bacterial lysates were analyzed on SDS-PAGE with a 12.5% running gel and 3.5% concentration of the stacking gel (20). A uniformly prepared bacterial lysate from *E. coli* DH5б cells transformed with the native pGEX-2T plasmid was used as an internal control. The gel was stained with Coomassie.

Purification of *GST*-recombinant proteins

Transformed *E. coli* DH5a were cultivated in 20ml Luria-Bertani medium containing 100mg/ml ampicillin until OD_{600} reached 0.5-2. Expression of the fusion proteins was induced by 1 mM IPTG for further 2 hours. The cultures were centrifuged for 10 min at 4°C, 5000 rpm. The cell pellets were resuspended in 50ml/ml ice-cold 1xPBS buffer. The cells were sonicated after adding 5mM PMSF and centrifuged at 10 000 rpm for 30 min at 4°C. Purification of the fused proteins was conducted using columns containing 50% Glutathione Sepharose 4B resin (Amersham Biosciences). The supernatants were poured into the columns, incubated on ice and centrifuged 5 min at 4000 rpm. Using a buffer, containing 10mM reduced glutation and 50mM Tris-HCl, H=8.

Confirmation of recombinant proteins by immunoblot

Bacterial lysates were precipitated with 20% TCA (trichloroacetic acid) for one hour on ice and after centrifugation the protein pellets were washed in an ethanol/ether 1:1 solution and dried. The pellets were suspended in ddH_2O and analyzed with 12.5% SDS-PAGE. The proteins were transferred to a sheet of a nitrocellulose membrane. The immunoblot was carried out using a 200mM PBST washing buffer containing 50mM phosphate buffer with pH=8.5, 200mM NaCl and 0.05% Tween 20. Blocking was performed with PBST containing 0.5% Tween 20 followed by incubation with 3% albumin for one hour at 37°C. The immune reactions were performed with monoclonal mouse antibodies, kindly provided by Prof. Wilske (Max von Pettenkofer Institut, Munich, Germany): 1F11 - anti-OspA antibody (diluted 1:50) and 1F8 - anti-OspC (diluted 1:50) and a HRP conjugated anti - mouse rabbit IgG (diluted 1:1600). The sequential incubation with both antibodies was held at 37 °C for one hour. The substrates for the reaction catalyzed by the peroxidase were o-Dianizidine dihydrochloride 40mg/100ml and Na-nitropruside 100mg/ml (Sigma).

RESULTS

Major *B. burgdorferi* proteins, involved in the immune response of the organism, were cloned.

OspA and o*spC* genes were amplified by PCR using genome DNA from *B. burgdorferi* sensu stricto strain B31 using specifically designed primers. The primers contained at their 5'-end restriction site of *BamH*I, and at the 3'-end - the restriction site of *EcoR*I. After digestion with both these restrictases, the resulting product was cloned into the multiple cloning site of the pGEX-2T expression vector downstream of the *GST* gene. The successful construction of the recombinant plasmid pGEX-2T - *GST/OspA* and pGEX-2T - *GST/OspC* was confirmed by agarose electrophoresis (Figure 1, lanes 2-4).

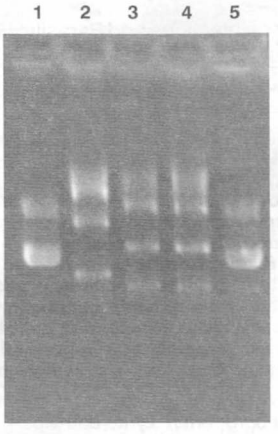

Fig. 1. Plasmid DNA agarose electrophoresis
Plasmid DNA was isolated from transformed *E.coli* DH5б and run on a 0.8% agarose gel in 0.5x TBE buffer. The resulting bands were visualized with ethidium bromide. Lane 1 and 5 - native pGEX-2T expression vector; lane 2 - pGEX-2T-*GST/flaB* plasmid; lane 3 - pGEX-2T/*OspA* plasmid; lane 4 - pGEX-2T/*OspC* plasmid

Expression of *ospA* and *ospC* in recombinant *E. coli* DH5б was induced by adding IPTG (isopropyl-в-D-thiogalactopyranoside). After induction, the whole cell lysates were examined on SDS-PAGE for the presence of the recombinant GST-OspA and GST-OspC fusion proteins. Figure 2 presents SDS gel - electrophoresis of protein lysates *E. coli* DH5б transformed with an empty pGEX-2T vector and with the pGEX-2T/OspA и pGEX-2T/OspC constructs after induction with IPTG.

OspA and OspC recombinant proteins were purified from glutation-S-transferase and transferred to a nitrocellulose membrane for confirmation by monoclonal antibodies in immunoblot. Fig 3 demonstrates results of the immunobloting of the two recombinant proteins by anti-OspA and anti-OspC monoclonal antibodies. Results of the immunoblots confirmed specificity of the recombinant proteins.

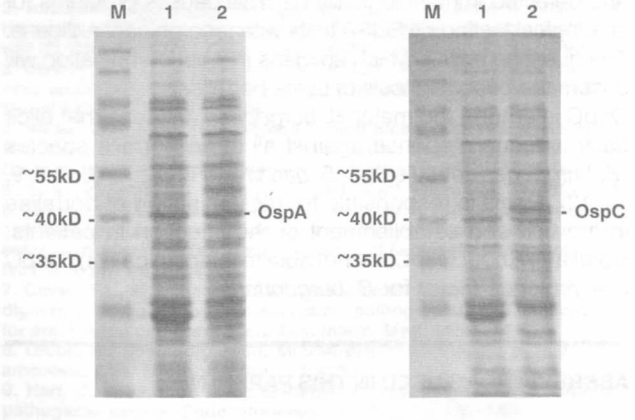

Fig. 2. SDS-PAGE Coomassie staining of cell lysates from E.coli DH5б transformed with the native pGEX-2T vector and the pGEX-2T/OspA and pGEX-2T/OspC constructs after IPTG induction.
M - Molecular weight marker; Lanes 1, A and B cell lysate from *E. coli* DH5б, transformed with the native pGEX-2T vector; Lane 2, A - cell lysate from *E. coli* DH5б, transformed with pGEX-2T-*GST/OspA*. Lane 2, B - cell lysate from *E. coli* DH5б, transformed with pGEX-2T-*GST/OspC*. New protein bands in the OspA and OspC resp. expressing transformants are clearly visible (arrow)

DISCUSSION

Introduction of ELISA tests with recombinant *B. burgdorferi* proteins noted substantial progress in development of serological diagnosis for Lyme borreliosis. Initial application of whole bacterial cells as antigens for ELISA has led in many cases to false positive results. *B. burgdorferi* cells integrate various antigens but not all of them are specific. Some of the immunodominant proteins are quite similar to structures in relative and even in unrelative microorganisms. That is why, it is important to use only specific *B. burgdorferi* proteins as antigens for the serological tests. This was achieved by development of ELISA tests with recombinant *B. burgdorferi* proteins. Aplication only of the specific poteins reduced sensitivity of the newly developed tests since some antigens, even not specific, are strong immunogens and could not be omitted. For example, flagellin is very similar with flagellar antigens of many microorganisms but antibody response against the flagellin develops in very early Lyme borreliosis. The question is which *B. burgdorferi* antigens would assure maximal diagnostic success. Currently, efforts of investigation groups are directed towards discovering *B. burgdorferi* antigens that are not expressed or are minimally expressed during in vitro cultivation but that are strongly involved in develolepment of humoral immune response of the patient. Examples for such antigens are VlsE and DbpA. Both antigens are strong immunogens but antibodies could not be detected by whole-cell *B. burgdorferi* antigens.

As a first step in developing a second generation ELISA tests for serological diagnosis of Lyme borreliosis in Bulgaria, we cloned and expressed in *E. coli* major protein antigens of *B. burgdorferi* - OspA and OspC. It is well known that OspC is the main antigen recognized in early Lyme borreliosis. OspC plays the main role in establishment the infection in the host. During the later stages of the diseases, expression of OsC is repressed but those of OspA is derepressed, antibodies to flagellin are continuosly detected in all stages of the disease.

Specificity of the obtained recombinant proteins was proven by monoclonal antibodies. Existence of additional bands on immunoblot strips is most probably due to degradation products of the proteins as they were detected only by monoclonal antibodies but were abscent in transformed with an empty vector *E. coli*. Schulte-Spechtel et al (21) also detected similar bands and proved that they are specific by appearing of immune reaction only with the specific serum but absence of reactivity with serum against *E. coli*.

The produced this way proteins could be used as antigens for ELISA or immunoblot. Besides diagnostic goals, they could be applied for investigations of antibody response during *B. burgdorferi* infection as well as for studies on pathogenesis of Lyme borreliosis.

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REFERENCE

1. Akin, E. et al. Infect. Immun. 1999, 67, 173-181.
2. Magnarelli, L. et al. J. Clin. Microbiol. 1990, 28, 1276-1279.
3. Hansen, K., P. Hindersson, and N. S. Pedersen. J. Clin. Microbiol. 1988, 26, 338-346.
4. Carreiro, M. M., D. C. Laux, and D. R. Nelson. Infect. Immun. 1990, 58, 2186-2191.
5. Engstrom, S. M., E. Shoop, and R. C. Johnson. J. Clin. Microbiol. 195, 33, 419-427.
6. Gerber, M. et al. J. Infect. Dis. 1995, 171, 724-727.
7. Lam, T. T. et al. Infect. Immun. 1994, 62, 290-298.
8. Magnarelli, L. A. et al. J. Clin. Microbiol. 1996, 34, 237-240.
9. Padula, S. J. et al. J. Clin. Microbiol. 1994, 32,1733-1738.
10. Probert, W. S., and B. J. B. Johnson. Mol. Microbiol. 1998, 30, 1003-1015.
11. Simpson, W.J., M.Schrumpf, and T.G.Schwan. J. Clin. Microbiol. 1990, 28, 1329-1337.
12. Fung, B et al. Infect. Immun. 1994, 62, 3213-3221.
13. Wilske, B.et al. Med. Microbiol. Immunol. 1994, 183, 43-59.
14. Grimm D. et al. Proc. Natl. Acad. Sci. USA 2004, 101, 3142-3147.
15. Preac-Mursic V. et al. Infection 1992, 20, 342 349.
16. Gilmore R. D. et al. Infect. Immun. 1996, 64, 2234 2239.
17. Qiu W.G. et al. Hereditas 1997, 127, 203 216.
18. Wang I.N. et al. Genetics 1999, 151, 15 30.
19. Sambrook, J., E.F. Fritsch, and T. Maniatis. Cold Spring Harbor, NY. 1989.
20. Laemmli, U.K. Nature 1970, 227, 680-685.
21. Schulte-Spechtel U. et al. J. Clin. Microbiol. 41, 2003, 1299-1303.

