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

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ACKNOWLEDGEMENTS

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A RAPID AND VERSATILE ASSAY FOR SCREENING OF AAC(6')-IB-CR IN MULTIDRUG-RESISTANT ENTEROCBACTERIACEAE

I. N. Ivanov[1], S. Sabtcheva[2], T. Saga[3], Y. Ishii[3], M. Kaku[4], T. Kantardjiev[1]

[1] National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria
[2] Laboratory for Clinical Microbiology, University specialized hospital for active treatment in oncology (National oncology centre), Sofia, Bulgaria
[3] Department of Microbiology and Infectious Diseases, Toho University School of Medicine, Tokyo, Japan
[4] Department of Infection Control and Laboratory Diagnostics, Tohoku University Graduate School of Medicine, Sendai, Japan

ABSTRACT

Plasmid-mediated quinolone resistance has emerged as an important clinical problem due to the rapid spread in clinical isolates of *Enterobacteriaceae*. Despite conferring only low level of resistance to ciprofloxacin and norfloxacin the plasmid-mediated ACC(6')-Ib-cr acetyltransferase is able to promote the selection and accumulation of high-level resistant chromosomal mutants during quinolone treatment. Therefore, in order to guide therapy, rapid assays for its detection are necessary. This study investigates one such method which is based on the BccI-Restriction Fragment Length Polymorphism (RFLP). It removes the disadvantages of previously used techniques and is considered cost-effective and versatile.

KEYWORDS:

Enterobacteriaceae, quinolone resistance, AAC(6')-Ib-cr, BccI-RFLP

ADDRESS FOR CORRESPONDENCE:

Ivan N. Ivanov, PhD
National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance
National Center of Infectious and Parasitic Diseases
26 Yanko Sakazov blvd
1504 Sofia, Bulgaria
Phone: +359 2 9446999 ext. 208
e-mail: ivanoov@gmail.com

INTRODUCTION

Quinolone resistance has initially been associated with stepwise accumulation of chromosomal mutations in the type II topoisomerases genes, efflux pumps or dysfunction of porin channels (1). Since 1998 however, despite the fully synthetic nature of quinolones, three additional mechanisms of plasmid-mediated quinolone resistance (PMQR) have been reported: Qnr-mediated target protection, modification of ciprofloxacin and norfloxacin by the AAC(6')-Ib-cr enzyme (encoding gene is also designated *aacA4-cr*), and active efflux due to QepA and OqxAB (2). AAC (6')-Ib-cr is a bifunctional variant of an aminoglycoside acetyltransferase active on kanamycin, tobramycin, netilmicin and amikacin but also able to acetylate ciprofloxacin and norfloxacin, which both have piperazinyl secondary amines (3). The other quinolones lacking an unsubstituted piperazinyl nitrogen remain unaffected. The AAC(6')-Ib-cr has two unique amino acid changes, Trp102Arg and Asp179Tyr, which together are necessary for the enzyme ability to acetylate sufficiently those fluoroquinolones. It has been shown that both mutations must be present for a fourfold increase in ciprofloxacin MIC, whereas Asp179Tyr alone could confer up to twofold MIC increase (3). Although Robicsek et al. and subsequent studies did not report naturally occurring single mutation hybrid alleles (e.g Arg102 combined with Asp179), both hybrid allele combinations are currently present in NCBI (MG516909, HG314123), some even being designated as *cr* variants (KC542812) (Fig. 1).

Various screening techniques for *aacA4-cr* have been reported e.g. BstF5I/BtsCI PCR-Restriction fragment length polymorphism (RFLP) (4), gap-ligase chain reaction (Gap-LCR) (5), Real-time PCR followed by simultaneous High-resolution melting curve analysis (HRM) (6). However, all of them have been designed to solely detect one of the two mutations misidentifying the hybrid variants and inevitably leading to false positive results as well as probable over-reporting of *aacA4-cr* (Fig. 1C). Phenotypic disc-based screening methods have also been reported but their applicability is limited since *aac(6')-Ib-cr* almost exclusively co-exists with other fluoroquinolone resistance mechanisms that might interfere with interpretation (7, 8). So far, DNA sequencing is the only tool which can

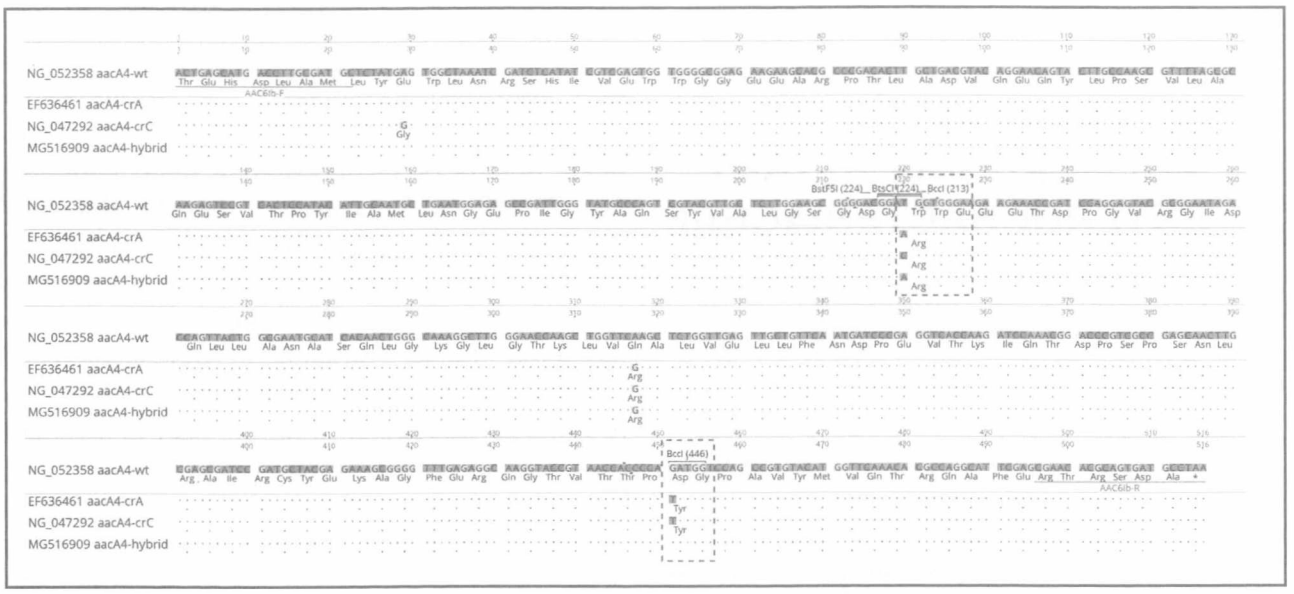


Figure 1. Alignment of various aac(6')-Ib variants showing primer positions, BccI, BtsCI and BstF5I restriction positions and Trp102Arg and Asp179Tyr mutations. Note that none of the restrictases except BccI cuts at Asp179.

unambiguously confirm the *cr* variant of *aac(6')-Ib* gene .

This study aims at resolving this problem by reporting a versatile and cost effective screening approach for *aac(6')-Ib-cr* differentiating all hybrids.

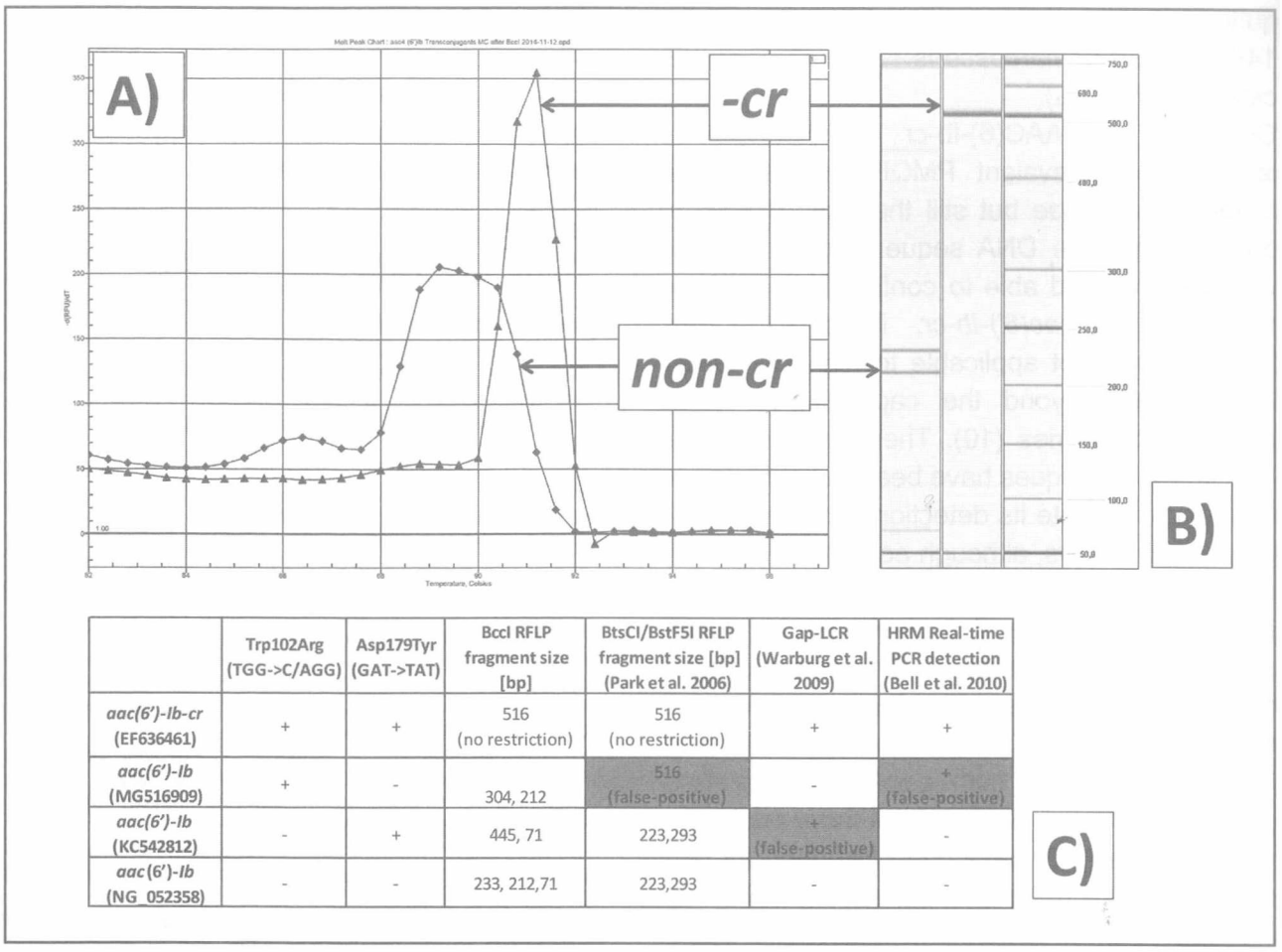
MATERIALS AND METHODS

BccI-PCR-RFLP. Bacterial DNA was extracted by boiling for 10 min in 10% Chelex100 (Bio-rad). Real-time PCR (15µl) was performed with primers (0.25µM each) AAC6Ib-F 5'-ACTGAGCATGACCTTGCGATGC and AAC6Ib-R 5'-TTAGGCATCACTGCGTGTTCG (9) generating a 516bp amplicon with the following reagents : 1x PCR buffer with 2.25mM $MgCl_2$, 0.2mM of each dNTP, 0.8x EvaGreen (Biotium Inc., USA), PerfectTaq (5Prime, UK) and 2µl (~20ng) DNA. The thermal program consisted of 95°C initial denaturation for 4 min followed by 35 cycles of 94°C for 30s, 61°C for 30s and 72°C for 35s (fluorescence detection) on a IQ5 Real-time PCR system (Bio-Rad). Positive reactions were supplemented with 5 µl restriction mix containing 1.5 µl 10x CutSmart buffer, 1.5U BccI (New England Biolabs, USA) and 3.5 µl ddH_2O, homogenized and incubated at 37°C for 60 min. Melting curve analysis was performed by increasing the temperature in 0.4°C steps for 10 seconds fluorescence from 72°C to 96°C. Restriction fragments were either resolved on 2.5% agarose gels or by QiAxcel Capillary electrophoresis system (Qiagen) using the 0M500 pre-programmed method. DNA sequencing verification was performed as described previously (9).