PCR AS A METHOD FOR PRESUMPTIVE DIAGNOSTICS OF INVASIVE CANDIDOSIS

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SUMMARY

***Candida spp.* are ubiquitous yeasts, which is known as a most frequent causative agents of systemic fungal infections in humans. Systemic candidosis is life-threatening infection which occurs in severely immunocompromised individuals, and most of the cases have an lethal outcome. The rapid detection and identification of *Candida spp.* in clinical laboratories are extremely important for the management of patients with systemic candidosis. The routinely used culture and immunological species identification are time-consuming and lack the required sensitivity and specificity. Molecular methods for diagnostics of systemic candidiasis are mostly PCR-based and use serum or blood specimens. However, there are many issues due to insufficient sensitivity of the method, and the occurrence of false-positive results. In this study we developed conventional PCR - based diagnostic method, using universal primers for detection of *Candida spp.* in serum specimens, which significantly improves the sensitivity of such methods, without any false-positive results.**

Keywords: Candida, candidemia, universal fungal primers, polymerase chain reaction (PCR), transitory fungaemia

INTRODUCTION

Under natural conditions *Candida sp.* are saprophytic yeasts. Some of the species are known as commensals of human skin and gastrointestinal tract. Some of the species are pathogenic in immunosuppressed host and are the most common causative agents of life-threatening invasive mycoses. Among the patients on highest risk are neutropenic individuals, patients on immunosuppressive or broad-spectrum antibiotic therapy, solid organ transplants (1).

Invasive candidosis progresses rapidly and has unspecific simptomatics. For treatment fluconazole and ampthotericin B are used, but newer broad-spectrum azoles (voriconazole and itraconazole) and newly introduced echinocandins (caspofungin and micafungin) currently are the most effective drugs. Although newer drugs were introduced, there is increasing number of drug-resistant *Candida albicans* isolates due to continuous treatment or prophylaxis with amphotericin B and fluconazole. Other species, such as *C. krusei* are inherently resistant to fluconazole; *C .glabrata* and *C. rugosa* develop resistance during the treatment (2).

ABBREVIATIONS USED IN THIS PAPER:

PCR - Polymerase chain reaction; SDA - Sabouraud dextrose agar; EDTA - ethylenediaminetetraacetate
ITS - internally transcribed spacer

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Epidemiological data shows that in Bulgaria for the period 1997-2003, *Candida spp.* are the fifth most commonly isolated species (4,3 %) from haemocultures (4).

Rapid and proper diagnostics is of critical importance for initiating of antimycotic therapy. Routinely used methods for diagnostics of invasive candidosis are based on morphological and cultural examination of isolated microorganism, and lack rapidness and precision (3).

For rapid detection and identification of the causative agent, diagnostic methods for detection of *Candida* - specific DNA in blood and serum were developed (5, 6). Polymerase chain reaction, based on genus-specific fungal primers was developed (7), as well as *Candida albicans*-specific and universal fungal primers in combination with species-specific probes (8, 9). Newer publications demonstrate the potential of Real-Time PCR for diagnostics of invasive candidosis in blood or serum, as well as for species identification in cultures. (10,11).

In the present work, we investigated the potential of PCR as method for rapid detection of invasive candidosis, in order to improve the diagnostics and therapy of such kind of infections. The main goal of the study was development of universal fungal primers-based PCR-assay for detection of *Candida*-specific DNA in blood or serum. The assay allows identification of six of the most-common species, causing invasive candidosis: *Candida albicans, C. parapsilosis, C. glabrata, C. krusei, C. guilliermondii, C. lusitaniae*. Although *Candida albicans*, *C. parapsilosis* and *C. guilliermondii* are usually susceptible to treatment with fluconazole, *C. lusitaniae* is intrinsically resistant to amphotericin B. *C. glabrata* and *C. krusei* isolates are commonly resistant to azoles, but are susceptible to voriconazole and amphotericin B.

MATERIALS AND METHODS

Referent strains

The following referent strains were used: *Candida albicans* ATCC 22019, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 90030, *C. guilliermondii* 40201, *C. lusitaniae* 40205. The strains were lyophilized, and in order to DNA extraction, were sub cultured on Sabouraud dextrose agar media (SDA) (BBL, Division of Becton Dickinson, Cockeysville, MD,USA), at 25° C / 24 hours. Clinical strains isolated for haemocultures were sub cultivated on SDA under the same conditions and preserved on Skimmed milk media at - 20° C in freezer.

Clinical materials

Eight to ten ml blood samples from 21 patients with clinics of sepsis and underlying diseases, were obtained and haemoculture bottles were inoculated (BacT/Alert®, Becton-Dickinson, USA). The bottles were cultivated at 35-37° C for 5 days, or when positive. Microorganisms from positive haemocultures was sub cultivated on SDA and pathogens species were identified by using automated biochemical systems VITEK® 2 (Bio Merieux, France) and API 20 C (Bio Merieux, Hazelwood, MO,USA).

Additionally, 5 ml blood samples were obtained from the patients (2 ml-samples from children) which transported by using EDTA-coated Vacutainer® tubes (Becton-Dickinson, MD,USA). Serum was separated by centrifugation at 10 min./2000 rpm and stored at - 20° C.

DNA extraction

DNA extraction from cultured yeast cells was performed by using GenomicPrep® Cells and tissue DNA Isolation Kit (Amersham Biosciences, Little Chalfont Buckinghamshire, UK) in accordance to the instructions of the manufacturer.

DNA extraction from serum samples was performed by using PureExtreme® Genomic DNA Purification Kit (Fermentas Life Sciences, Lithuania) according to the manufacturer's instructions.
Extracted DNA samples in both cases were diluted in 30 ul 10 mM TE-buffer (Tris - EDTA, pH 8.0) and were stored at - 20° C.

Primers and polymerase chain reaction (PCR)
Fungal universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (12) were used. The selected primer pair is capable of amplifying part of both 18S r DNA and 28S r DNA genes, including the internal transcribed spacer region 1 (ITS1). The primers were produced by LKB® GmbH, Austria.
Polymerase chain reaction was performed in 0.5 mL thin-walled tubes (Greiner Co., Germany) in total volume of 40 μL and master mix consisting of 1 X PCR buffer, 1 U Taq DNA polymerase, 2 pmol each of ITS1 and ITS2 primers, and 0,2 mM each of dNTPs.
All the reagents used were produced by Invitrogen Corporation, Carlsbad CA, USA.
The reaction was carried out in PCR thermocycler (GeneAmp® PCR system 9700, Applied Biosystems®, Foster City, CA, USA) under the following conditions: denaturation at 94° C for 40 s, hybridization at 66° C for 40 s, primer extension at 72° C for 1 min, as well as initial denaturation at 95° C for 3 min and final extension at 72° C for 5 min. Best results was achieved when the reaction was performed for 40 cycles using both referent culture and serum DNA.
PCR products were separated by using agarose gel-electrophoresis (13), visualized under UV-light and photographed, and for amplicon identification 50 bp DNA-ladder was used (Fermentas Lifesciences, Lithuania).
In order to avoid contamination, recommendations of Kwok and Higuchi were observed (14). Master-mix was prepared in room, physically separated from the room where the PCR and DNA extraction was performed. Appropriate negative control samples containing DNA extracted from serum samples obtained from healthy individuals were used.

RESULTS

PCR of serum samples and comparison with results from cultural examination
The results from comparative PCR analysis of serum samples and clinical isolates are presented on Fig. 1-4. Clinical data of the patients are presented in Table 1.
By using ITS1-ITS2 primer pair DNA fragments with species-specific molecular weights 147 bp (*C. lusitaniae*), 182 bp (*C. krusei*), 218 bp (*C. albicans*), 228 bp (*C. parapsilosis*), 246 bp (*C. guilliermondii*), 485 bp (*C. glabrata*) were generated after PCR amplification.
Any of the serum samples obtained from healthy individuals were positive after PCR analysis.
From 21 patients with signs suspicious for invasive mycosis, 17 were with positive haemocultures. Species identification was determined by using automated biochemical systems VITEK 2 and API 20 C, as well as by PCR. Results obtained by the two groups of methods were identical in 100 % of the isolates tested. Comparative PCR test of serum and cultural DNA samples showed identical results in 16 of 17 (94%) patients with positive haemocultures. At the same time analysis of serum and cultural samples showed different DNA fragments in only one of the cases, where in serum sample *Candida albicans*-specific DNA fragment was detected, and in the culture sample was detected *C. glabrata* - specific DNA. At the same time, in 4 of the patients, where haemoculture bottles remained negative PCR analysis of serum samples detected *Candida* specific DNA. In two of these patients *Candida guilliermondii* - specific DNA was detected, and in the other two - DNA specific for *C. parapsilosis*.
In summary, 17 from 21 haemoculture bottles from patients suspicious for invasive mycosis, became positive after cultivation and sub cultivation of the pathogen was possible. When tested with PCR, in 16 of the cases DNA fragments from serum sample was identical to those from the clinical yeast isolates. In 4 of the cases where the inoculated haemocultures remained negative and performing of biochemical tests was impossible, PCR successfully detected *Candida* - specific DNA when respective serum samples were tested.