A total of 99 ESBL- and AAC(6')-Ib-producing isolates of *Enterobacteriaceae*, including 52 isolates of *Escherichia coli* (n=47), *Citrobacter freundii* (n=2), *Klebsiella pneumoniae* (n=1), *Enterobacter aerogenes* (n=1) and *Morganella morganii* (n=1), previously confirmed to harbour *the aac(6')-Ib-cr* gene by PCR and sequencing were studied with the newly developed method (9). In addition to this, 100 consecutive, non-duplicate, ESBL- and 16S rRNA methyltransferase (16S-RMTase)-producing isolates of *Enterobacteriaceae* were studied.

RESULTS

The BccI-RFLP method is versatile and could be coupled with different detection formats. By adding the fluorescent dye EvaGreen in PCR mastermix all detection steps could be performed on Real-time PCR systems followed by final HRM analysis after BccI restriction (Fig. 2A). Alternatively, conventional PCR could be used and restriction fragments are readily separated by capillary electrophoresis (Fig. 2B) or standard 2.5% agarose gels (data not shown). Moreover the *aacA4* and *aacA4-cr* co-existence (heterozygote variants) can be differentiated by BccI-RFLP resulting in incomplete restriction electrophoretic pattern (presence of the 516-



	Trp102Arg (TGG->C/AGG)	Asp179Tyr (GAT->TAT)	BccI RFLP fragment size [bp]	BtsCI/BstF5I RFLP fragment size [bp] (Park et al. 2006)	Gap-LCR (Warburg et al. 2009)	HRM Real-time PCR detection (Bell et al. 2010)
aac(6')-Ib-cr (EF636461)	+	+	516 (no restriction)	516 (no restriction)	+	+
aac(6')-Ib (MG516909)	+	-	304, 212	516 (false-positive)	-	+ (false-positive)
aac(6')-Ib (KC542812)	-	+	445, 71	223,293	+ (false-positive)	-
aac(6')-Ib (NG_052358)	-	-	233, 212,71	223,293	-	-

Figure 2. Various detection formats of the BccI-RFLP method and comparison with previously published ones: A) Melting curve analysis after BccI restriction; B) Capillary electrophoresis after BccI restriction; C) Comparison of various methods to detect both mutations.

bp amplicon together with the three restriction fragments). The full procedure could be performed in 4 hours for 96 samples which is useful in high throughput screening studies.

The preliminary testing of BccI RFLP on a panel of 99 well characterised strains showed 100% concordance with the DNA sequencing results (100% sensitivity and 100% specificity). The method was further tested on 100 recent clinical isolates. Overall, thirty-six (36%) isolates were positive for *aac(6')-Ib*, from which 26 (26% of all) had the *cr* variant. The distribution of *aac(6')-Ib-cr* was as follows: 14/29 (48%) of *K. pneumoniae*, 11/26 (42%) of *Serratia marcescens*, 7/15 (47%) of *E. cloacae*, 3/7 (43%) of *C. freundii* and 1/7 (14%) of *E. coli* isolates. Co-existence of *aacA4* and *aacA4-cr* was not evidenced.

DISCUSSION

AAC(6')-Ib-cr-mediated fluoroquinolone resistance was discovered in 2006 but numerous retrospective studies proved that it has been present at least since 1999 (1, 4). In Bulgaria, PMQR was first reported from clinical isolates of *Enterobacteriaceae* collected at the cancer hospital in 2000 to 2005 (9). The *aac(6')-Ib-cr* gene was present in 36 % (52/163) of ESBL-producing enterobacteria, including 47/94 (50 %) *E. coli*, 2/10 (20 %) *C. freundii*, 1/40 (2.5 %) *K. pneumoniae* and 1 each of *E. aerogenes* and *M. morganii* isolates. It was disseminated manly by CTX-M-15 plasmids and associated with *qnrB* in two *C. freundii* strains. Most of the isolates were resistant to ciprofloxacin, reflecting probably its ability to promote higher-level quinolone resistance mutations (9).

In this study, we approved the newly developed method by confirming correctly all of the *aac(6')-Ib-* and *aac(6')-Ib-cr*-positive isolates reported in our previous study (9). In addition to this, we found that thirty-six (36%) of the 100 ESBL- and 16S-RMTase-producing isolates of *Enterobacteriaceae* were positive for *aac(6')-Ib*, from which 26 had the *cr* variant, distributed

quite distinctly, mainly among *K. pneumoniae* 14/29 (48%), *S. marcescens* 11/26 (47%) and *E. cloacae* 3/7 (43%).

Currently, the AAC(6)-Ib-cr acetyltransferase is the most prevalent PMQR mechanism in *Enterobacteriaceae* but still the most difficult to detect. While the DNA sequencing is the sole published method able to confirm both required mutations in *aac(6')-Ib-cr*, it is expensive, laborious and not applicable to high throughput studies and beyond the capacities of small hospital laboratories (10). Therefore, a number of different techniques have been published in an attempt to facilitate its detection. The phenotypic disc-based assays, although accessible, are only effective when *aacA4-cr* is the only mechanism of expressed resistance (7, 8). However, in hospital settings clinical isolates of *Enterobacteriaceae* often harbour multiple determinants such as 16S rRNA methylases, Qnr proteins etc. and even the co-existence of both *aacA4* and *aacA4-cr* genes is observed. Some of the more applicable molecular methods have been designed for only one of the two mutations. As shown in Fig. 2C previously published methods detect either one of the two required mutations and might provide false positive results for the *cr* variant in case of hybrid variants with the Arg102/Asp179 genotype. The BccI-RFLP assay presented here overcomes all of the mentioned disadvantages and has already applied successfully to detect the *aacA4-cr* in various multidrug-resistant *Enterobacteriaceae* while expressing additional aminoglycoside and quinolone resistance determinants.

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PUTATIVE AGGREGATION-RELATED SURFACE PROTEINS IN *BIFIDOBACTERIUM LONGUM*1/2

T. Yungareva, Z. Urshev, Z. Dimitrov, M. Michaylova

LB Bulgaricum PLC, Sofia, Bulgaria

ABSTRACT

Earlier observations have shown that *Bifidobacterium longum* 1/2 demonstrates auto-aggregation properties or co-aggregates with some enteropathogenic bacteria. In this study we aimed to collect initial data that will point to the potential factors that promote the aggregation properties of this strain. An overnight culture was grown in modified TS broth, washed and treated with different solutions with or without proteases to test the effect on the aggregation of cells. Surface protein extracts obtained with 5.0M LiCl were separated by SDS-PAGE. The genome of *B. longum* 1/2 was used to perform *in silico* analyses of candidate cell surface proteins that may be involved in aggregation. Trypsin, chymotrypsin and pepsin treatments drastically reduced the extent of aggregation pointing to the proteinaceous nature of the factor involved in cell-to-cell interaction. For cells treated with LiCl a partial loss of auto-aggregation and co-aggregation abilities was observed that correlated with the extraction of a specific protein with MW of approximately 13 kDa. In *B. longum* 1/2 four sortase genes were found, only one of which was part of a putative pilus-encoding gene cluster as it contained the typical major (FimA) and minor (FimB) pilin subunit genes. The results from this initial study suggest that the aggregation phenotype of *B. longum* 1/2 may be attributed either to a LiCl-extractable13 kDa surface protein or to the presence of pili-encoding gene cluster in the genome of this strain.

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

KEYWORDS:
aggregation, *Bifidobacterium longum*,cell surface proteins,pili

INTRODUCTION

Bifidobacteria are the most abundant group of lactic acid-producing bacteria in children and adults (1,2). There are different mechanisms that contribute to the health-promoting (probiotic) effect of bifidobacteria and lactobacilli in the human gut such as bacteriocin production (3, 4, 5), protection of the epithelial layer by strong adherence (2, 6) and biofilm formation (7), competitive exclusion of pathogens from the intestinal surface(8), production of organic acids inhibiting the growth of undesired bacteria (9, 10), indirect immunomodulating effect by activation of immunocompetent cells or inducing their tolerant state (2).

Some strains of probiotic bacteria show strong ability to auto-aggregate or co-aggregate with other bacterial species. The aggregation phenotype is strain-specific and has an important role in the interaction of probiotic bacteria with the host and potentially pathogenic bacteria. Due to their aggregation and co-aggregation abilities probiotic bacteria can prevent colonisation by pathogens and assist in their removal from the human mucosal surfaces (11-16). Enteropathogens may cause a number of socially important diseases (17, 18). Another issue that urges the application of probiotic cultures is the growing number of antibiotic-resistant pathogen strains (19).

The factors/molecules determining the aggregation can be of various nature. Several bifidobacterial aggregation factors with proteinaceous nature are reported. In the genome of *Bifidobacterium bifidum* PRL2010 Turoni et al. (20) identified three pilus-encoding gene clusters. Two of them are important factors that promote bacterial aggregation and adhesion to the human enterocytes (20, 21).

González-Rodríguez et al. (22) found that the transaldolase is a specific aggregation factor and mucin-binding protein in *B. bifidum* A8 (22). Despite the fact that in general this enzyme has a cytoplasmic location it also can be found on the cell surface and be readily extracted with other surface proteins. The surface-exposed transaldolase appears to perform an additional, "moonlighting" function as aggregation factor in this strain (22).

Bifidobacterium longum 1/2 was selected after performance of aggregation and co-aggregation assays. The strain demonstrated auto-aggregation and co-aggregation abilities with the enteropathogenic bacteria *Escherichia coli*, *Salmonella* Typhimurium and *Salmonella* Enteritidis, provided by the National Reference Laboratory (NRL) "Enteric Infections" at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia (Yungareva, unpublished results).*B. longum* 1/2 is of human origin with a previous history of immunomodulation and industrial production.

The aim of the present work was to collect initial evidence and data that will point to the potential factors that promote the aggregation and co-aggregation properties of *B. longum* 1/2.

MATERIAL AND METHODS

Bacterial culture

B. longum 1/2 was isolated form a healthy adult and was maintained in the culture collection of LBBulgaricum PLC (Sofia, Bulgaria). The bacterial culture was grown at 37°C overnight in modified Trypticase Soy Broth (TSB without dextrose, Becton Dickinson, BBL) supplemented with (g/l): tween 80 – 1.0, glucose – 7.5, L-cysteine – 0.2, yeast extract – 2.0; pH 7.0.

Enzymatic and chemical treatments of bacterial cells

An overnight culture was grown in modified TS broth, harvested by centrifugation and washed twice with PBS buffer, pH 7.2. Aliquot of washed cells was treated for 30 min at 37°C with 5.0M Li Clorfor 1 h at 37°C with one of the following solutions: 0.1M citrate-phosphate-0.1 M NaCl (pH 4.5) (buffer A); buffer A containing 0.05M sodium iodate or 0.05M sodium periodate; 0.05M Tris-HCl-0.1M NaCl (pH 8.0) (buffer B); buffer B containing trypsin or chymotrypsin (5mg/ml); 0.05M glycine-HCl-0.1M NaCl (pH 2.2) (buffer C) and buffer C containing pepsin (5mg/ml). After incubation the cells were washed twice with PBS and then they were used for the aggregation and co-aggregation assay (23). Washed and untreated cells were used as a positive control.

Aggregation and co-aggregation assay

Both aggregation and co-aggregation assays were performed following the methods described by Savvidou(24)with some modifications (25). Different strains of *E. coli*, *S.* Typhimurium and *S.* Enteritidis were used in the co-aggregation assay. Aggregation and co-aggregation were evaluated by microscope observation.

Electrophoretic separation of cell surface protein extracts

To compare the protein fractions that are specifically extracted with 5M LiCl and Buffer B, the supernatants of cells treated with these two solutions, as described above, were concentrated twenty times on an ultrafiltration spin column with molecular weight cut-off of 1000 Da (Sartorius Stedim Biotech). The concentrated proteins were treated and separated by SDS-PAGE on 12% acrylamide gel according to Laemmli (26). Protein broad range molecular markers (Promega, USA) of 10, 15, 25, 35, 50, 75, 100, 150 and 225 kDa were used for determination of the molecular weight. Proteins were visualised by Coomassie blue R-350 (Amersham) staining.

In silico analyses of candidate cell surface proteins of *B. longum* 1/2 that may be involved in aggregation

The genome of *B. longum* 1/2 was used to screen for the presence of pilus-encoding gene clusters and to extract the aminoacid sequence of the transaldolase in this strain. The corresponding proteins were analysed and compared with the aminoacid sequence database of similar proteins in other bifidobacteria using different programs and tools – BLASTP (27),(http://blast.ncbi.nlm.nih.gov); MEGA 6; CLC Sequence Viewer (www.clcbio.com).