DISCUSSION

In present study PCR-based assay for diagnostics of invasive candidosis was developed and proposed. The assay is based on universal fungal primers and allows identification of six of the most important pathogenic *Candida* species. The diagnostic potential of the method was proved by examination of clinical isolates, serum samples and referent *Candida* strains as a positive controls. We were used a primer pair that flanks the genes, coding ribosomal DNA in fungi (r DNA genes). Independently of that there are many diagnostic studies, based on other molecular markers (15, 16), r DNA - based assay is much more appropriate. Because of fact that fungal r DNA genes are presented in many copies (40-100) per haploid genome (16), PCR - assays based on multicopy target sequences are more sensitive than those based on single-copy targets (15, 16). Additionally, in fungi an variable ITS1 and ITS2 (internally transcribed spacer) regions are located between the highly conserved r DNA genes, which allows amplicons with different length and determines good specificity of the method. Some of the diagnostic assays described include post-amplifications stages such as restriction-enzyme treatment (17, 18), using of radioactively- or fluorescently-labeled species-specific probes (5, 19, 20, 21), and sequencing (22). Most of the assays listed require additional time for hybridization and use radioactive isotopes for DNA labeling. Unlike many other methods, our assay require only 6-8 hours and does not include post-amplification stages or using of radioactive substances, and allows rapid identification of six clinically important *Candida sp.*
Blood samples from 21 patients suspicious for invasive mycosis were obtained and haemocultural bottles were inoculated (Table 1). In 17 of the cases blood cultures were positive and sub cultivation and identification of fungal isolates was performed by cultural methods and proposed PCR method. All 17 clinical isolates were successfully identified by both PCR and cultural methods, and the results were identical in 100%. When compared to serum PCR, DNA fragments in 16 from 17 serum samples (94%) were identical to those from clinical isolates (Fig.1-3). In only one of the cases (isolate 328) PCR of the serum sample resulted in DNA fragment, specific for *C. albicans*, while PCR of the clinical isolate showed *Candida glabrata* - specific DNA fragment. Such differences occur frequent and it is thought that they arise from so called transitory fungaemia - the strain which initially causes fungaemia is consequently removed by another strain or species which has become resistant during the applied antimycotic therapy (if any), or just the second strain or species is more adaptable than the initially occurring one (23,24). Because of that, the lack of consistence in this case was not accounted as a mistake or disadvantage of the proposed method.
Electrophoresis in agarose gel with concentration 3 % was performed in order to achieve optimal separation of PCR products with similar molecular weights.

Table 1.

№ of the sample	Laboratory №	Patient	Age	Underlying disease	Microorganism, isolated from haemoculture
1	637	Т.Т.	47	Pneumonia	*C. albicans*
2	122	Р.Х.	43	Pancreatitis	*C. albicans*
3	328	И.Т.	45	Postgastrectomia	*C. glabrata*
4	539	В.В.	51	Cerebral insult	*C. albicans*
5	168	Т.Й.	23	Sepsis	*C. parapsilosis*
6	308	И.П.	36	Thoracic contusion	*C. parapsilosis*
7	297	И.Ч.	40	Traumatic shock	*C. parapsilosis*
8	4486	П.Д.	39	Sepsis following liver transplantation	*C. krusei*
9	406	Д.В.	-	Fetal infection	*C. albicans*
10	190	А.Н.	35	Ileuses	*C. albicans*
11	129	Л.З.	49	Ceccal carcinoma	*C. albicans*
12	85	Й.Г.	35	Valve prosthesis endocarditis	*C. albicans*
13	213	Р.Л.	29	Sepsis	*C. albicans*
14	407	Д.Н.	-	Fetal infection	*C. albicans*
15	830	Г.И	47	Sepsis	*C. parapsilosis*
16	790	А.Г.	55	Pancreatitis	*C. parapsilosis*
17	5783	А.В.	39	Traumatic shock	*C. parapsilosis*
18	10785	В.Н.	44	Sepsis	-
19	10124	С.С.	28	Thoracic contusion	-
20	10022	Ц.И.	30	Sepsis	-
21	5784	Л.Г.	42	Ileuses	-

Amplification of DNA from healthy serum samples remained negative in consistence with scientific data from other authors (20, 21). Four from haemoculture bottles (19 %) remained negative after the stage of incubation and it was not possible to isolate pathogenic agent, while PCR test of the respective serums samples showed *Candida parapsilosis* - specific DNA fragment (in 2 of the cases) and in the other 2 - *C.* guilliermondii-specific DNA. Regardless of negative haemocultures and respectively impossibility for performing the cultural test by using the described PCR assay, we were able to detect *Candida* - specific DNA, and to identify the pathogenic species. This finding is supported by several other publications where PCR-based methods demonstrate significantly higher sensitivity in comparison with cultural methods for diagnostics of invasive candidosis (21, 25).

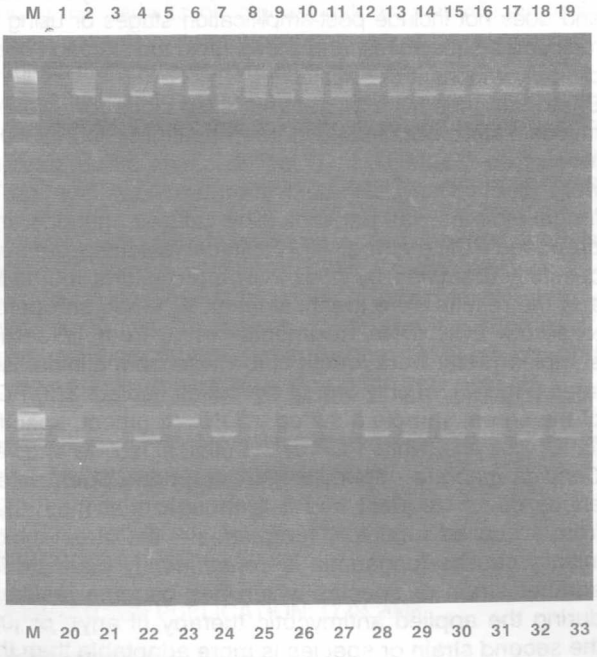

Fig. 1. M - 50 bp ladder; 1 - negative control sample; 2 - C. albicans; 3 - C. krusei; 4 - C. parapsilosis; 5 - C. glabrata; 6 - C. guilliermondii; 7 - C. lusitaniae; 8 - 637K (clinical isolate); 9 - 637S (serum sample); 10 - 122K; 11 - 122S; 12 - 328K; 13 - 328S; 14 - 539K; 15 - 539S; 16 - 168K; 17 - 168S; 18 - 308K; 19 - 308S; 20 - *C. albicans*; 21 - *C. krusei*; 22 - *C. parapsilosis*; 23 - *C. glabrata*; 24 - *C. guilliermondii*; 25 - *C. lusitaniae*; 26 - 4486K; 27 - 4486S; 28 - 297K; 29 - 297S; 30 - 406K; 31 - 406S; 32 - 190K; 33 - 190S

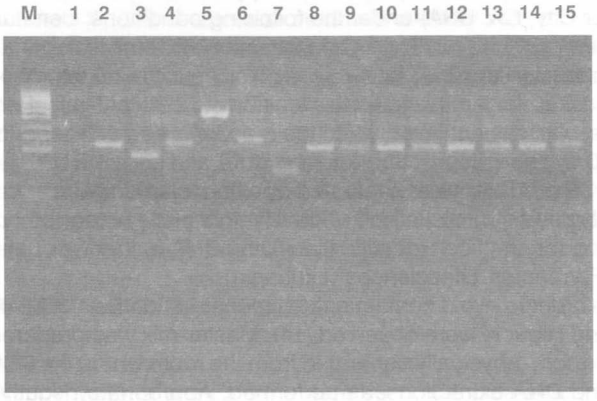

Fig. 2. M - 50 bp ladder; 1 - negative control sample; 2 - *C. albicans*; 3 - *C. krusei*; 4 - *C. parapsilosis*; 5 - *C. glabrata*; 6 - *C. guilliermondii*; 7 - *C. lusitaniae*; 8 - 129K; 9 - 129S; 10 - 85K; 11 - 85S; 12 - 213K; 13 - 213S; 14 - 407K; 15 - 407S

Non-specific symptomatics and the aggressive nature of invasive candidosis as well as commonly occurring drug-resistant isolates (26), define high level of lethal outcome in case of mixed infection or misidentification of the pathogen. Special caution must be taken to many scientific works where false-positive results and cross-contamination are reported (27). In some of these studies where im-

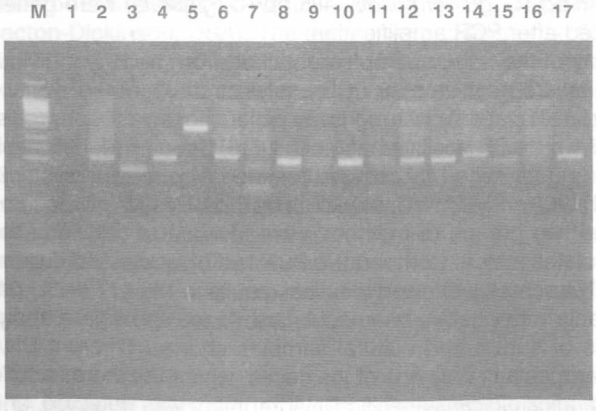

Fig. 3. M - 50 bp ladder; 1 - negative control sample; 2 - *C. albicans*; 3 - *C. krusei*; 4 - *C. parapsilosis*; 5 - *C. glabrata*; 6 - *C. guilliermondii*; 7 - *C. lusitaniae*; 8 - 830K; 9 - 830S; 10 - 790K; 11 - 790S; 12 - 5783K; 13 - 5783S; 14 - 10785S; 15 - 10124S; 16 - 10022S; 17 - 5784S

munological diagnostic tests have been applied, lack of correlation between antigen titres and the results from cultural tests have been observed (27).

In conclusion, PCR - based method for diagnostics of six from the most commonly causing candidaemia species (*Candida albicans, Candida krusei, C. parapsilosis, C. glabrata, C. guilliermondii* and *C. lusitaniae*) was developed. The proposed method showed high sensitivity and specificity when performed with *Candida* clinical isolates and serum samples from patients with suspected invasive candidosis. The method could be successfully used for early diagnostics of candidosis when timely starting of appropriate antimycotic therapy is of crucial importance.