RESULTS AND DISCUSSION

Enzymatic and chemical treatments of bacterial cells

For cells treated with LiCl a partial loss of auto-aggregation and co-aggregation abilities was observed compared to the untreated control. We suggest that this phenomenon was due to the previous extraction by LiCl solution of a loosely bound surface structure of *B. longum* 1/2 involved in aggregation. Both trypsin and chymotrypsin (dissolved in buffer B) and pepsin (dissolved in buffer C) treatments drastically reduced the extent of aggregation and co-aggregation pointing to the proteinaceous nature of the factor involved in cell-to-cell interaction (Fig.1). Protease-free buffers B and C did not affect cell aggregation.

After treatment with sodium iodate and sodium periodate the cells precipitated and could not be used for the subsequent aggregation/

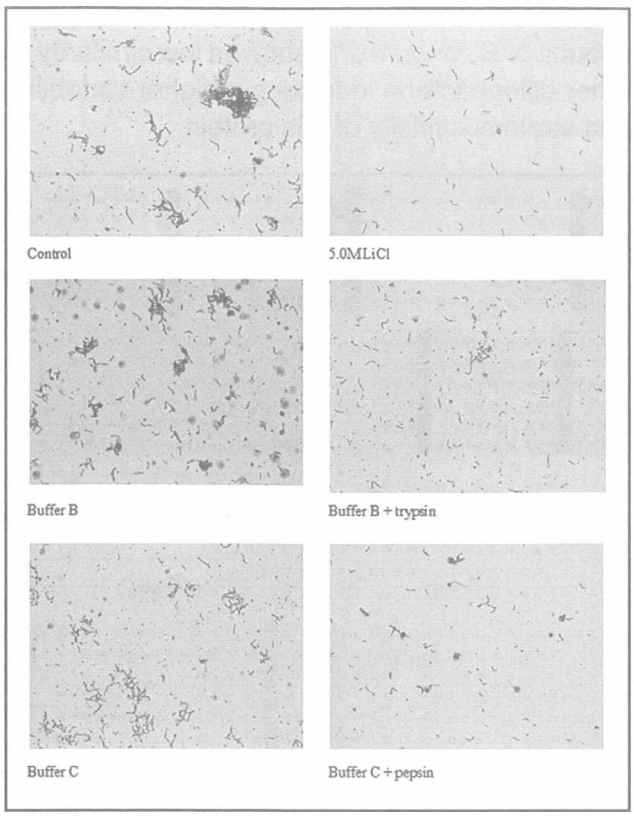

Fig. 1. Effect of different buffer and enzyme treatments on the aggregation ability of *B. longum* 1/2 cells.

co-aggregation assay. Moreover, buffer A itself had inhibiting effect on cell aggregation. Therefore, the role of cell surface carbohydrates in aggregation could not be tested under our experimental conditions.

Different surface proteins have been described to play essential role in the aggregation of lactobacilli. Examples of such proteins are a surface protein in *Lactobacillus gasseri* 2459 (28), a 32 kDa protein in *L. gasseri* 4B2 (29), the aggregation factors in *Lactobacillus johnsonii*(30) and *Lactobacillus crispatus* M247 (31),*Lactobacillus acidophilus* NCFM (32), the S-layer proteins of *L. crispatus* ZJ001 (33) and *Lactobacillus κefiri* CIDCA 8321 (34). In many cases these proteins interact with carbohydrates and teichoic acids (29, 31). Aggregation properties may be also dependent on the production of exopolysaccharides (35).

***In silico* analyses of candidate cell surface proteins of *B. longum* 1/2 that may be involved in aggregation**

The genome of *B. longum* 1/2 was screened for pilus-encoding gene clusters as such genes are associated with the aggregation phenotype in this species (20, 21). In *Bifidobacteria* such clusters encompass genes encoding a major pilin subunit (FimA or FimP), one or two ancillary minor subunits (FimB and FimQ) and an associated sortase-encoding gene (21).

In *B. longum* 1/2 four sortase genes were found. Only one of them was part of a putative pilus-encoding gene cluster. The cluster consisted of three genes that showed similarity to earlier annotated sequences as follows: „possible cell surface protein similar to FimA fimbrial subunit of *Actinomyces naeslundii*" (FimA), "Internalin-like protein (LPXTG motif) Lmo0409 homolog" (putative FimB) and „Sortase A, LPXTG specific" (SrtA). The three encoded proteins had a similar size with the corresponding proteins from the pilus-encoding gene clusters in*B. bifidum* PRL2010 *pil*1, *B. longum* subsp. *longum* NCC2705 and *B. longum* subsp. *longum*DJO10A (21).

Table 1. Length of the three genes (sortase, *fim*A and *fim*B) comprising the pilus-encoding cluster in different bifidobacteria.

Strain	Lenght (bp)		
	Sortase	*fim*A	*fim*B
B. bifidum PRL2010 *pil*1	327	530	2574
B. longum subsp. *longum* NCC2705	328	525	2573
B. longum subsp. *longum*DJO10A	327	539	2573
B. longum 1/2	325	611	2321

The sortase of the putative pilus-encoding gene cluster in *B. longum* 1/2 belongs to the class C family as all sortases in other pili-encoding genetic loci.In Gram-positive bacteria the sortases from class C are involved in the formation of pili, extracellular structures built from multiple pilin subunits. Such structures contribute to microbial adhesion and biofilm formation. In most cases class C sortase genes are included in clusters that contain the genes that encode their substrates (36, 37).

The protein sequences of the sortase, FimA and FimB were compared with similar proteins from different species of *Bifidobacteria* and the conservative amino acids were found with MEGA 6 software package (Fig.2).The Fim Agene of *B. longum* 1/2 was identified as it contained typical major pilin-subunit motifs and domains – the sortase recognition site (CWSS motif), the pilin-like motif (TVXXK) and the E-box (Fig.2)(21, 38, 39). The sortase recognition site and the pilin-like

motif are involved in the generation of covalent bonds between the major subunits, while the E-box is responsible for the interaction between the major and minor pilin subunits. (21).The aminoacid sequence of Fim B, the minor pilin subunit of *B. longum* 1/2, showed low similarity to other bifidobacteria indicating a higher variability and strain specificity of this protein.

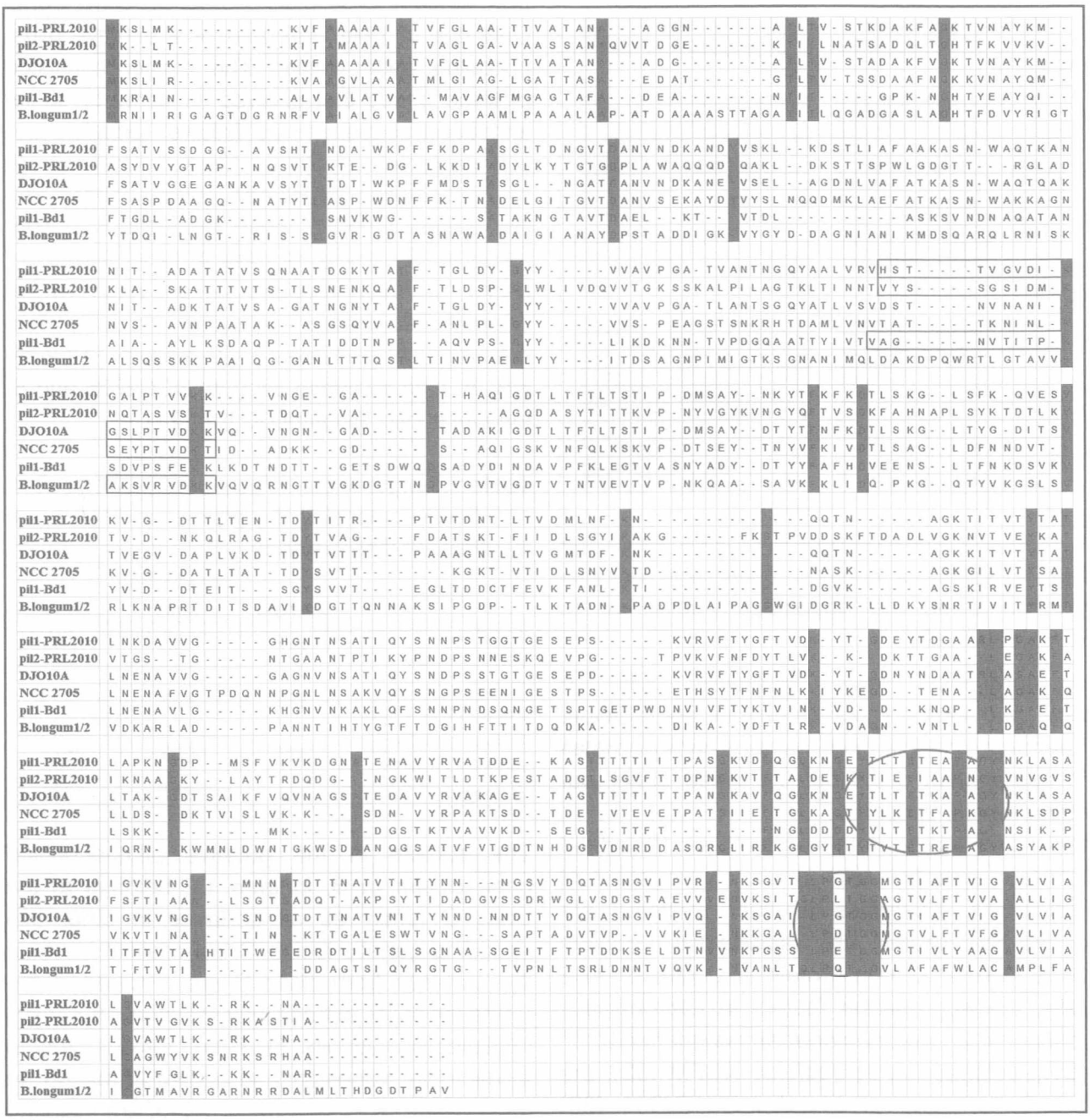

Fig. 2. Alignment of the FimA protein in different bifidobacterial strains and *B. longum* 1/2. The conserved amino acids are presented in dark background. The E-box and LPXTG motif are marked with circles. The pilin motif is marked with a rectangle (MEGA 6 software package). FimA-pil1-PRL2010 and pil2-PRL2010 – alternative FimAs in *B. bifidum* PRL2010; DJO10A – *B. longum* DJO10A; NCC 2705 – *B.longum* NCC 2705; pil1-Bd1 –*B. dentium*Bd1.

A single copy of the transaldolase gene was identified in the genome of *B. longum* 1/2. The aminoacid sequence of the *B. longum* 1/2 transaldolase was aligned with the same enzyme in *B. bifidum* A8. With only seven aminoacid substitutions the transaldolases of these two bacteria appear almost identical. In *in vitro* experiments Gonzalez-Rodriguez et al. (22) demonstrate that the transaldolase of *B. bifidum* A8 is functioning as an aggregation and adhesion

factor. Their study also suggests that proteins are not the only factors involved in the adhesion phenotype of *B. bifidum* A8 but also there is a partial role of sugar moieties (40). The results reported by these authors are the first evidence that the transaldolase of bifidobacteria, although it is an intracellular enzyme, also plays the role of an extracellular factor in aggregation and mucin binding. The mechanisms by which enzymes contribute to aggregation may be different. As an example, the glycosyltransferase of *Lactobacillus reuteri* TMW1.106 and other Gram-positive bacteria plays a key role in the process of aggregation due to the synthesis of glucans(41, 42, 43, 44), while transpeptidases such as sortase A in *Lactobacillus plantarum* CMPG5300 and other lactobacilli are essential for adhesion and aggregation as they anchor different sortase-dependent proteins to the cell surface (7, 45, 46).

Electrophoretic separation of cell surface protein extracts

The comparison of the proteins extracted by 5.0M LiCl and Buffer B showed a specific protein with MW of approx. 13 kDa that was extracted only with 5.0M LiCl (Fig. 3). As auto-aggregation of *B. longum* 1/2 was abolished only after LiCl treatment and not Buffer B, we can speculate that this 13kDa protein may be involved in the aggregation process. The molecular mass of the transaldolase was estimated to be approx. 39kDa, however both extracts had identical protein bands within this range. Therefore, the loss of aggregation after LiCl extraction of surface proteins in *B. longum* 1/2 may not be associated with the presence or absence of transaldolase on the cell surface of this strain.

Conclusions

The results from this initial study point to the proteinaceous nature of the factor(s) involved in the occurrence of the aggregation phenotype of *B. longum* 1/2. One such factor can be a 13 kDa protein, the extraction of which with LiCl correlates with the loss of the aggregation property. Also, in the genome of *B. longum* 1/2 a pili-encoding gene cluster is present with the corresponding proteins sharing similarity to pilin proteins in other bifidobacteria suggested to be involved in adhesion and aggregation. Although the transaldolase of this strain is almost identical to that of other strains of bifidobacteria, there is still no evidence that this enzyme is related to the aggregation of *B. longum* 1/2.

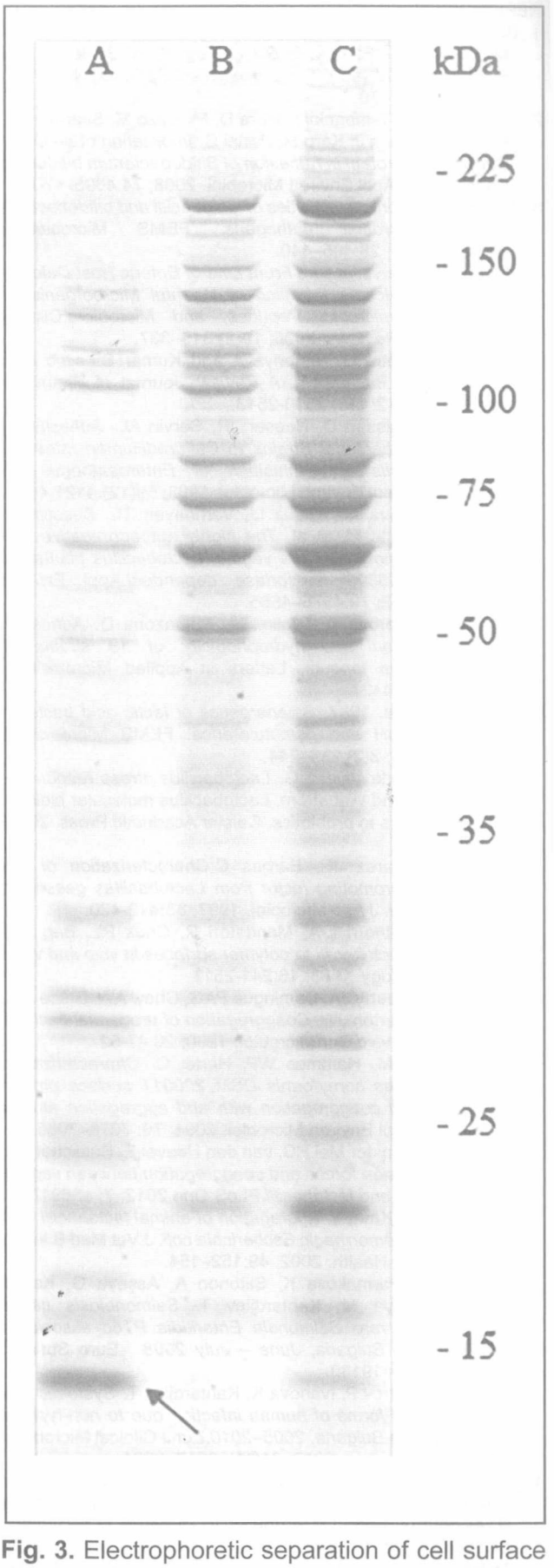

Fig. 3. Electrophoretic separation of cell surface proteins of *B.longum* 1/2 after incubation in 5.0M LiCl (A), PBS (B) and buffer B (C). The arrow indicates a 13 kDa protein specific for the LiCl extract.

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MICROBIAL DIVERSITY OF MALE GENITOURINARY TRACT MICROBIOME IN DNIPRO CITY, UKRAINE

[1]Sklyar T.V., [1*]Voronkova O.S., [2]Papiashvili M.H.

[1]Oles Honchar Dnipro National University, Dnipro, Ukraine
[2]Independent Laboratory INVITRO LLC, Dnipro, Ukraine

ABSTRACT

The most common causes of male reproductive system disorders are infectious and inflammatory diseases of the genitourinary tract. Obligate and opportunistic pathogens are involved as causative agents of different pathological conditions and can provoke complications, raising the need for a complex therapy with antibiotics. By performing real-time PCR assay using the "Androflor" test-system, the composition of genitourinary tract microbiota was determined in 67 men. Of them, 16% of cases were healthy individuals. The following obligate pathogens were detected: *Chlamydia trachomatis* (3.0%), *Mycoplasma genitalium* (6.0%), *Trichomonas vaginalis* (1.5%) and *Neisseria gonorrhoeae* (1.5%). Opportunistic microorganisms were identified in 71.6% of cases. Among them, staphylococci were the most commonly isolated (75.0%) with prevalence of *Staphylococcus epidermidis* (58.3%). We also identified *Enterococcus faecalis* and members of *Enterobacteriaceae* (52.1%). In 22.4% of cases there were associations of more than 2 pathogens or opportunistic microorganisms. The number of microorganisms exhibited some deviations as compared with microbiota of healthy people. The majority of microorganisms or their associations had quantity of 2.5 – 4.9 lg CFU/ml, which is a significant level indicating microbial disorder. More than 74% of opportunistic microorganisms were susceptible to fluoroquinolones and cephalosporins. Low susceptibility was determined for penicillins, macrolides and tetracyclines. The use of molecular genetic methods significantly speeds up the identification process, which in turn enables early initiation of treatment.

KEYWORDS:
microbiome, male genitourinary tract, PCR, Androflor, antibiotic resistance

ADDRESS FOR CORRESPONDENCE:
*Olga S. Voronkova
72 Gagarin Ave., Dnipro, Ukraine, 49050
email: voronkova_olga@i.ua,
tel. +380962250463

INTRODUCTION

The microbiome of human body is one of the most significant factors affecting health. Diversity of bacteria composition determines the state of functioning of different organs and systems as a whole. The presence of saprophytic bacteria supports the functional activity, whereas opportunistic or obligate pathogens are implicated in disorders (1, 2). Therefore, it is necessary to understand the state of human microbiota and to determine the presence of certain microorganisms by accurate and rapid methods (3).

Nowadays, among the most common causes of male reproductive system disorders are infectious and inflammatory diseases of the genitourinary tract (4, 5, 6, 7).

The infectious process and its consequences can affect various organs of the genitourinary system: the prostate, seminal vesicles and their ducts, testicles, etc. (8, 9, 10, 11). Infection often leads to a chronic process in the gonads that provokes infertility (12, 13). In the last years, the number of inflammatory diseases of male genital organs caused by opportunistic microorganisms has increased significantly (14, 15, 16, 17). Besides pathogens, other factors such as stress, overcooling, trauma, intoxication etc. can lead to inflammation as they disrupt the proper functioning of the immune system mechanisms and thus opportunistic microbes can cause pathological conditions (14, 15, 18).

According to Kungurov et al. (17), the most common causes of non-specific inflammatory processes of the genitourinary tract of men are *Staphylococcus epidermidis* – 55.2%, *S. aureus* – 8%, *Streptococcus* spp. – 6%, *Enterococcus* spp. – 6%, *Escherichia coli* – 4.3%, *Proteus vulgaris* – 3.4%, *Pseudomonas aeruginosa* – 2%, *Klebsiella* spp. – 0.3%. The bacteriological method is commonly used for studying the microbiome composition but its performance is labour-intensive and time-consuming. For this reason, the application of rapid methods

for microbial identification becomes necessary. In this sense, molecular genetic methods are considered a prospect because they are highly sensitive and rapid (19).

The aim of this research was to analyse the qualitative and quantitative composition of the male genitourinary tract microbiome in healthy individuals and patients with pathological conditions, using the "Androflor" test system.

MATERIAL AND METHODS

The research was conducted by the Department of Microbiology at the LLC "Independent Laboratory InVitro" (Dnipro city, Ukraine). Genitourinary tract microbiota composition was determined in 67 men aged 20 to 60 years. Samples scraped from the urethra were studied with multiplex real-time polymerase chain reaction (PCR) assay using the DT-96 / DT-322 amplifier (LLC "DNA-technology", Russia) and "Androflor" test-system (LLC "DNA-technology", Russia) for detection of microbial DNA/RNA, regardless of cultural and morphological features. Furthermore, the structure of microbiologically relevant biotope was characterised in order to assess the pathological role of each group of microorganisms in a particular patient. The application of a highly sensitive, specific and rapid to perform (1–1.5 h) method, makes obtaining of results and diagnosis of unculturable microorganisms by real-time PCR technology clinically meaningful for the study of infectious diseases. The survey was conducted according to the recommendations provided in the manufacturer`s guide (20). 24 markers were determined beginning with human genomic DNA to assess the adequacy of the material. The absence of human genomic DNA in the test sample indicates the absence of epithelial cells lining the genitourinary tract, the surface of which is colonised by microorganisms. Total bacterial mass along with the presence of *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp. was also determined. The listed bacteria are representatives of the normal microbiome in the male genitourinary tract, accounting for approximately 60% of the total bacterial mass. Decrease in this proportion is a sign of dysbiosis. The "Androflor" test-system successfully identifies and performs qualitative analysis of the obligate pathogens *C. trachomatis, M. genitalium, T. vaginalis, N. gonorrhoeae*; also of opportunistic microorganisms as *G. vaginalis, Ureaplasma* spp., *M. hominis, Bacteroides* spp., *P. aeruginosa*, members of the *Enterobacteriaceae* family, *Candida* spp. etc. When there is no inflammatory process, total bacterial mass does not exceed 10^4 g/ml. Normally, in the genitourinary tract of men obligate pathogens such as *C. trachomatis, M. genitalium* and *N. gonorrhoeae* are not present. Lactobacilli should not exceed 10% of all isolated microorganisms. In a number of cases, transient microbiome can cause inflammatory diseases of the lower sections of the male genitourinary tract (21).

Identification of isolated enterobacteria, staphylococci and streptococci was carried out with culture methods in accordance with features of bacteria, listed in the Bergey's Manual of Determinative Bacteriology (22). Antibiotic susceptibility was determined with the disc diffusion method using the following standard discs (HiMedia, India): oxacillin (1 µg), cefotaxime (30 µg), ceftriaxone (30 µg), erythromycin (15 µg), ofloxacin (5 µg), levofloxacin (5 µg), gentamicin (10 µg) and chlortetracycline (30 µg).

RESULTS AND DISCUSSION

By using the "Androflor" test-system, microbiota composition of the genitourinary tract was determined in 67 men aged 20 to 60 years (Fig. 1). Of them, 11 (16%) were healthy. Results showed the presence of non-pathogenic staphylococci, streptococci and corynebacteria. Obligate pathogens were also detected in 8 men (12%) with *C. trachomatis* – 2 cases (3.0%), *M. genitalium* – 4 cases (6.0%), *T. vaginalis* – 1 case (1.5%) and *N. gonorrhoeae* – 1 case (1.5%).

Results from the analysis of 48 samples collected from men with prevalence of opportunistic microorganisms and without infectious pathology showed that staphylococci were the most commonly isolated microorganisms - 36 strains (75.0%). Among them, the following coagulase-negative staphylococci were identified: *S. epidermidis* – 21 strains (58.3%), *S. saprophyticus* – 5 strains (13.9%), *S. haemolyticus* – 6 strains (16.7%) and 4 *S. aureus* strains (11.1%). The second most frequently isolated microorganism was *Enterococcus faecalis* – 17 strains (35.4%). As for Gram-negative bacteria, 5 *E. coli* strains (10.4%) were identified; *Proteus* and *Klebsiella* were isolated