REFERENCES

1. Maschmeyer G. The changing epidemiology of invasive fungal infections: new threats
International Journal of Antimicrobial Agents 27S (2006) S3-S6
2. Enoch, D.A., Ludlam, H.A., Brown, N.M. 2006. Invasive fungal infections: a review of epidemiology and management options, J. Med. Microbiol. 55: 809-818
3. Hazen,K.C. New and emerging yeast pathogens. Clin.Microbiol. Rev. 8: 462-478
4. BulSTAR's annual report on frequency of microorganisms, isolated from haemocultures, 2005
5.Einsele, H., Hebart, H., Roller, G., et al. Detection and identification of fungal pathogens in blood by using molecular probes. J. Clin. Microbiol. 1997; 35: 1353-1360)
6. van Burik, J-A, Myerson, D., Schreckhise, R.W., et al. Panfungal PCR assay for detection of fungal infection in human blood samples. J. Clin. Microbiol. 1998; 36: 1169-1175)
7. van Deventer, A.J.M, Goessens, W.H.F., van Belkum, et al. Improved detection of Candida albicans in blood of neutropenic mice with systemic candidosis. 1995. J. Clin. Microbiol. 33: 625 628
8. Elie, C.M., Lott, T.J., Reiss, E., et al. Rapid identification of Candida species with species specific DNA-probes. J. Clin. Microbiol. 1998; 36: 3260-3265)
9. Shin, J.H., Nolte, F.S., Holloway, B.P., et al. Rapid identification of up to three Candida species in a single reaction tube by a 5' exonuclease assay using fluorescent DNA probes. J.Clin.Microbiol. 1999; 37: 165 170
10. Maaroufi, Y., Heymans C., de Bruyne, J.- M., et al. Rapid identification of Candida albicans in clinical blood samples by using a TaqMan - based PCR assay. 2003. J.Clin. Microbiol., 41(7): 3293-3298
11. Guiver, M., Levi. K., Oppenheim, B.A. Rapid identification of candida species by TaqMan PCR, , J.Clin.Pathol., 2001; 54: 362-366
12. White, T.J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification an direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. In M.A. Innis, D.H. Gelland, J.J.Shinsky, T.J. White (ed.) PCR protocols - a guide to methods and applications. Academic Press, Inc. San Diego, Calif.
13. Khan Z and Mustafa A. Detection of Candida species by polymerase chain reaction (PCR) in blood samples of experimentally infected mice and patients with suspected candidemia. *Microbiol Res*, 2001; 156: 95 - 102
14. Kwok S and Higichi R (1989) Avoiding false positives with PCR. *Nature* (London) 339: 237-238
15. Mitchell, T. G., R. L. Sandin, B. H. Bowman, W. Meyer, and W. G. Merz. 1994. Molecular mycology: DNA probes and applications of PCR technology. J. Med. Vet. Mycol. 32(Suppl. 1):351 366.
16. Reiss, E., K. Tanaka, G. Bruker, V. Chazalet, D. Coleman, J. P. Debeaupuis, R. Hanazawa, J. P. Latge, J. Lortholary, K. Makimura, C. J. Morrison, S. Y. Murayama, S. Naoe, S. Paris, J. Sarfati, K. Shibuya, D. Sullivan, K. Uchida, and H. Yamaguchi. 1998. Molecular diagnosis and epidemiology of fungal infections. Med. Mycol. 36(Suppl. 1):249-257
17. Morace, G., L. Pagano, M. Sanguinetti, B. Posteraro, L. Mele, F. Equitani, G. D'Amore, G. Leone, and G. Fadda. 1999. PCR-restriction enzyme analysis for detection of *Candida* DNA in blood from febrile patients with hematological malignancies. J. Clin. Microbiol. 37:1871-1875.
18. Williams, D. W., M. J. Wilson, M. A. Lewis, and A. J. Potts. 1995. Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. J. Clin. Microbiol. 33:2476-2479
19. Fujita, S., B. A. Lasker, T. J. Lott, E. Reiss, and C. J. Morrison. 1995. Microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species in blood. J. Clin. Microbiol. 33:962 967.
20. Kan, V. L. 1993. Polymerase chain reaction for the diagnosis of candidemia. J. Infect. Dis. 168:779-783.
21 Wahyuningsih, R., H. J. Freisleben, H. G. Sonntag, and P. Schnitzler. 2000. Simple and rapid detection of *Candida albicans* DNA in serum by PCR for diagnosis of invasive candidiasis. J. Clin. Microbiol. 38:3016-3021.
22. Niesters, H. G., W. H. Goessens, J. F. Meis, and W. G. Quint. 1993. Rapid, polymerase chain reaction-based identification assays for *Candida* species. J. Clin. Microbiol. 31:904-910
23. Sendid, B., Poirot., J.L., Tabouret, M., Bonnin, A., Caillot, D., Camus, D., Poulain., D. 2002. Combined detection of mannanaemia and anti-mannan antibodies as a strategy for the diagnosis of systemic infection, caused by pathogenic Candida species. J. Med. Microbiol., 51(5): 433-442
24. Pasqualotto, A., Antunes, A.G., Severo, L. 2006. *Candida guilliermondii* as the aethiology of candidosis. Rev. Inst. Med. trop., 48(3): 123-127
25. Chryssanthou, E., B. Andersson, B. Petrini, S. Lofdahl, and J. Tollemar. 1994. Detection of *Candida albicans* DNA in serum by polymerase chain reaction. Scand. J. Infect. Dis. 26: 479-485.
26. Gulshan, K., Moye-Rowley, S. 2007. Multidrug resistance in fungi. Eucar. Cell, doi:10.1128/EC.00254-07
27. Alexander, B., Pfaller, M. 2006 Contemporary methods for the diagnosis and management of invasive mycoses, Clin. Infect. Dis., 43: S15-S27

ANTI-INFLUENZA VIRUS EFFECTS OF A PLANT POLYPHENOLIC EXTRACT AND *E*-AMINOCAPROIC ACID APPLIED ALONE AND IN COMBINATION

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ABSTRACT

Influenza is a major public health problem because of its wide spread, high morbidity rate and considerable social and economic implications. Despite the achievements of antiviral chemotherapy, the need of new potent antiviral agents continues to exist alongside with the necessity of novel methods and approaches. The combined application of natural and synthetic viral inhibitors may be used successfully to potentate the antiviral efficacy of the plant preparations and may enable dose reduction of their toxic components. The aim of the study is to investigate the combined *in vitro* and *in vivo* anti-influenza virus effects of a plant polyphenol-rich extract, obtained from *Geranium sanguineum* L. (PC) and the protease inhibitor *e*-aminocaproic acid (ACA). The *in vitro* antiviral activity was determined by the difference in the infectious titres of control and treated viruses and the combined effect was defined on the base of infectious viral yields. The combined protective activity *in vivo* was determined on the base of the reduction of mortality rates. The combined use of PC with ACA resulted in additive to synergistic inhibition of the A/Aichi/2/68 (H3N2) (A/Aichi) virus replication in MDCK cells; however some of the combinations were antagonistic. The joint application of PC with ACA in the murine experimental influenza A/Aichi-a virus infection produced a synergistic protective effect with no enhancement of host toxicity. Survival rate and mean survival time were markedly increased; the index of protection reached 91.2%. The presented results support the combined application of natural and synthetic viral inhibitors with different modes of action.

Key words: influenza virus, combined antiviral effect, protease inhibitors

INTRODUCTION

Influenza is a major public health problem because of its wide spread, high morbidity rate and considerable social and economic implications. Despite the achievements of antiviral chemotherapy, the need of new potent antiviral agents continues to exist alongside with the necessity of novel methods and approaches. The data from the combined application of natural and synthetic viral inhibitors suggest that this could be a promising approach in the control of viral infections (2, 3, 6, 8, 9, 24). Our group has a substantial experience in this field of research (4, 5, 11, 15, 16, 17, 19, 23).

The aim of the present study is to investigate the combined *in vitro* and *in vivo* anti-influenza virus effects of a plant polyphenol-rich extract, obtained from *Geranium sanguineum* L. (PC) and the protease inhibitor *e*-aminocaproic acid (ACA).

ABBREVIATIONS USED IN THIS PAPER:
ACA - *e*-aminocaproic acid

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MATERIALS AND METHODS

Substances. PC was prepared as described previously (20) and kindly provided by Dr. S. Ivancheva, Institute of Botany, Bulg. Acad. Sci. *e*-aminocaproic acid (ACA) and ribavirin (Rib) were purchased from Sigma, Diesenhofen, Germany.

Cells and Viruses. MDCK cells, media and influenza viruses A/chicken/Germany/34, strain Rostock (H7N1) (A/Rostock), A/Aichi/2/68 (H3N2) (A/Aichi) and A/Aichi/2/68 (H3N2), adapted to mice lungs (A/Aichi-a) were as in (1).

Mice. Male and female (16-18 g) ICR mice were obtained from the Experimental Animal Station of the Bulgarian Academy of Sciences, Slivnitza, Sofia. They were quarantined for 24 h prior to use and maintained on standard laboratory chow and tap water *ad libitum* for the duration of the studies.

Cellular toxicity was monitored as described in Serkedjieva and Hay, 1998. The 50% toxic concentrations, the dose required to cause visible changes in 50% of intact cultures (TC_{50}) were determined.