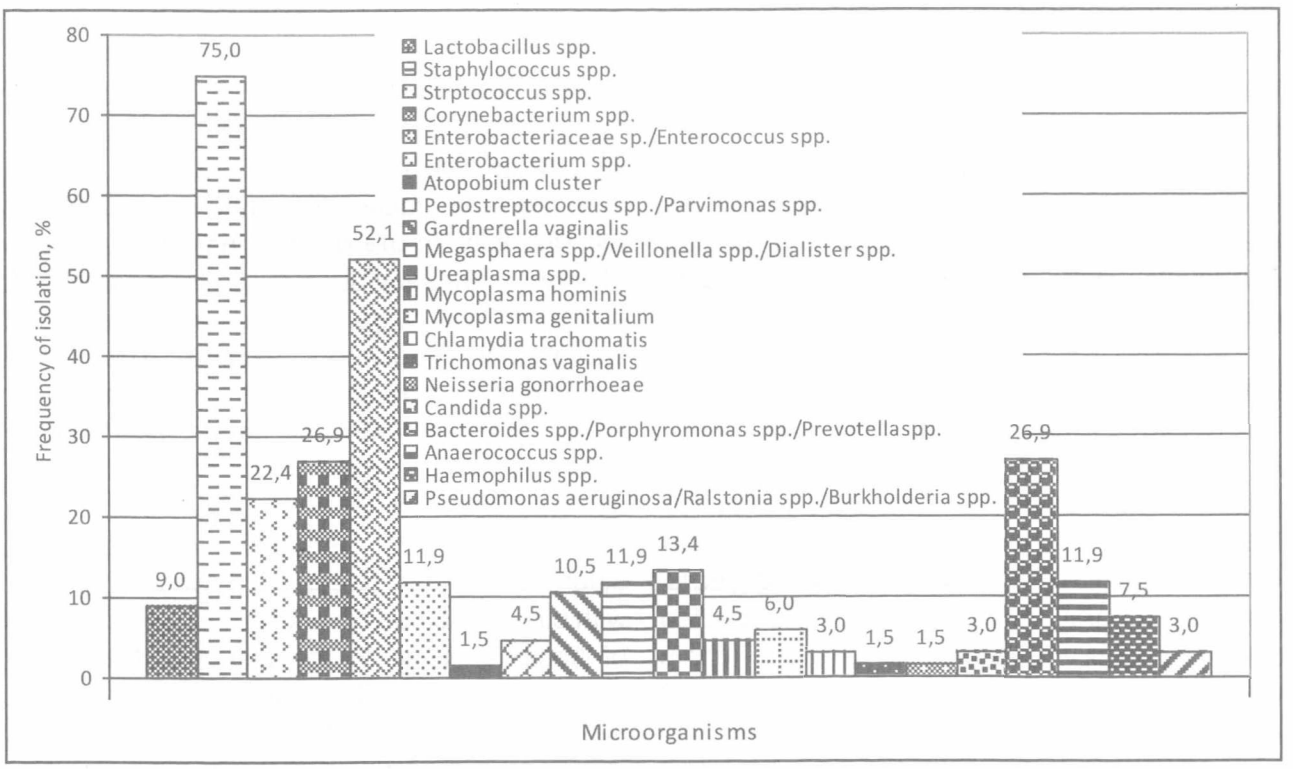

Fig. 1. Composition of microbiota of male genitourinary tract using test-system "Androflor"

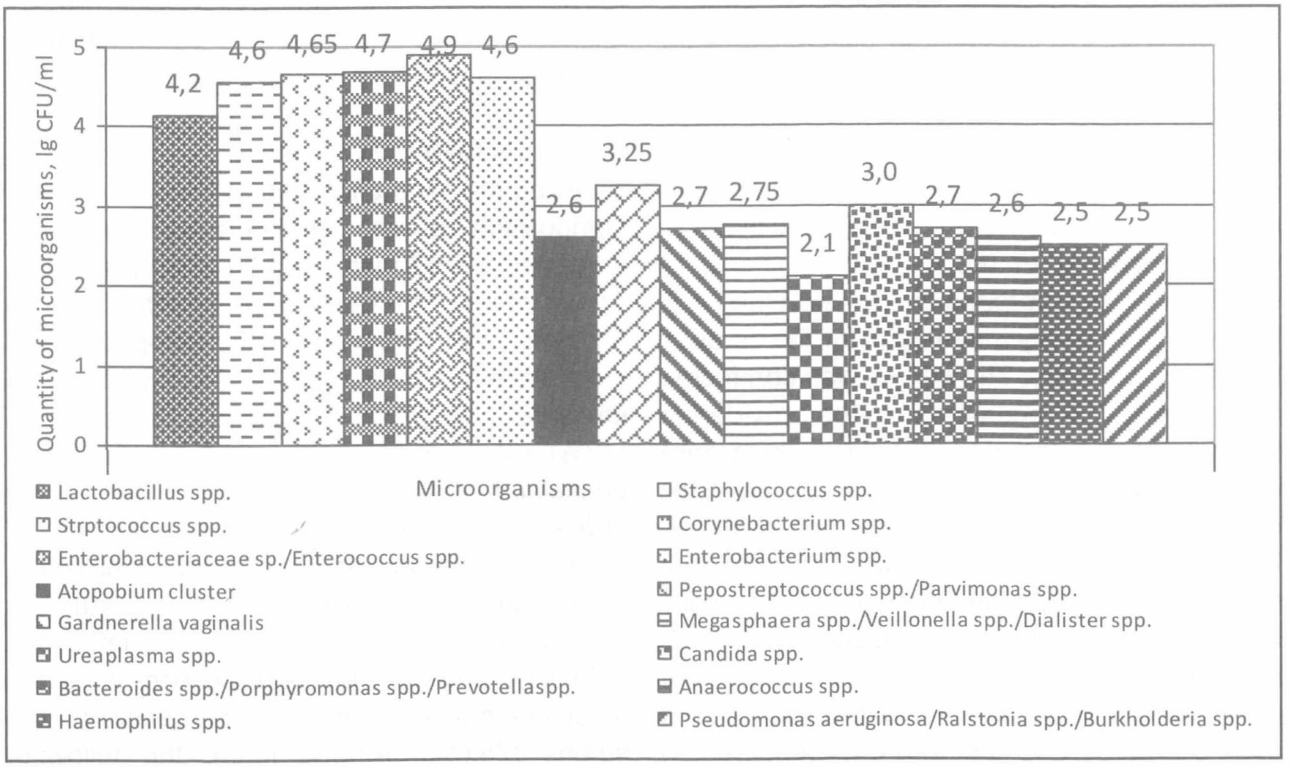

Fig. 2. Quantity of microorganisms of male genitourinary tract using test-system "Androflor"

in a significantly smaller number of cases - 4.2% and 2.1%, respectively. *Candida* species were rarely identified – 2 strains (3.0%).

In 15 cases (22.4%) more than 2 strains were isolated, which is a common feature of genitourinary tract microbiota (23). Thus, staphylococci were co-isolated most often with streptococci and members of the *Enterobacteriaceae* family. Microbial associations involving 2 and 3 microorganisms were identified in 14.9% and 7.5% of cases, respectively. The highest proportion in the first group belongs to

Staphylococcus / Streptococcus – 69%, as well as *Staphylococcus / Enterobacteriaceae* – 31%. *Staphylococcus-Enterobacteriaceae-Candida* dominated among the triple associations (40%). Our data is in accordance with results from a number of authors (8, 15) pointing to the predominance of associations with coagulase-negative staphylococci. The microbial association is not constant and uniform (6, 12, 17, 23) as it can vary depending on the immune status of the body, drug therapy and other factors (14, 24). According to our data, the microflora of the urogenital tract varied with the age of patients. Gram-positive cocci dominated both in associations and in monomicrobial cultures from young men (25 to 35 years old), and Gram-negative bacteria – in individuals of middle and senior age (40 to 60 years old). This pattern is due to age-related deviations of urine outflow from the bladder associated with benign prostatic hyperplasia (14).

The number of microorganisms exhibited some deviations as compared to the microbiota of healthy people (5, 10, 17). The majority of identified microorganisms or their associations had quantity of 2.5 – 4.9 lg CFU/ml, which is a significant level indicating microbial disorder.

The presence of associations of pathogenic and opportunistic microorganisms in the genitourinary tract microbiota was observed in all cases with pathological conditions, significantly complicating treatment, since each pathogen often displays different spectrum of resistance to antibiotics (15, 24).

At the next stage was determined the antibiotic susceptibility of opportunistic microorganisms isolated from patients with inflammatory disease of the genitourinary tract. Staphylococci were susceptible to fluoroquinolones (ofloxacin – 77.8%) and cephalosporins (ceftriaxone – 83.3%). More than half of the isolated staphylococcal strains were resistant to penicillins (oxacillin – 63.9%) and macrolides (erythromycin – 58.3%), and 36.1% were resistant to tetracyclines. Streptococcal strains were susceptible to cephalosporins (cefotaxime – 76%, ceftriaxone – 80%) and fluoroquinolones (ofloxacin – 68%, levofloxacin – 72%). Up to 52% of streptococci were susceptible to oxacillin, whereas resistance to aminoglycosides, tetracyclines and macrolides was determined for gentamicin – 72%, chlortetracycline – 80% and erythromycin – 68%. Members of the *Enterobacteriaceae* family had low susceptibility to macrolides and tetracyclines in 87.5% of cases. The highest level of susceptibility was determined for fluoroquinolones (ofloxacin – 75%), which is in correlation with other studies (25, 26, 27). Furthermore, isolates from *Enterobacteriaceae* had different levels of resistance to antimicrobials. *Klebsiella* spp. and *Proteus* spp. strains were resistant to a higher number of antibiotics compared to *E. coli* strains, which is consistent with data from other surveys (24, 28, 29).

The conducted investigation demonstrates the necessity of continuous monitoring of opportunistic pathogens isolated from the urogenital tract of men with inflammatory diseases of urogenital organs. In order to solve effectively this problem, real-time PCR using the "Androflor" test-system as a qualitative and quantitative analysis of male urogenital tract microflora allows the detection of microorganisms, regardless of cultural and morphological features and in particular, microorganisms that cannot be cultivated. This serves as the basis for developing rational antibiotic therapy schemes aiming to reduce antimicrobial resistance.

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SELECTIVE PRESSURE ANALYSIS OF HUMAN RESPIRATORY SYNCYTIAL VIRUSES CIRCULATING IN BULGARIA

I. Georgieva, S. Angelova, N. Korsun

National Reference Laboratory "Influenza and ARD", Department of Virology,

ABSTRACT

In order to investigate the genetic and evolutionary diversity of co-circulating genotypes of human respiratory syncytial viruses (HRSV) in Bulgaria, we conducted selective pressure analysis of the second hypervariable region of the attachment G protein of HRSV. The study included representative numbers of all HRSV-positive nasopharyngeal samples collected from children aged under 5 years, during two consecutive winter seasons (2014/2015 and 2015/2016). The HRSVs were detected using Real-timeRT-PCR assay. The G genes were partially sequenced and tested for positive selection. For HRSV group A, selection pressure analysis revealed purifying selection in the ON1 genotype. Three codons at position 274, 297 and 298 were positively selected by at least two different methods. For HRSV group B, no positive selection site was detected in the BA9 genotype. This is the first report describing the selective pressure analysis of HRSV genotypes circulating in Bulgaria.

KEYWORDS:

respiratorysyncytial virus, selective pressure

INTRODUCTION

Human respiratory syncytial virus (HRSV) is one of the most common viruses inducing acute respiratory infections in infants and young children. The HRSV infection often involves lower respiratory tract and is the most frequent cause of hospitalisation of children under five years of age (1, 2). In Europe and Bulgaria HRSV infection occurs as well-defined seasonal epidemic outbreaks during winter months that coincide with the influenza season (3).

HRSV is an enveloped virus with single-stranded negative-sense RNA genome. The envelope of the virus contains three surface glycoproteins: G (glycoprotein), F(fusion protein) and SH (small hydrophobic protein). Based on genetic and antigenic variations in structural proteins human respiratory syncytial viruses have been subdivided into two major antigenic groups - A and B, and these group shave been further classified into genotypes based on genetic divergence within the G gene (4, 5, 6). The G protein has an essential role in attachment of the virion to the host cell; it is rich in serine and threonine residues and is therefore highly glycosylated. The external domain of the protein contains two hypervariable regions (HVRs) flanking the putative receptor-binding site (7). The second hypervariable region (HVR2) is a hotspot for mutations making itsuitable for molecular epidemiological analyses (8, 9).

HRSV G gene evolves rapidly in a manner which is similar to the HA gene of influenza A viruses (10, 11). Genetic variations occur in HRSV due to mutation especially in the second hyper variableregion of the G protein gene and include amino acid substitutions in the mature protein. This antigenic variation may contribute to the susceptibility of some individuals to repeated infection especially in the early years of life (12). Sites demonstrating negative and positive selection have been found in the second hypervariable region of the G gene (10). Positively selected sites may benefit the survival of the viruses and may enable HRSV to escape from immune responses, while negatively selected sites can further sustain functions of viruses (13). Thus, it is important to analyse the G gene in order to obtain a better understanding of the properties of HRSV. In our study we conducted selective pressure analysis in addition to a previous phylogenetic analysis of the HRSV viruses circulating in Bulgaria.

MATERIAL AND METHODS

Clinical samples

Respiratory samples were collected as part of the National Influenza Surveillance Program.

ADDRESS FOR CORRESPONDENCE:

Irina Georgieva
National Centre of Infectious and Parasitic Diseases,
1233 Sofia, Bulgaria
44A Stoletov Blvd.,
E-mail: georgieva@irrito.com;
Tel/Fax: +359 29318132

Nasopharyngeal swabs from children at the age offive years or younger, ambulatory treated or hospitalised for influenza-like illness or acute respiratory illness,were enrolled in the present study. All samples were obtained between October 2014 and May 2016 from different regions of the country.

Extraction of nucleic acids, Real-time RT-PCR and nucleotide sequencing

Viral nucleic acids were automatically extracted from respiratory specimens using a commercial *ExiPrepDx Viral DNA/RNA Kit (Bioneer)* according to the manufacturer's instructions.