Antiviral assays. The antiviral effect was studied in multicycle experiments of viral growth. The virus-induced cytopathogenic effect (CPE) and the production of infectious virus were used as measures of viral growth. CPE-reduction assay was as in (14). The concentration reducing CPE by 50% (EC_{50}) with respect to virus control was estimated from graphic plots. The selectivity index (SI) was determined from the ratio TC_{50}/EC_{50}. Infectious virus yield (IVY) reduction assay was as in (14). Virus titers were determined by endpoint titration (10) and expressed in 50% tissue culture infectious doses ($TCID_{50}$/ml). The concentrations that reduced virus infectivity by 90% (1 $\log_{10}TCID_{50}$/ml) were determined (EC_{90}). PC (0.15 - 10.0 μg/ml) and ACA (0.75 - 50.0 mg/ml) were applied simultaneously with viral inoculation. The type of the combined antiviral effect was found according to the method of (21), based on virus yields. The fractional yield of the compound A (Y_A) was defined as the viral titer in the presence of the compound divided by the titer obtained in the absence of the compound. The same was done for the second compound (Y_B) and the combination (Y_{AB}). Then Y_C was calculated from the formulae $Y_C=Y_A x Y_B$; if $Y_C>Y_{AB}$ - the effect was synergistic; if $Y_C=Y_{AB}$ - the effect was indifferent; if $Y_C<Y_{AB}$ - the effect was antagonistic.

Experimental influenza virus infection (EIVI) was induced under ether anesthesia by the intranasal inoculation of A/Aichi-a, which causes hemorrhagic pneumonia in mice. To produce lethal infection mice were challenged with 10 LD_{50}. PC was applied by intranasal instillation 3 h before the infection in the dose 5 mg/kg in the volume of 0.05 ml PBS under light ether anesthesia. ACA was administered orally -24, -2, +24, +48, +72 h post infection (p.i.) in the dose 25 mg/kg in the volume of 0.2 ml PBS. Rib was inoculated in parallel as positive control orally -24, -2, +24, +48, +72 h p.i. in the dose 10 mg/kg in the volume of 0.2 ml PBS. Experimental groups were of 12 animals each. The protective effect was estimated by the reduction of the rate of mortality, the increase of the index of protection (PI) and prolongation of mean survival time (MST) as in (15). PI was determined from the equation (PR-1)/PRx100, where PR (ratio of protection) was $M_{control}/M_{experiment}$ and M was mortality. Mice were observed for death daily for 14 days. After the end of the experiments surviving mice were sacrificed by cervical dislocation. Toxicity controls (4 mice per combination) were run in parallel. The combined effect was evaluated according to (25). The effect of the combination ($E_{1,2}=PI_{1,2}/100$) and the effects of the individual substances ($E_1=PI_1/100$ and $E_2=PI_2/100$) are related in the equation $E_{1,2}=E_1+E_2-E_1 x E_2$; the combined effect is synergistic if $E_{1,2}$ is >, indifferent if $E_{1,2}$ is = and antagonistic if $E_{1,2}$ is $<E_1+E_2-E_1 x E_2$.

Statistical methods. Results are given as arithmetic mean values. Student's *t*-test was used to evaluate differences in virus titres. $p<0.05$ was accepted for statistical significance.

RESULTS AND DISCUSSION

Influenza A remains a major burden on mankind with annual epidemics of disease. WHO has recently decreed that „another influenza pandemic is inevitable and possibly imminent", an alarming prospect, considering the devastating previous pandemics. Influenza infection is usually self-limiting, culminating in a local and systemic reaction. However, there remains a significant proportion of patients who develop severe illness and complications, such as the elderly, the very young and the immunocompromised. Even with the development of killed virus vaccines the need for effective anti-influenza therapies still exists.

Current anti-influenza drugs (M2 ion channel blockers and neuraminidase inhibitors) target viral components. Recently cellular proteins are emerging as potential targets for new antiviral drugs. The principal idea is to affect the mechanisms underlying virus-cell interactions and favouring viral replication in virus-infected cells. In the case of influenza viruses, the virulence of a particular influenza virus strain depends on the ability of its haemagglutinin precursor (HA0) to be cleaved post translation to subunits HA1 and HA2 by trypsin-like proteases of the host (7, 26). Thus it seems reasonable that the prevention of this cleavage would result in inhibition of subsequent rounds of viral replication and spread in the respiratory tract.

On the other part it is well accepted that one of the possible ways of enhancing the effect of antiviral substances is to use them in appropriate combinations. This provides a prospect to potentate the inhibitory effect of the substances, to reduce their toxic action and to prevent the appearance of resistant virus mutants. The data on the combined inhibitory activity of natural and synthetic antiviral agents, though scarce, suggest that this could be a promising approach in the control of viral infections. Our data together with the evidence provided by others (2, 3, 6, 8, 9, 24) suggest that the combined application of natural and synthetic viral inhibitors may be used successfully to potentate the antiviral efficacy of the plant preparations and may enable dose reduction of their toxic components.

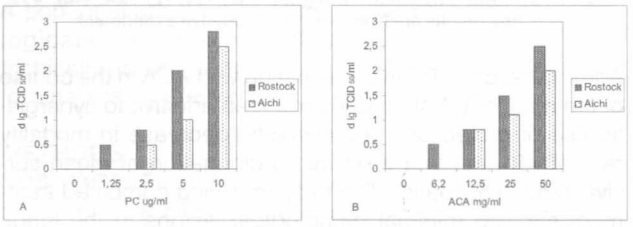
We have studied intensively the mode of the anti-influenza virus activity of the semi-standardized polyphenol-rich extract, isolated from *Geranium sanguineum* L. (PC) (14). It was shown that its *in vitro* virus-inhibitory effect was strain-dependent, consistent with a selective antiviral action. PC affected the synthetic stages of A/Rostock viral replication; virus-specific RNA and protein synthesis were selectively inhibited. In addition the selectivity of inhibition was confirmed by the generation of PC-resistant mutants of A/Rostock. We have demonstrated that the plant preparation markedly protected mice from mortality in the murine EIVI (12, 13). The anti-influenza virus effect *in vivo* was associated with restoration of the balance between the lung protease and the protease-inhibitory activities of influenza virus-infected mice (20). It should be noted that *in vitro* PC demonstrated a distinct inhibition of trypsin protease activity in two complementary assays (20).

The anti-influenza virus effect of *e*-aminocaproic acid, an inhibitor of plasminogen activation, has been intensively studied by Lozitski *et al.*, 1987 (7) *in vitro* and in animal models. It was shown to reduce the HA-cleavage and virus proteolytic activation in cultured cells, in chick embryos and in the lungs of infected mice.

All these considerations persuaded us to carry out an investigation on the combined anti-influenza virus effects of PC and ACA *in vitro* and *in vivo*. Here we present the results from these experiments.

As a first approach we examined the drug-susceptibility of A/Aichi virus to both substances and the results are presented in Figs. 1A and 1B. EC_{90}-s were found: 5.2 μg/ml for PC and 25.0 mg/ml for ACA. From dose-response curves the TC_{50}-s of PC and ACA for MDCK cells were estimated - 75.0 μg/ml and 60.5 mg/ml resp. The EC_{50}-s for A/Aichi were evaluated as described in Materials and Methods - 2.2 μg/ml and 11.2 mg/ml resp. and accordingly the selectivity indices were 34.1 and 5.4 resp. We have inspected also the drug-susceptibility of A/Rostock virus to PC and ACA for comparison. The EC_{90}-s for PC and ACA were 3.0 μg/ml and 20.5 mg/ml resp. Thus the virus-inhibitory effect of both substances was strain-specific and consistent with anti-viral selectivity.

Fig. 1. Drug-susceptibility of A/Rostock and A/Aichi viruses to PC (A) and ACA (B) in MDCK cells[a]



VC - virus control, PC - polyphenolic complex, ACA - *e*-aminocaproic acid, [a]the results are the mean of 3-6 experiments

Further we applied PC and ACA in doses, which by themselves do not suppress significantly the replication of influenza virus A/Aichi in cell cultures. The results from these experiments are shown in Table 1. In the most of cases the combined use of PC with ACA resulted in synergistic enhancement of the inhibition of A/Aichi virus in MDCK cells. There were found also some antagonistic combinations (PC 2.5 μg/ml+ACA 0.7 mg/ml; PC 0.31 μg/ml+ACA 12.5 mg/ml; PC 0.31 μg/ml+ACA 1.5 mg/ml; PC 0.31 μg/ml+ACA 0.7 mg/ml; PC 0.15 μg/ml+ACA 12.5 mg/ml). The combinations did not exhibit any virucidal effect and the cellular toxicity was not enhanced.

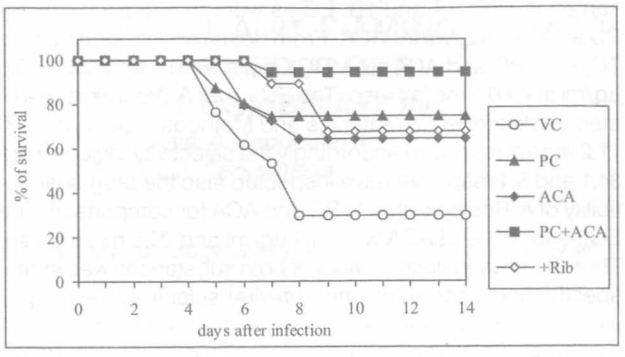
Table 1. Effects of individual and combined samples of PC and ACA on the replication of A/Aichi influenza virus in MDCK cells (checkerboard schedule)[a]

PC ACA	0	EC_{90} (5 μg/ml)	$EC_{90}/2$	$EC_{90}/4$	$EC_{90}/8$	$EC_{90}/16$	$EC_{90}/32$
0	7.2 $\log_{10}$ $TCID_{50}$/ml	5.9	6.3	6.7	6.8	6.7	7.0
EC_{90} (25 mg/ml)	6.1						
$EC_{90}/2$	6.3		4.5	5.1	4.0	5.0	6.0
$EC_{90}/4$	6.5		5.5	4.5	4.9	5.4	6.2
$EC_{90}/8$	7.0		5.8	4.9	5.2	5.6	6.5
$EC_{90}/16$	7.0		5.9	4.9	5.6	6.6	6.3
$EC_{90}/32$	7.0		6.7	5.4	5.7	6.7	7.0

VC, PC, ACA, d - as in Fig. 1, the type of the combined antiviral effect was determined according to Schinazi *et al.* 1982, as described in Materials and methods, bold - synergistic combinations

It is obvious that the *in vitro* assays could not be completely reliable to predict the effectiveness of combination therapy. The results from animal experiments could help to provide consistent evidence about the appropriate usage of combined treatment. Therefore the combined treatment with PC and ACA was tested in the EIVI. The intranasal inoculation of A/Aichi-a virus to mice produces a damaging infection of the lungs, causing viral pneumonitis. The combined protection from mortality of mice by the simultaneous treatment with PC and ACA is presented in Fig. 2.