Detection of HRSV viruses was carried out by Real-time RT-PCR assay based on specific primers/probe in singleplex reactions with the use of *SuperScript® III Platium® One Step Quantitative RT-PCR System* kit and *AgPath-ID One Step RT-PCR* kit *(Applied Biosystems, Thermo Fisher Scientific, USA)*.Subtyping was performed for HRSV-positive samples by multiplex RT-PCR assay using subgroup-specific primes and probes targeting F and N genes, as previously described (14).

A representative number of HRSV-positive samples were selected for amplification of the second hypervariable region of the Gprotein gene. RSV-A- and RSV-B-specific oligonucleotide primers used for sequencing of the G geneand the conditions of the PCR reactions were previously described (9).The amplicons were purified using a *PureLink® Quick Gel Extraction Kit (Invitrogen)*, according to the manufacturer's instructions. The purified amplicons were sequenced commercially *(BioNeer, Korea)* in both directions using a *BigDye Terminator v. 3.1 sequencing kit (Applied Biosystems, ThermoFisher Scientific, USA)*.

Phylogenetic analysis

A multiple sequence alignment of the second hypervariable region sequences of the Bulgarian HRSV strains included in this analysis had previously been done and the results were reported (14, 9).

Selective pressure analysis

Selective pressure on the G gene was estimated by calculating synonymous (dS) and non-synonymous (dN) substitution rates at every codon with single-likelihood ancestor counting (SLAC) method,fixedeffects likelihood (FEL) method (15),the mixed effects model of evolution (MEME) (16), and fast unconstrained Bayesian approximation (FUBAR) (17) on the *Datamonkey server*(18). In order to avoid excessive false-positive rate, sites with SLAC, FEL and MEME p-values of 0.1 and a FUBAR posterior probability of >0.90 were accepted as candidates for selection. The positively selected sites were considered as the sitesunder weak selection pressure by at least two different methods.

RESULTS AND DISCUSSION

A total of 610 nasopharyngeal swabs from children, aged 5 years or younger were enrolled in the present study. Human respiratory syncytial viruses were detected in 157 samples. Among the positive samples HRSV-A was identified in 35% (55 samples) and HRSV-B in 51% (80 samples). Co-infection with both subgroups was also detected in 8 (5%) of the positive samples. For 14specimens (9%), the subgroup could not be determined due to low viral load (9).

It is known that the amino acid sequences of the G protein have both conservative and hypervariable regions (19). The second hypervariable region is a target of intensive molecular studies because it contains multiple epitopes for neutralising antibodies(20). Amino acid substitutions within this region may lead to antigenic changes which is associated with selective pressure due to host immune responses (13, 10, 21). These changes are strongly linked to re-infections (22).

The second hypervariable region of the G gene was successfully sequencedin 46 out of the 157 HRSV-positive samples. The phylogenetic analysis showed that all 21 sequenced Bulgarian HRSV-A strains belonged to the ON 1genotype with the characteristic duplication of 72 nucleotides starting after the residue 850 of the G gene and resulting in duplication of 24 amino acids. The nucleotide position is according to RSV-A2 prototype virus, GenBank accession number M11486.The vast majority (24/25) of the studied RSV-B strains were classified as genotype BA9 (9) containing the characteristic of all BA viruses duplication of 60 nucleotides. This insertion results in duplication of 20 amino acids motif,starting from position 240. The amino acid numbering corresponds to strain BA/4128/99 (GenBank: Nucleotide AY333364), protein position 219-315.

The selective pressure analysis was done for both groups of Bulgarian HRSV. The HRSV-A data set contained 21 ON1 sequences of Bulgarian strains and reference ON1(Accession

numbers: JN257693; JN257694; KM402627; KP284649; KJ710366; KF030154) and NA genotypes (GenBank: AB470478; JQ844856). Sequences with internal stop codons or deletions were excluded from the HRSV-B data set, so the analysis was done for 9 Bulgarian BA9 strains,and reference BA9 (Accession numbers: DQ227395; KM402711; KT371593; KJ710409) and BA strains (GenBank: Nucleotide AY333364).

A global analysis of selective pressure made using the SLAC model indicated an estimated overall dN/dS ratio of 0.649for HRSV-A and 0.575for HRSV-B. This relatively low dN/dS ratio indicates purifying selection, as described by some authors (23, 24) and suggests that the codons positions were relatively conserved. However, the site-specific analyses identified sites under positive and negative selection in the HRSV-A alignment (Table 1).

Table 1. Positive and negative selection sites for HRSV-A identified in this study.

Method	Positive	Negative
FEL	273, 297, 298	213, 226, 238, 248, 264, 277, 280, 299
SLAC	274, 298	280
MEME	297	None
FUBAR	274, 298	213, 226, 238, 248, 264, 280

One site (298) was under positive selection by at least three of the methods used. Two sites (274 and 297) were identified as being under weak positive selective pressure by two different models. These mutations were found in the duplicated region in ON1 genotypes showing that this region is under strong evolutionary pressure. It is known that the amino acid substitution at position 274 is associated with antibody escape (25, 13, 24, 7) suggesting that the effect of this mutation on the antigenic properties of HRSV-A viruses may provide evolutionary advantage of the ON1 genotype.

Only onesite (232) exhibited positive selection in the Bulgarian genotype BA9 according to mixed effects model of evolution (MEME). We detected two (221, 312) negative selection sites only by FUBAR method. These resultssuggested that strainswith genotypes BA 9 did not experiencestrong selective pressure in the host (26, 27).

Our results from selective pressure analysis revealed that the genes of Bulgarian HRSV have predominantly negatively or neutrally selected evolving sites thus suggesting that forces driving the evolution of HRSV are mainly stochastic (25). Investigations of sites under positive or negative selection are an important addition to the phylogenetic analysis of viruses circulating in Bulgaria and might be a successful follow-up study for other viruses like parvovirus B19 (28). Continued molecular epidemiological surveillance of HRSV is essential for further understanding of HRSV evolution and its mechanisms of immune evasion.

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RT-PCR SYSTEMS FOR DETECTION OF ARBOVIRUSES

I. Trifonova, I. Christova, E. Panayotova, T. Gladnishka*

National reference laboratory ofvector-borne infections, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria

ABSTRACT

The number of vector-borne viruses (tick- and mosquito-borne) is huge. They have widespread distribution and potential to cause conditions ranging from mild self-limiting illnesses to severe diseases with fatal outcome. Viruses of particular importance that belong to this group are Tick-borne encephalitis virus (TBEV), West Nile fever virus (WNV), causative agents of dengue fever (DENV), yellow fever (YFV), Japanese encephalitis (JEV), as well as zika (ZIKV) and Toskana (TOSV) viruses. Among them, TBE and WNV are known to cause infections in Bulgaria. However,on a global scale the possibility of importing infections is an actual issue and the preparedness for fast and accurate diagnosis is very important. In order to improve diagnostic capabilities for early and reliable detection of arboviruses, genetic methods for direct detection were developed and tested with clinical samples, reference materials and samples received from external quality assessment. In recent years, a number of new PCR protocols were implemented for routine diagnostics in our laboratory, including genetic methods for WNV (Real-time PCR detection of lineage 1 and 2), ZIKV (conventional and Real-time PCR) and Real-time detection protocols for TBEV, DENV, YFV, TOSV, ZIKV and Usutu virus. Reliability of the introduced methods was confirmed by international external quality assessment controls as well as detection of the specific RNAs in two patients with WNF and two patients with imported dengue fever.

KEYWORDS:

arboviruses, genetic methods

ADDRESS FOR CORRESPONDENCE:

*Iva Trifonova, PhD
Department of Microbiology
National Centre of Infectious and Parasitic Diseases
26 Yanko SakazovBlvd.
1504, Sofia, Bulgaria
Tel. + 359 885 440 557, Fax: +3592 943 3075
E-mail: iva_trrifonova@abv.bg

INTRODUCTION

The number of viruses transmitted by mosquitoes, ticks and phlebotomus as vectors is enormous. Vector-borne viruses can cause conditions ranging from mild self-limiting diseases to severe illness with fatal outcome.

Pathogenic vector-borne viruses of major concern belong to several families. The *Flaviviridae* family comprises a large number of viruses among which are the tick-borne encephalitis virus (TBEV), West Nile fever virus (WNV), dengue virus (DEN), yellow fever virus (YFV), Zika virus (ZIKV), and Japanese encephalitis virus (JEV). In addition, mosquito-borne Chikungunya virus (CHIKV) and Toscana virus (TOSV) transmitted by phlebotomus belong to the families *Togaviridae* and *Phleboviridae,* respectively.

TBE and WNV are known to cause infections in Bulgaria. However, nowadays the import of infections on a global scale with travels from endemic regions is a fact and the preparedness for timely diagnosis is of paramount importance. In addition, the spread of these viruses is ubiquitous and related to distribution of their vectors. Dengue is the most common arbovirus infection in the world with over 390 million cases per year in tropical and subtropical areas. Yellow fever and Chikungunya infections are endemic in many areas of Africa and South America.

Genetic methods for direct detection of viral nucleic acid in clinical materials are the major diagnostic method, especially in acute infections, before the development of immune response with increased amount of specific antibodies.

For the National reference laboratory of vector-borne infections, it is very important to provide fast and reliable diagnostics for all of these infections. Therefore, numerous RT-PCR systems were developed and introduced in the laboratory practice.

MATERIAL AND METHODS

Multiple PCR protocols, both conventional and Real-time, have been systematically introduced for routine diagnostics of vector-borne viruses.

Conventional and Real-time RT-PCR for West Nile fever virus lineages 1 and 2, for ZIKV, as well as Real-time RT-PCR systems for TBEV, DENV, YFV, TOSV, Usutu virus (USUV) and CHIKV were developed and tested. Reference samples and clinical materials were used to check reliability. The genes detected in each reaction are as follows:

- *5'-UTR (untranslated region)and part of the capsid gene* for WNFV lineage 1 and 2- conventional PCR and Real-time PCR
- *envelope gene* for ZIKV - conventional PCR and Real-time PCR
- *3' noncoding region* for TBEV - Real-time PCR
- *5'-UTR (untranslated region)* for DENV - Real-time PCR
- *5'-UTR (untranslated region)* for YFV - Real-time PCR
- *N-gene (S-segment)* for TOSV - Real-time PCR
- *NS5 gene* for USUV - Real-time PCR
- *nSP1 gene* for CHIKV - Real-time PCR

RESULTS AND DISCUSSION

Vector-borne infections are of increasing interest in the last decade due to several outbreaks in non-endemic areas, especially in European countries around the Mediterranean Sea. The import of such infections from endemic regions together with availability of competent vectors contributes to the possible disease dissemination with further autochthonous cases.

The rapid invasion and distribution of *Aedes albopictus* (tiger mosquito) in Europe provides conditions for emergence of tropical infections. Severe mosquito-borne viral infections imported from tropical countries in Europe are increasingly recognised – dengue, chikungunya, West Nile fever, zika virus infection. As a consequence of imported infections, epidemic outbreaks of dengue and chikungunya occurred due to local transmission of the virus. Such examples are the autochthonous dengue outbreak in South France in 2015 and the epidemic of chikungunya in Northeast Italy with 254 cases, of which 78 were laboratory-confirmed. The total number of autochthonous cases of dengue and chikungunya in Europe (Italy, France, Croatia, Madeira Island) for 2007-2012 are 2237 and 231, respectively (1). According to the European Centre for Disease Prevention and Control, the total number of imported zika virus infections in Europe until July 2016 is 940 registered in 18 European countries.

Undoubtedly, West Nile fever remains the main problem, being transmitted by the common mosquito genus *Culex* (2). In recent years, West Nile fever cases are reported in all neighbouring countries of Bulgaria. In Greece, a total of 262 cases, of which 197 with encephalitis, meningoencephalitis and meningitis, were diagnosed in 2010 and in 33 (17%) of them the infection was fatal (3). In the next years, annually about 80-100 new cases of West Nile fever were reported in Greece. In Romania, following the big outbreak in 1998 with 393 cases in the lower reaches of the Danube River (4), again in 2010, 57 new West Nile fever cases were registered, 10% of which were fatal (5).About 10-30 new cases per year are diagnosed, mostly in the region of Bucharest. In Turkey, specific neutralising antibodies against West Nile fever virus were confirmed in healthy blood donors (6), confirming the circulation of the virus among people in this country. In Serbia, 52 cases in 2012, mostly encephalitis (85%) and less often meningitis (15%), and in 2013 a very large outbreak with 302 cases, were observed. In 2014, West Nile fever cases were registered in Bosnia and Herzegovina (7).