Fig. 2. Effects of PC, ACA and their combination on the survival rate in the murine EIVI[a]



VC - virus control (A/Aichi-a), PC - polyphenolic complex, 5 mg/kg, i.n., -3 h, ACA - *e*-aminocaproic acid, 50 mg/kg, p.o., -24, -2, +24, +48, +72 h, Rib - ribavirin, 10 mg/kg, p.o., -24, -2, +24, +48, +72 h, [a]the results are from one representative experiment

Administration of PC in combination with ACA in the course of the murine A/Aichi-a EIVI produced additive to synergistic rise of protection - a synergistic decrease in mortality rate (IP=91.3%) and a distinct prolongation of mean survival time (+5.2 days). The mice receiving combined treatment showed minimal pathological lesions in the lungs whereas control untreated animals had total hemorrhagic pneumonia. Thus the combined use of PC and ACA had a synergistic therapeutic effect in the animals, inoculated with a high dose of influenza A/Aichi-a virus.

Earlier we have studied the combined virus-inhibitory effects of a proteinaceous protease inhibitor, produced by *Streptomyces* sp. 225b (BF) and PC on the replication of influenza virus A/Rostock in MDCK cells. Although the simultaneous application of BF and PC resulted predominantly in antagonistic virus-inhibitory effects, there were found also some synergistic combinations (19).

CONCLUSION

The present paper clearly indicates that combination therapy provides an advantage over single-agent therapy and suggests that the combined treatment may provide an effective basis for prophylactics and therapy of influenza virus infection. In addition the presented paper supports the consideration that blocking specific host-cell functions that are required for viral replication might be an useful approach in antiviral therapy.

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REFERENCES

1. Angelova L., M. Remichkova, I. Ivanova *et al.* J. Basic Microbiol., 2006, 46, 504.
2. Barquero A.A., S.M. Villamil. Rev. Argent. Microbiol., 1997, 29, 32.
3. Corina P., S. Dimitris, T. Emanuil *et al.* Oftalmologia, 1999, 46, 55.
4. Dzeguze D., M.K. Indulen, N. Manolova *et al.* In: Abstracts Vth Int. Symposium on Antiviral Substances, Riga, 6-8 September, 1982, p. 117.
5. Gegova G., N. Manolova, J. Serkedjieva *et al.* Acta Microbiol. Bulg., 1993, 30, 37.
6. Kahlon J.B., M.C. Kemp, N. Yawei *et al.* Mol. Biother., 1991, 3, 214.
7. Lozitsky V.P., A.S. Fedchuk, L.E. Puzis *et al.* Voprosi Virusologii, 1987, 32, 413 (in Russian).
8. Kurokawa M., K. Nagasaka, T. Hirabayashi *et al.* Antiviral Res., 1995, 27, 19.
9. Musci I., Z. Gyulai, I. Beladi. Acta microbiol. Hung., 1992, 39, 137.
10. Reed L.J., H. Muench. Am. J. Hygiene, 1938, 27, 493.
11. Serkedjieva J. Phytother. Res., 2000, 14, 571.
12. Serkedjieva J. In: Recent Progress in Medicinal Plants, Govil J.N. Ed., 2008 (in press).
13. Serkedjieva J., N. Manolova. Basic Life Sci., 1992, 59, 705.
14. Serkedjieva J., A.J. Hay. Antiviral Res., 1998, 37, 221.
15. Serkedjieva J., E. Ivanova. Acta Virol., 1997, 41, 65.
16. Serkedjieva J., I. Zgorniak-Nowosielska. Acta Virol., 1993, 37, 258.
17. Serkedjieva J., N. Manolova, V. Maximova *et al.* Acta Microbiol. Bulg., 1986, 19, 18 (in Bulgarian).
18. Serkedjieva J., I. Roeva, M. Angelova *et al.* Acta Virol., 2003, 47, 53.
19. Serkedjieva J., I. Roeva, I. Ivanova. Biotechnol. Biotechnol. Eq., 2005, 19, Suppl., 6.
20. Serkedjieva J., R. Toshkova, T. Stefanova *et al.* Antivir. Chem. Chemother. 2007, 18, 75.
21. Schinazi R.F., J.C. Peters, C. Williams *et al.* Antimicrob. Ag. Chemother., 1982, 22, 499.
22. Steinhauer D.A. Virology, 1999, 258, 1.
23. Uzunov S., J. Serkedjieva, M. Lidaks. Acta Microbiol. Bulg., 1991, 27, 57.
24. Weaver, E., G.M. Arou. Canad. J. Microbiol., 1998, 44, 702.
25. Webb L. In: Inhibitors of Enzymes and Methabolism (General Principles of Inhibition), Mir Publishing House, Moskow, 1966, p. 484.
26. Zhirnov O.P., A.V. Ovcharenko, A.G. Bukrinskaja. Voprosi virusologii, 1985, 30, 207 (in Russian).

ANALYZING AND EVALUATION OF VARIOUS ANTIGENS FROM TRICHINELLA SPIRALIS LARVAE USING WESTERN BLOT

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SUMMARY

The accurate diagnosis of trichinellosis depends largely on the antigens used in serological tests. Nowadays there are two kinds of antigens, isolated from *T. spiralis* larvae which are used in diagnostic methods. One kind is crude extract from T.spiralis larvae and the other - excretory/secretory products from T.spiralis larvae, cultivated in virto.

The aim of the present study was to analyze these two kinds of antigens, obtained from *T. spiralis larvae* by SDS-PAGE and Western blot. Electrophoresis was performed in 4% stacking and 12% separating gel under reducing conditions. Western blot was performed in Towbin's buffer on nitrocellulose paper.

The results obtained showed a very complex antigen profile. Crude extract (CE) yielded in Western blot 7-11 bands with molecular weight (MW) between 28-198 kDa, while excretory/secretory antigen (E/S) revealed bands with MW between 35-208 kDa.

The presence of antigen fractions with molecular weight 45-55 kDa corresponds with the opinion of most authors who consider that these fractions are specific for *Trichinella spiralis*.

These results implicate that the antigens examined in our study are suitable for evaluation of antibody response in trichinellosis.

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Key words: Western blot, Trichinella spiralis, diagnosis, antigen

INTRODUCTION

Trichinella spiralis is a parasitic nematode which infects a wide variety of mammals, including humans, by invading epithelial cells that line the small intestine. Although the immune response against the parasite is very strong, *Trichinella* larvae make their way to the muscle cells and encapsulates there. For understanding the basis of protective immunity against *T. spiralis*, it is necessary to identify the antigens that evoke these responses. *T. spiralis* antigens, which are mostly glycoproteins, are key modulators or targets of the host immune system (1). In recent years Western blot became a method for confirmatory diagnosis of trichinellosis. In earlier studies about application of Western blot for diagnosis of trichinellosis crude preparations of *Trichinella* muscle larvae were used as antigen. Bands with molecular weight (MW) of 43-44, 64 and 127 kDa (5) were considered as highly specific to *T.spiralis*. Recently excretory/secretory (ES) products of *T.spiralis* muscle larvae (TSL), cultivated in vitro, were utilized as antigen. Using monoclonal antibody (7C2C5) which recognizes epitope specific for TSL, Sofronic-Milosavljevic et al. (2004) accepted as specific band triad of 45, 49 and 53 kDa, which is presented in ES antigen (7).

The aim of the present study was to analyze in Western blot two antigens, obtained from Trichinella spiralis larvae - crude extract and excretory/secretory products.

ABBREVIATIONS USED IN THIS PAPER: NONE

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MATERIALS AND METHODS

MATERIALS

***Trichinella spiralis* larvae (TSL)** were recovered from experimentally infected guinea pigs after a standard pepsin digestion method. Following washing of the larvae in water soluble whole extract were submitted to a cycle of thawing and freezing and sonicated. The crude extract (CE) was centrifuged at 10000 x g and the supernatant was used as antigen and stored at -70° C (2 batches).

Excretory/secretory products (ESP) were collected from TSL cultivated in vitro in culture media (DMEM), following the method of Gamble (1985) (2). The culture fluid was harvested, filtered and stored at -70° C until used (4 batches). Protein content of all antigen batches was quantified spectrophotometrically.

Sera samples

A pool of serum samples from 10 patients with epidemiologically, clinically and serologically confirmed trichinellosis was prepared as positive control and used for analysis by Western blot of obtained antigens.

METHODS

SDS-PAGE. Electrophoresis was performed in 4% stacking and 12% separating gel under reducing conditions according to the method of Laemmli (1970) (4). Coomassie blue R250 and silver staining methods were used for visualization of the fractions.

Western blot (WB) was performed in Towbin's buffer on nitrocellulose paper (8). The dilution of serum was 1:50 in TBS/0.5 % skimmed milk and the dilution of the conjugate was 1:200. DAB (Sigma) was used as a substrate.

Prestained protein standards (low range and broad range, Bio-Rad) were used as molecular weight markers.

RESULTS AND DISCUSSION

The results obtained showed a very complex antigen profile. Different number and molecular weight (MW) bands were demonstrated. Both batches of crude extract (CE_1 and CE_2) showed similar profile although number of bands obtained was different (table 1, fig. 1, lanes 2, 3). In the first batch CE_1

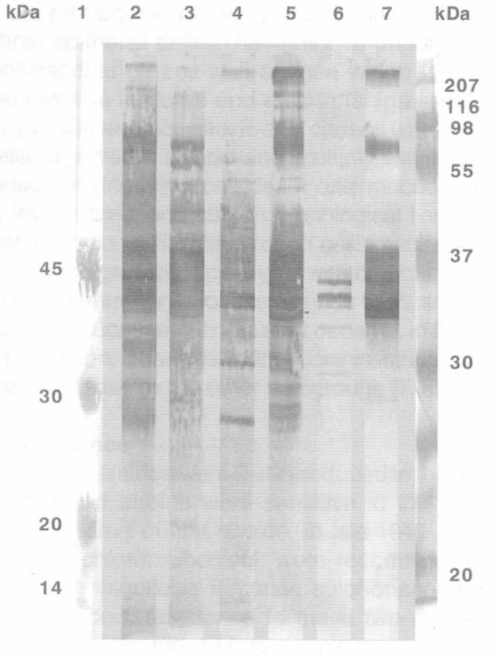

Fig.1. Western blotting analysis of *T. spiralis* preparations: 1. Low range marker; 2. CE_1; 3. CE_2; 4. ESP_1; 5. ESP_2; 6. ESP_3; 7. ESP_4; 8. Broad range marker

Tabl. 1. Range of bands in Western blotting analysis of *Trichinella* antigens

№	Used antigens	Number of bands	Range of bands, kDa
1.	CE_1 (5 mg/ml)	7	198, 104, 62, 55, 46, 36, 34
2.	CE_2 (2 mg/ml)	11	198, 112, 102, 83, 60, 55, 51, 45, 35, 31, 28
3.	ESP_1 (9.8 mg/ml)	10	104, 102, 75, 64, 55, 51, 45, 38, 34, 14
4.	ESP_2 (2.8 mg/ml)	14	196, 162, 104, 98, 90, 78, 75, 60, 50, 45 40, 38, 36, 34
5.	ESP_3 (2.7 mg/ml)	5	64, 55, 51, 45, 37
6.	ESP_4 (3.4 mg/ml)	6	208, 107, 64, 53, 45, 35

with used serum sample were recognized 7 antigen fractions with MW 198, 104, 62, 55, 46, 36 and 34 kDa. In the second batch CE_2 the obtained bands were 11 with MW of 198, 112, 102, 83, 62, 53, 51, 45, 36, 31 and 28 kDa. These results were not unexpected, because in previous studying we established by SDS-PAGE 10 fractions in crude extract from T.spiralis larvae with similar MW (Rainova et al., 1999) (6). Yera et al. (2003) obtained in Western blot with crude extract and sera from 60 patients with trichinellosis 11 bands, which confirmed that crude preparations from T.spiralis larvae yielded large number of antigenic fractions and most of them were recognized with sera samples from patients with trichinellosis (9). Authors chose as a specific profile bands with MW 43, 44 and 64 kDa. Batches of excretory /secretory products (ESP) showed some differences in obtained bands after testing in Western blot. ESP_1 and ESP_2 exhibited large number of bands - 10 in ESP_1 and 14 in ESP_2 with MW between 14 and 196 kDa and their analysis was similar to the bands obtained in CE (Tab.1, fig.1, lines 4, 5). In Western blot ESP_3 and ESP_4 yielded 5 and 6 antigenic bands with MW 64, 55, 51, 45 and 37 for ESP_3 and 208, 107, 64, 53, 45 and 35 for ESP_4 (table 1, Fig. 1, lanes 6, 7). According to Gamble (1985) specific in E/S preparations from Trichinella larvae was band triad with MW 45, 49 and 53 kDa (2). Recently Sofronic-Milosavljevic et al. (2004) using Western blot implicated that the existence of 45, 49 and 53 kDa bands could be a criterion for evaluation of anti - T.spiralis antibodies in sera of infected hosts (7). According to the literature most authors consider that the major antigenic fraction, obtained in the preparation from T.spiralis larvae has a MW of 45 kDa (3). Antigens with MW of 49 and 53 kDa, isolated from Gamble by affinity chromatography produced in mice significant levels of immunity against T.spiralis larvae (2).

All of our preparations from trichinella larvae (crude extract end excretory/secretory products) revealed bands with MW between 45 and 55 kDa, which correspond with the opinion of most authors that these fractions are specific for *Trichinella spiralis*. CE yielded bands with MW 55-53, 51, 46-45 kDa and E/SP - bands with MW 55-53, 51-50, 46-45 kDa. These results implicate that both kinds of antigens (crude extract and excretory/secretory products) are suitable for application in Western blot for diagnosis of trichinellosis.

REFERENCES

1. Alcantara, P. et al. Int. J. Parasitol.,1993, 23, 657-660.
2. Gamble, H.R. Exp. Parasitology, 1985, 59, 398-404.
3. Homan, W.L., et al. Parasitol. Res., 1992, 78, 112-119.
4. Laemmli, U.K. Nature, 1970, 227, 680-685.
5. Nunez, G. G., et al., J. Parasitol., 2000, 86, 1121-1124.
6. Rainova I., et al. Problems of Infectious and Parasitic Diseases, 1999, 2; 23-25.
7. Sofronic-Milosavljevic et al. Turk. J. Immunol, 2004, 9-2 Suppl.,152.
8. Towbin, H., et al. Proc. Natl. Acad. Sci. USA, 1976, 76, 4350-4354.
9. Yera, H., et al. Clin. Diagn. Lab.Immunol, 2003, 10, 793-796.

SHIGELLOSIS: PECULIARITIES AND MANAGEMENT

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SUMMARY

Infectious diseases kill about 11 million children each year and 99 per cent of these deaths occur in the developing countries. Notably, of the 1 1 million deaths, 4 million die within the first year of their life1. Acute diarrhoeal diseases rank second amongst all deaths due to infectious diseases accounting for 3.1 million deaths in under 5 children; 80 per cent of these deaths occur in children below 2 yr of age. In recent years etiology of salmonelloses was profoundly described. (2) It was introduced in context of WHO-Global Salm Surveillance system. (3) Shigellosis is an important cause of diarrhoeal deaths. It has been reported that no less than 140 million cases of shigellosis occur worldwide with 600,000 deaths annually; 60 per cent of such deaths are seen in children under 5 (4). In this review, attempts have been made to highlight the molecular epidemiology, epidemic and pandemic potential, current case management strategies including drug resistance problem and preventive aspects of shigellosis.

Key words: dysentery, shigellae, shigellosis

Definition

Shigellosis, commonly known as acute bacillary dysentery, is manifested by the passage of loose stools mixed with blood and mucous and accompanied by fever, abdominal cramps and tenesmus (a symptom characterized by incomplete sense of evacuation with rectal pain).

Clinical features

In some cases, there may not be any symptoms (asymptomatic), while in others it may produce mild to moderate dysentery or even fulminating dysentery with fever, severe abdominal cramps and rectal pain. Children may. have high fever with convulsions, rectal prolapse and later develop malnutrition. Shigella sonnei produces mild dysentery. S. flexneri and S. dysenteriae type 1 typically produce severe dysentery, particularly the latter.

Complications

Shigellosis may be associated with a large number of mild to severe life-threatening complications(5), particularly due to S.dysenteriae type 1. Children may have high fever, rectal prolapse and convulsions. Arthritis and arthralgia are complained by some patients. Intestinal perforation, haemorrhage, toxic megacolon and protein loosing enteropathy may complicate a shigellosis case. Leukemoid reaction (WBC count > 50,000/ cmm) and haemolytic uraemic syndrome (a triad of microangiopathic haemolytic anaemia, thrombocytopaenia and renal failure) are see'n in S.dysenteriae type 1 infection and may be fatal(6).

Epidemiology

Shigellosis is a highly contagious disease caused by Shigella spp. and humans are the principal reservoir of infection. The organism is acid resistant and can easily pass the gastric acid barrier. The infective dose is as low as 10-100 organisms only. The disease is transmitted faeco-orally, the commonest modes being person-to-person contact and contaminated food and water. Infected food handlers can spread the disease. Sometimes consumption of raw vegetables harvested in fields where sewage is used as fertilizer can cause contamination. Flies can breed in infected faeces and contaminate food. Washing clothes and bathing in ponds, which is a common practice in rural India, can also enhance transmission of the disease if the water is contaminated with excreta of an infected person. It is a disease of overcrowding, insanitary conditions and poor personal hygiene, and affects mostly children of developing countries. However, travelers visiting endemic areas may be affected by this disease if they do not take proper precautionary hygienic measures.

Incubation period of the disease is 1-4 days which is usually followed by sudden onset of acute symptoms. In mild cases the disease may be self limiting but severe disease requires appropriate medication. The disease is communicable as long as an infected person excretes the organisms in the stool and this can extend up to 4 wk from the onset of illness. However, timely antibiotic therapy can reduce the period of communicability. Secondary attack rates can be as high as 40. per cent especially among household contacts.

Microbiology

Shigellosis is caused by Shigella spp. which can be subdivided into four serogroups - S. sonnei, S. boydii, S. flexneri and S. dysenteriae. Each of them has a number of serotypes, e.g., S. dysenteriae type 1-12, S. sonnei phase I and II, S. boydii type 1- 18, S. flexneri type 1-6. However, three predominant strains are responsible for majority of shigellosis cases viz., S. sonnei, S. flexneri 2a and S. dysenleriae type 1. Of these, S. sonnei is encountered mostly in industrialized countries, S. flexneri 2a in developing countries and S. dysenteriae type 1 is the only epidemic as well as pandemic strain. S. dysenteriae type 1, which produces severe disease, may cause life-threatening complications, is usually multi drug resistant and can cause large epidemics and even pandemics with high morbidity and mortality.

Shigellae are facultative non motile, Gram negative bacilli. They are pathogenic primarily due to their ability to invade intestinal epithelial cells. The virulence factor is a smooth lipopolysaccharide cell wall antigen which is responsible for the invasive features and a toxin (shiga toxin) which is both cytotoxic and neurotoxic and causes watery diarrhoea. Shigella organisms invade and multiply within the colonie epithelial cells causing cell death and mucosal ulcers but rarely invade the blood stream. Histological findings include cellular infiltration with mixed round cells, neutrophilic in majority and disorganization of crypts with branching and dilatation. The inflammatory process extends to muscularis mucosae and submucosae with resultant oedema. In S.dysenteriae type 1 infection, changes in the colonie mucosa are more severe than those by the other serogroups (7, 8).