It is established that all currently known viruses causing West Nile fever belong to two main phylogenetic lineages (8). Lineage 1includes viruses that circulate mainly in North America and viruses described also in Europe (Spain, Italy), Africa and the Middle East. Lineage 2 comprises mainly viruses considered until recently as causing mild, self-limiting disease. Viruses of this lineage circulate mainly in Africa, south of Sahara, and in Madagascar. However, they have been isolated in a number of European countries since 2004: Hungary, Austria, Russia, and later in Greece, Romania, Albania. It is also determined that they have potential to cause severe invasive neurological infections.

In Bulgaria, neutralising antibodies against the virus were detected in a patient with viral meningitis in 2011 (9). In 2015 it was recognised again serologically in a case of a child exposed to a number of mosquito bites in the North Park of Sofia. In 2016 the virus genome along with specific antibodies were detected in a man with severe and fatal encephalitis (10). Sequencing

of the virus showed that it belongs to lineage 2, close to the Greek strains and capable of causing severe infections of the central nervous system.

Closely related viruses that belong to one viral family (*Flaviviridae*) cause several arboviral diseases – Zika virus infection, West Nile fever, tick-borne encephalitis, yellow fever, Japanese encephalitis, etc. All of them are mosquito-borne with one exception – tick-borne encephalitis.

Zika viruses are of two major lineages – one with African strains, and the other with Asian and American strains from the last outbreak. Competent vectors to transmit the virus are *Aedes aegypti* and *Aedes albopictus*, the former being the main vector. *A. aegypti*is currently distributed only at the northeast Black Sea coast. However, it has recently been predicted that it might soon colonise southern European regions (11). *A. albopictus*is widely spread in southern Europe and it can hibernate and survive in cool temperature regions. Zika virus infection is mild, self-limiting, with influenza-like syndrome, but in some cases it is associated with Guillain-Barre syndrome or microcephaly, the latter being confirmed in more than 1600 cases only in Brazil.

Dengue fever is the most prevalent mosquito-borne viral disease in humans and has spread rapidly. According to WHO, dengue cases have increased dramatically over the past few decades. Currently, 390 million dengue fever cases are reported annually. The disease is endemic in more than 100 countries in tropical and subtropical regions, where 40% of the population is at risk. Etiological agents are 4 types of dengue viruses, transmitted by the rapidly expanding mosquito species *A. aegypti* and *A. albopictus*. Clinical manifestations include dengue fever, dengue haemorrhagic fever and dengue shock syndrome. Myalgia is pronounced and lasts several weeks or months. In Europe, several local outbreaks are described that started from imported cases. It is established, that all of these cases are transmitted by *A. albopictus* mosquitoes. The only exception is Madeira, which is a Portuguese territory, where the big dengue epidemic outbreak in 2012-2013 with more than 2100 cases was connected to *A. aegypti* mosquitoes.

Chikungunya fever is a mosquito-borne arboviral infection. Chikungunya virus belongs to the genus *Alphavirus* within the family *Togaviridae*. Since its discovery, chikungunya virus regularly causes larger or smaller epidemics and epidemic outbreaks in Africa and Southeast Asia with hundreds and thousands of patients. The virus re-emerged again between 2009-2010. Imported cases were reported in Europe. In addition, autochthonous cases intensify the infection in the countries of the Mediterranean Basin. During epidemics, the virus circulates in so-called "epidemic cycle" between vectors, *A. aegypti* and *A. albopictus*, and humans as a reservoir. Clinically, the disease is manifested by fever and headache, back pain, myalgia and arthralgia. The main problem for these patients is severe pain in the small joints of the hand and the foot. Complications are neurological and ocular.

Dengue, chikungunya and zika are exotic infections for Bulgaria, of which we do not take notice, but the establishment of *A. albopictus* mosquitoes in many regions of the country shows there are conditions for the spread of viral pathogens. Movement of large human masses to and from regions with widespread mosquito-borne infections creates a potential for the spread of these infections and requires testing by diagnostic methods.

Genetic methods for detection of viral RNA were introduced after thorough check of the published literature on PCR systems for each of the viral pathogens.

A panel for neuroinvasive infections based on detection of WNV, TBE, USUV and TOSV was introduced. Both conventional and Real-time RT-PCR systems for detection of lineages 1 and 2 were used to detect West Nile viruses. TBE, Usutu and Toscana viruses are detected by Real-time RT-PCRs. International external quality assessment round confirmed applicability, sensitivity and specificity of the PCR systems.

Triplex RT-PCR system is used for simultaneous detection of DENV, CHIKV and ZIKV. Thereby, the most common mosquito-borne infections can be specifically and reliably diagnosed. EQA for Zika virus and WNV, organised by INSTAND institute, was successfully passed this year.

Finally, RT-PCR system was introduced for reliable detection of various groups of yellow fever viruses. The system allows to distinguish between African and American types of YFV. Recently, EVD-LabNet supported by ECDC, organised EQA for YFV with 15 blind samples, including African and American YFV as well as

other flaviviruses. Reliability of the developed RT-PCR system for YFV detection was confirmed with 100% success rate.

The above mentioned PCR systems allow direct detection of the virus in clinical materials at early stages of infection, before development of an immune response and application of other diagnostic methods (12). Their availability at the National reference laboratory allowed early detection and prompt diagnosis of two cases of WNF and two cases of imported dengue fever in the last year, genetically confirmed by detection of the specific RNAs of the causative viruses.

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SEROPREVALENCE STUDY FOR WEST NILE VIRUS IN BULGARIA

I. Christova[1], I. Trifonova[1], E. Panayotova[1], S. Tchakarova[2], T. Gladnishka[1], E. Taseva[1]*

[1]National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
[2]Bulgarian Food Safety Agency

ABSTRACT

A nationwide seroprevalence study was conducted in order to assess local circulation and risk of human infections with West Nile virus (WNV) in Bulgaria. In total, 1451 residents of all 28 districts in Bulgaria were tested for WNV-specific IgG antibodies. The survey found overall seroprevalence of 1.5%. The highest WNV seroprevalence was found in Sofia Province and districts near the river Danube. The results showed that WNV infection seems to be more widespread in the country than it has been described.

KEYWORDS:

zoonoses, endemic infection, flavivirus

INTRODUCTION

West Nile virus (WNV) is an expanding neurotropic arbovirus, a member of the genus *Flavivirus* within the *Flaviviridae* family. Widespread *Culex* mosquitoes transmit WNV.

The majority of human infections with WNV are asymptomatic (about 80% of infections with WNV) (1). Around 20% of infections with WNV present as febrile syndrome and less than 1% manifest as neuroinvasive diseases such as encephalitis, meningitis or polio-like paralysis (1).

ADDRESS FOR CORRESPONDENCE:

Prof. Dr. Iva Christova, MD, PhD, DSc
Department of Microbiology
National Centre of Infectious and Parasitic Diseases
26Yanko Sakazov Blvd.
1504, Sofia, Bulgaria
Tel. + 359 885 440 557, Fax: +3592 943 3075
E-mail: iva_christova@yahoo.com

Bulgaria was not affected by WNV until 2015, when a few probable WNV human cases were detected and the first confirmed WNV neuroinvasive infection was reported (2). Full sequencing of the causative virus revealed WNV lineage 2 sharing common ancestor with Greek strains involved in the largest outbreak of WNV in Europe between 2010 and 2013 (3), and close to the virus that caused outbreak in Hungary in 2008, when the WNV lineage 2 emerged for the first time outside Africa (4).

A nationwide seroprevalence study was conducted in order to assess local circulation and risk of human infections with WNV.

MATERIAL AND METHODS

From May to October 2015, serum samples were collected prospectively from randomly selected persons visiting public biochemistry laboratories in the regional primary healthcare centres to follow up non-infectious diseases or for routine prophylactic check-up in all districts of Bulgaria: Blagoevgrad (n=64), Vidin (n=40), Gabrovo (n=63), Dobrich (n=52), Montana (n=78), Plovdiv (n=62), Targovishte (n=42) and 50 samples from each of the rest 21 districts. Oral consent was received from each participant. The laboratories registered information on age, sex and area of residence for each sampled person. Persons previously vaccinated against yellow fever were excluded from the study.

All serum samples were tested for WNV IgG antibodies by ELISA kits (Euroimmun, Lübeck, Germany). All IgG-positive samples were tested by ELISA for specific IgM antibodies and by avidity tests (from the same manufacturer) to distinguish between acute and non-acute WNV infections. In addition, all IgG-positive samples for WNV were further examined by microneutralisation assay (MNTA) for the presence of neutralising antibodies against WNV and the closely related Usutu virus (USUV)(5).

RESULTS

In total, 1451 residents of all 28 districts in Bulgaria (total population of 7,2 million) were tested for WNV-specific IgG antibodies. The mean age ± standard deviation was 53.2 ± 18.8 years. The study panel included 622male and 829 female persons.

WNV IgG antibodies were detected in 22 (1.5%) participants tested by ELISA. Neutralising

antibodies against WNV were present in 6/22(27.3%) IgG ELISA-positive samples with a range of the titre between 1:10 and 1:100. Two out of the 22 IgG-positive samples were also found positive for IgM antibodies. One of the IgM-positive samples was also MNTA-positive (titre 1:100) and its IgG avidity index was48%, whereas for the rest of the MNTA-positive samples, the IgG avidity index was between 70% and 97%. The mean IgG avidity index for all samples was 59%, range 14%-97%. None of the tested samples was positive for USUV.

The districts of Sofia Province and Vidin had the highest seroprevalence – 10% and 7.5%, respectively, followed by districts of Ruse and Silistra- 6% each (Table 1). There was no significant difference between the positive male (1.9%) and female (1.2%) groups (OR 1.61, 95% CI 0.69-3.76, p=0.264). No association between age and WNV infection was present (OR 1.13, 95% CI 0.91-1.42, p=0.251).

The MNTA-positive samples originated from 4 districts (Table 1). No significant association between gender or age and MNTA-positive rates (p=0.163;p=0.942) was found.

Table 1. WNV seroprevalence in Bulgaria.

District	Number of positive/ number of all samples tested	% of positive samples	Number of MNTA*-positive samples
Blagoevgrad	0	0	0
Burgas	0	0	0
Dobrich	1/52	1.9 %	0
Gabrovo	0	0	0
Haskovo	0	0	0
Kardzhali	0	0	0
Kyustendil	1/50	2 %	0
Lovech	0	0	0
Montana	0	0	0
Pazardzhik	0	0	0
Pernik	0	0	0
Pleven	1/50	2 %	0
Plovdiv	1/62	1.6 %	0
Razgrad	0	0	0
Ruse	3/50	6 %	1
Shumen	0	0	0
Silistra	3/50	6 %	3
Sliven	0	0	0
Smolyan	1/50	2 %	0
Sofia	0	0	0
Sofia Province	5/50	10 %	1
Stara Zagora	0	0	0
Targovishte	0	0	0
Varna	0	0	0
VelikoTarnovo	0	0	0
Vidin	3/40	7.5 %	1
Vratsa	2/50	4 %	0
Yambol	1/50	2 %	0

*MNTA - microneutralisation assay

DISCUSSION

The first and nationwide seroprevalence survey on WNV circulation in Bulgaria found overall seroprevalence of 1.5%. However, district analysis showed WNV seroprevalence up to 7.5%-10%. The great variability of IgG avidity indices was suggestive of recent and past infections.

The WNV seroprevalence rates in Bulgaria, even though lower than identified in the endemic European countries (Greece, northern Italy, southern France) (4,6,7), illustrated widespread circulation of WNV in the country. Notably, the highest WNV seroprevalence was detected in the district around the capital, namely Sofia Province, where the first confirmed neuroinvasive case appeared in 2015 (2) and another one was confirmed in 2016 (data not published). Furthermore, WNV IgM antibodies were detected in samples only from this district. The area should be considered as endemic and more cases should be expected in the future. In addition, reactive samples were detected in almost all districts near the river Danube, the northern border of Bulgaria. This could be related with excellent conditions for mosquito reproduction. WNV outbreaks in Romania in 1996-1997 and 2010 appeared in areas close to the Bulgarian border (8). Reactive samples were also found from residents of some central districts, near wetlands along the big rivers Maritsa and Tundzha, as well as in a southern district, close to the border with Greece, where the last WNV outbreak, 2010-2012, was confirmed as a consequence of a recent introduction of WNV lineage 2 strain (9). In the last years, WNV expanded also in other Balkan states (10).