Drug resistance

When sulphonamides were first introduced in the early 1940s, all the Shigella strains were sensitive to this drug, which became the drug of first choice. In late 1940s, tetracycline followed by chloramphenicol, were recommended for the treatment of shigellosis because sulphonamides became ineffective. Soon, resistance to these two drugs was observed. Ampicillin and co-trimoxazole came to the rescue (9, 10). They were found to be clinically highly effective. However, during the epidemic in eastern India in 1980s, the isolated S. dysenteriae type 1 strains were found to be resistant

ABBREVIATIONS USED IN THIS PAPER: None

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to most of the antibiotics (11) except nalidixic acid which was found to be clinically highly effective (12) But later, S. dysenteriae type 1 strains isolated from an outbreak in Tripura were even resistant to nalidixic acid (13). In the late 1980s, fluoroquinolones (norfloxacin, ciprofloxacin and ofloxacin) were introduced and were found to be very effective in the treatment of shigellosis cases (14-18) including those caused by multi drug resistant S. dysenteriae type 1 strain.
Mechanisms of antibiotic resistance will be analyzed in another review.

Diagnosis

Diagnosis of shigellosis is made clinically by the typical features of bacillary dysentery with blood and mucus in stool although some cases may present with mild to moderate watery diarrhea initially. Dehydration is usually not a conspicuous feature. Microscopic examination of faecal smear stained with iodine shows presence of plenty of faecal leucocytes (> 10/high power field). Confirmation is made by stool culture, serological and biochemical tests (19).
Collection, transportation and culture of stool specimen
Specific diagnosis of shigella in stool specimens depends on the appropriate collection and transportation to the laboratory. Fresh stool samples collected from patients before initiation of therapy are preferred for microbiological tests because the chances of recovering the organisms are higher. For microbiologie cultures, fresh stool is preferred to rectal swabs in which the pathogens are less in number. Samples that cannot be cultured immediately should be kept in buffered glycerol-saline transport medium. Cary-Blair medium is the second option. Direct inoculation of culture plates at the bedside is the most efficient means of isolating shigella from the dysentery patients.
Stool specimens for isolation of shigella should be plated on both moderately selective medium such as MacConkey or deoxycholate citrate agar (DCA), and a highly selective medium such as xylose- lysin deoxycholate (XLD), Hektoen enteric (HE) or Salmonella- Shigella (SS) agar. Since the Shigella isolates growing in these plates do not change the colour of the pH indicator due to its inability to ferment lactose, it is easy to pick up the typical colonies.
Further identification can be made by using triple sugar iron (TSI) agar or Kligler iron agar (KIA), on which Shigellae are non- motile, produce an alkaline slant and acid butt due to inability to ferment lactose aerobically in the slope and the anaerobic fermentation of glucose in the butt, and fail to produce hydrogen sulphide or other gas. After tentative identification, strains can be speciated by serological methods, using grouping antisera. Rapid methods for the diagnosis of S. dysenteriae type 1 by means of fluorescent antibody staining have been established50. Till date, no reliable rapid method is commercially available and none are in use routinely anywhere.

Management

Diagnosis of shigellosis can be made clinically by the typical features of the disease. Sometimes laboratory confirmation is necessary especially for antimicrobial resistance pattern as it varies from place to place and from time to time. In case management of shigellosis antibiotics play a central role. Use of appropriate antibiotic hastens recovery, shortens the duration of excretion of pathogen in stool and possibly prevents complications. However, these should be chosen carefully considering the sensitivity pattern of the circulating strains. Presently in India, the antibiotics of choice are norfloxacin (400 mg) or ciprofloxacin (500 mg) or ofloxacin (200 mg) twice daily for 3-5 days(20). These drugs are not yet recommended for children and pregnant women although their use has shown that they are probably safe in children (21).

Although dehydration is not a common feature of shigellosis infection, but if it occurs or the stools are watery, patients should be given the oral rehydration salt (ORS) recommended by WHO / UNICEF. In severe dehydration, intravenous fluids preferably Ringer's lactate solution is recommended. However, clinical experience indicates that ORS is beneficial in all cases of shigellosis if given as routine fluid intake.
Anorexia poses a major problem for feeding especially in children. They should be encouraged to take small, frequent and easily digestible meals. This is easily achieved after an effective antibiotic is started when appetite improves and the patient is able to take food.

Prevention and control

Since the main route of transmission of shigellosis is through water, food and also person-to-person contact, the prevention and control strategies essentially include provision of safe water supply and adequate sanitation facilities, maintenance of good personal hygiene and food safety. Hand washing with plenty of water and soap is the most important single effective preventive strategy against shigellosis61. It is emphasized that hands should be washed before eating, before feeding children, after defeacation and after disposal of children's excr\eta. These measures are further reinforced in epidemic situations, when because of the very low infective dose of the organism and its potential for rapid spread, stringent control measures need to be instituted through simple but effective health education messages to the common masses.

CONCLUSION

Shigellosis is an important public health problem with high morbidity and also mortality mainly among children in developing country situations where overcrowding and poor personal hygiene are rampant. Of all the Shigellae spp, S. dysenleriae type 1 is notorious for producing not only large scale epidemics but also pandemics which are characterized by multiple drug resistance and several serious complications including haemolytic uraemic syndrome (HUS). The mainstay of treatment is appropriate antibiotics but development of drug resistance poses a serious therapeutic challenge. Preventive long-term measures like improved sanitation and personal hygiene may be a difficult task to achieve in the near future specially among the impoverished urban and rural communities. Thus, an alternative preventive strategy in the form of suitable vaccines against S.dysenteriae type 1 and S.flexneri 2a is urgently required to save mankind from the scourge of this dreaded disease.

REFERENCES

1. http://www.who.int/infectious-disease-report accessed on 20th April 2004.
2. Petrov P., G. Asseva, T. Kantardjiev. Etiology of salmonelloses in Bulgaria, 1986-1997. Probl. Inf. Parasit. Dis., 2001, 29 (2), 28-33.
3. Petrov P., G. Asseva. WHO Global Salmonella Surveillance. Infectology, 2007, XLIV, 92-93.
4. http://www.globalhealth.org accessed on 20th April 2004.
5. Barrett-Connor E, Connor JD. Extraintestinal manifestations of shigellosis. Am J Gastroenterol 1970; 53 : 234-45.
6. Sinha AK, Bhattacharya SK, Sen D, Sengupta PG, Pal SC. Leukemoid reaction in Shigel/a dysenleriae type 1 infection. Indian J Med Res 1987; 85 : 500-2. :
5. Mendizabal-Morris CA, Malta I, Gangarosa EJ, Guzman G. Epidemic Shiga-bacillus dysentery in Central America. Derivation of the epidemic and its progression in Guatemala. Am J Trop Med Hyg 1969; 20 : 927-33.
6. Pal SC, Sengupta PG, Sen D, Bhattacharya SK Deb BC. Epidemic shigellosis due to Shigella dysenteriae type 1 in South Asia. Indian J Med Res 1989; 89 : 57-64.
7. Anand BS, Malhotra VS, Bhattacharya SK, Datta P, Datta D, Sen D, et al. Rectal histology in acute bacillary dysentery. Castroenterology 1986; 90 : 654-60.
8. Mathan MM, Mathan VI. Ultrastructural pathology of the rectal mucosa in Shigella dysentery. Am J Pathol 1986; 123 : 25-38.
9.Nelson JD, Kusmiesz H, Jackson LH. Comparison of trimethoprim/ sulfamethoxazole and ampicillin therapy for s h ige l los is in ambulatory patients. J Paediatr 1976; 89 : 491.

10. Ross S, Controni G, Khan W. Resistance of shigellae to ampicillin and other antibiotics. J Am Med Assoc 1972; 221 : 45.
11. Bose R, Nashipuri JN, Sen PK, Datta P, Bhattacharya SK, Datta D, et al. Epidemic of dysentery in West Bengal: Clinicians' enigma. Lancet 1984; ii : 1160.
12. Bhattacharya SK, Datta P, Datta D, Bhattacharya MK, Sen D, Saha MR, et al. Relative efficacy of trimethoprimsulphamethoxazole and nalidixic acid for acute invasive diarrhoea. Antimicrob Agents Chemother 1987; 31 : 837.
13. Sen D, Dutta P, Deb BC, Pal SC. Nalidixic acid resistant Shigella dysenteriae type 1 in eastern India. Lancet 1988; : 911.
14.Chunder N, Bhattacharya SK, Biswas D, Niyogi SK, Kumar R. Isolation of a fluoroquinolone resistant Shigella dysenteriae 1 strain from Calcutta. Indian J Med Res 1997; 106 : 494-6.
15. Bhattacharya SK, Bhattacharya MK, Dutta P, Sen D, Rasaily R, Moitra A, et al. Randomized clinical trial of norfloxacin for shigellosis. Am J Trop Med Hyg 1991; 45 : 683-7.
16. Bhattacharya MK, Nair GB, Sen D, Paul M, Debnath A, Nag A, et al. Efficacy of norfloxacin for shigellosis: A double-blind randomised clinical trial. J Diarrhoeal Dis Res 1992; 10 : 146-50.
17. Bhattacharya SK, Bhattacharya MK, Dutta D, Dutta P, Paul M, Sen D, et al. Single-dose ciprofloxacin for shigellosis in adults. J- Infect 1992; 25 : 117-9.
18. Sen D, Niyogi SK, Ghosh AR, Pal SC. Susceptibility of epidemic strains of shigella to ofloxacin and other anti-microbials. Indian J Pathol Microbiol 1988; 31.:. 306-10.
19. World Health Organisation. 1987. Manual for laboratory investigation of acute enteric infections. CDD/83.3. World Health Organisation, Geneva, Switzerland.
20. Bhattacharya SK, Sur D. An evaluation of current shigellosis treatment. Expert Opin Pharmacother 2003; 4 : 1315-20.
21. Bhattacharya SK, Bhattacharya MK, Dutta D, Dutta S, Deb M, Das KP, et al. Double-blind, randomized clinical trial fr safety and efficacy of norfloxacin for shigellosis in children. Acta Paediatr 1997; 86 : 319-20.