The results of this first WNV seroprevalence study in Bulgaria showed that the virus infection seems to be more widespread in the country than it has been described so far. The level of WNV seroprevalence found in Bulgaria is evidence that some viral encephalitis or meningoencephalitis cases in the country are underdiagnosed and underreported (11, 12). Taking into consideration the known trend for expansion of WNV in the last decade, more clinical cases and higher seroprevalence rates are likely to be expected in the near future.

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CHALLENGES IN THE DIAGNOSIS AND TREATMENT OF ALLERGY TO *ALTERNARIA ALTERNATE* AND OTHER MOULD SPECIES

D. Hristova, MD

Department of Immunology
National Centre of Infectious and Parasitic Diseases

ABSTRACT

Moulds are involved in the pathogenesis of a number of respiratory diseases. It is estimated that 2-6% of the general population is sensitized to moulds. Terms such as sensitization, colonisation and invasive infection can be discussed in regardto mould-sensitive individuals. Clinical manifestationsare predominantly determined by the immunological status. *Alternaria* is among the most common moulds in the world.The incidence of asthma is three times higher among mould-sensitizedindividuals and up to 70% of them show positive skin tests to *A. alternata*. However, cross-reactivity among moulds makes theaccuratediagnosedifficult. Alt A1 is a marker for actual sensitization to *A. alternata*. The additional testing ofenolase and Mn superoxide dismutase is useful in assessing the sensitization to *Pleosporaceae.*

KEYWORDS:

Alternaria allergens, fungal sensitization, allergen cross-reactivity, recombinant allergens

Alternariaalternata parasitize in plants in warm climate regions. This mould is also typically found in unventilated roomsand is implicated in theincrease inunfavourable outcomes for respiratory health by 30%. Recent reviews confirm that damp indoor environment is a risk factor for asthma development. What is not known is whether reducing mould exposure would decrease that risk. Some precautions taken especially at home may reduce indoor exposure to allergenic fungal species. Cleaning to prevent the accumulation of dust, protectingbuildingsfrom dampness, avoiding indoor smoking are just some of the protective measuresthat may significantly decrease indoor fungal growth (4, 5, 6, 7, 9,15,21).

People permanently inhale various fungal spores and particles (1, 2, 5, 12, 20,21, 22). *Alternaria* spores are prevalent during the whole year in tropical and subtropical areas. Small enough to be inhaled, spores are released on rainy,windy days (3, 7, 11, 16, 18, 19). In Bulgaria, peak spore counts occur in summer (June, July, August) (4, 9, 11, 13, 16, 17). Spores with a size of less than 5 μmin diameter can reach the small airways.Moulds can release huge amounts of small fragments of 0.03 μm to 2 μmin diameter. It is estimated that the spore amount enough to provoke symptoms differ from species to species with 3000 spores/m^3 for *Cladosporium spp.* and100 spores/m^3 for *A. alternata*. *Alternaria*is among the most commonmoulds worldwide and it is not surprising that up to 70% of the mould-sensitized individuals are positivefor*A. alternata.*

Alternaria-specific IgE antibodies increase during childhood and then seem to decline over the years. *A. alternata* is the third most common cause of sensitization after Der p and grass pollens in individuals at 4 years of age (18).

Sensitivity to *Alternariaalternata*is considered to be a risk factor for asthma development (6, 7, 13, 19, 23, 24, 25, 26, 27, 28). Fungal sensitization might also contribute to the persistence of active symptoms of asthma. Jamieson et al. describe that there is an "allergic phenotype" of COPD and Nazarenko et al. found specific IgE antibodies against Alt a 1 among this population. D'Amato shows that the frequency of positive skin test varies from country to country. For instance, it ranges from less than 1% in Austria to 50% in Arizona, USA (7,8,9,22)

Allergens of *A.alternata*.Cross-reacting allergens.

A. alternata is a source of many IgE-binding molecules, differing in clinical significance (Table 1) (4, 5, 10, 13, 14, 18, 23, 29, 30, 31, 32).

ADDRESS FOR CORRESPONDENCE:
Diana Hristova
Postal address: 26 Yanko Sakazov Blvd.
Sofia1504, Bulgaria
E-mail: did_hris@abv.bg
Telephone: 0887143275

Table 1. *Alternariaalternata* allergens.

Name	Protein classification	Biochemical function	IgE binding	Investigator
Alt a 1	Alt a1 protein is a two-chain dimer linked by disulfide bonds with a molecular weight of approximately 30 kDa; Alt a 1 is a unique β-barrel which comprises 11 β-strands and forms a "butterfly-like" dimer.	Unknown	80-90 % of individuals allergic to *Alternaria*	Agarwalet al. were able to detect Alta 1 in the atmosphere. Yunginger et al. isolated a major allergenic fraction of Alt a 1
Alt a 2	25 kDa protein	Unknown	60 % of individuals allergic to *Alternaria*	Bush et al. identified it as a major allergen; Asturias et al. concluded that Alt a 2 is a minor allergen
Alt a 3	Heat shock protein of 70 kDa; homologous to a region of (HSP-70) from another mould, *C. herbarum*. Highly conserved across both prokaryotic and eukaryotic organisms	Protects cells from thermal and oxidative stress	5 %	De Vouge et al.
Alt a 4	57 kDa, disulfideisomerase; contains thioredoxin domain	Disulfide oxidation, reduction, isomerization	42 %	Achatz et al.
Alt a 5	11 kDa 60S acidic ribosomal protein P1	Structural constituent of the large ribosomal subunit	8–14 %	De Vouge et al.
Alt a 6	Enolase. Enolase is found from bacteria to mammals, and its sequence is highly conserved throughout evolution	Catalysing conversion of 2-phospho-D-glycerate (2PGA) into phosphopyruvate (PEP)	50%	Lohman and Meyerhof; Baldo and Baker
Alt a 7	22 kDa protein highly homologous to YCP4 yeast; YCP4 is similar in sequence and structure to flavodoxins—proteins that have been detected in highly purified mitochondria	Transcriptional regulator	7%	Achatz et al.
Alt a 8	29 kDa enzyme - NADP-dependent mannitol 2- dehydrogenase	Catalyse the reversible conversion of mannitol to fructose	41 %	Schneider et al.
Alt a 9	43 kDa; unknown structure	Unknown	5%	Not in the official database of the WHO/IUIS Allergen Nomenclature
Alt a 10	53 kDa aldehyde dehydrogenase	ALDH metabolises ethanol-derived acetaldehyde and has a broad substrate specificity which includes a variety of aldehyde structures	2%	Achatz et al.
Alt a 12	11 kDa 60S acidic ribosomal protein P2	Protein synthesis		
Alt a 13	26 kDa glutathione-S-transferase (GST); a cross-reactive allergen in fungal extracts	Biotransformation of endogenous and xenobiotic compounds	82 %	McGoldrick et al., Shankar et al.
Alt a 70 kDa	Unknown structute. Glycoprotein which forms 13% of the dry weight of *Alternaria* extracts	Unknown	Causes positive skin test results in 87 % (n= 14/16) of *Alternaria*-sensitive patients	Portnoy et al.
Alt a 14	24 kDa; manganese-dependent superoxide dismutases (MnSODs). They are phylogeneticallyconserved enzymes.	Antioxidant enzyme	11.5%	Postigo et al.
Alt a 15	Serine proteases (SPs) panallergens		10.2%.	Gabriel et al.
Alt a NTF2	Nuclear Transport Factor 2; 28 kDa small homodimeric protein	Cytosolic factor for protein import into the nucleus through the nuclear pore complex		Weichel et al.
Alt a TCTP	Highly conserved histamine-releasing factor	Cell cycle proliferation, survival, and malignant transformation of cells	4 %	Rid et al., Kim et al.

An allergen is considered major when there is IgE-binding in at least 50% of individuals sensitive to that allergen as determined by radioimmune electrophoresis, and minor if IgE-binding is present in less than 50% of allergic individuals (1, 14).

The immune system can recognise foreign proteins. Cross-reactivity occurs when specific antibodiesproduced against particular allergen bind to another proteinwhich may cause an allergic reaction. Homolog sequence of at least 50% is needed for cross-reactivity. Cross-reactive proteins are a conserved group of allergens which includes, among others, enolase, heat shock proteins, thioredoxins, Mn superoxide dismutase and disulphide isomerases. Cross-reactions are observed between*A. alternata* allergens and *Cladosporium, Candida* and *Aspergillus* allergens. Reports in scientific literature describe theMn superoxide dismutase protein family to cross-react with human Mn superoxide dismutase (MnSOD). This may maintain chronic inflammation (5,13).

Allergen based-component diagnosis: a new era of *Alternaria* allergy diagnosis

Diagnosis of fungal allergic diseases requires confirmation of allergen-specific IgE antibodies by skin tests, *in vitro* tests and provocation tests. The fungal panelfor skin tests used in Bulgaria includes: *Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium chrysogenum, Mucor racemosus, Neurospora sitophila, Rhizopus nigricans, Fusarium lini* (17). Mould-sensitized patients generally develop symptoms in damp dwellings, basements, libraries and old houses. Some questions might be more sensitive than others in evaluation of symptoms from mould exposure. Nasal symptoms during the night seem to be more characteristic than symptoms during the day. The combination of skin prick tests and determination of allergen-specific IgE levels in the serum is currently recommended for a reliable assessment of *Alternaria* sensitization. Provocation tests can confirm the diagnosis of mould allergy (6, 16, 17, 27, 32, 33, 34). Standardisation and purification of fungal allergens is very difficult.However, the usefulness of these extracts in diagnostics and therapy is limited. Different commercial allergen extracts demonstrate variable potency. On the other hand, the presence of cross-reactivity greatly complicates the accuracy of the diagnosis(4, 5). However, studies indicate that immunotherapy with *Alternaria* species extract reduced combined symptom and medication scores in children with rhinitis and asthma (13, 17, 23, 25).

It was demonstrated that Alt a1 expression depends on fungal growing time, culture media and strain. Martínez found a high variability of Alt a 1 expression in different strains of *A. alternata*. He concluded that measurement of Alt a 1 concentration during early stage of allergen production is needed for the process of extract production.

Component-resolved diagnosis (CRD) is the right step to overcome the limitations of crude extracts. Recombinant allergens allow thediscrimination between true sensitization and cross-reactivity. This is decisive for choosing the strategy inimmunotherapy. Postigoet al. suggest that Alt a 1,enolase, and MnSOD are equally important for the diagnosis of allergy to *Pleosporaceae*. Unfortunately, only rAlt a 1, rAlt a 6 are available on the market.

The use of recombinant allergens, obtained by genetic engineering, makes it possible to wash out the impurities present in allergen extracts (1, 4, 5, 8, 10, 31).

In reality there are even more complicated issues. The role of mould sensitization is widely considered in uncontrolled asthma conditions. Patients are treated for prolonged time with corticosteroids and antibiotics during severe exacerbations andin these cases individuals are immunocompromised. As already stated in the scientific literature, *Aspergillus* and *Candida*, for example, are widely discussed in the pathogenesis of allergic diseases. They are also one of the most common causes of systemic infections that occur with non-specific symptoms and can be life-threatening. Rapid and accurate diagnosis is required for rapid and accurate treatment. Therefore,there is need to perform diagnostic tests specific to invasive mycoses such as microscopic detection, indirect immunofluorescence (IIF), detection of 1, 3–β D-glucan, latex agglutination, sandwich ELISA, pulse-field gel electrophoresis of chromosomal DNA (PFGE) and amplified fragment length polymorphism (AFLP) analysis. The choice is determined by theparticular limitations of the assay and the patient`s condition. Unfortunately,

there is no clear algorithm forimplementation of these toolsin follow-up and treatment of uncontrolled asthma (31, 35,36,37,38).

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METHODS FOR DIAGNOSIS OF SYSTEMIC CANDIDOSIS

L. Boyanova

National Centre of Infectious and Parasitic Diseases

ABSTRACT

The aim of this review is to present the common laboratory methods for diagnosis of invasive candidosis, a fungal disease caused by species of the genus *Candida*.

We are conducting a study which is part of the surveillance system BulSTAR investigating the etiological structure of infections in Bulgaria. The extracted BulSTAR data evaluating *Candida* blood culture isolates from Bulgarian hospitals showed higher number of isolates than previous years. The most frequently isolated species were: *Candida albicans*, followed by *Candida parapsilosis, C. glabrata, C. krusei, C. tropicalis* and other *Candida* non-*albicans* strains (19). The current study confirmed the increase in the number of isolated *C.* non-*albicans* strains.

The most common methods in the diagnosis of systemic candidosis are blood culture, antigen and antibody detection in body fluids (serum, plasma), histopathology (biopsy) and PCR-based molecular techniques.

KEYWORDS:

systemic candidosis, *Candida*

Invasive fungal disease is a leading cause of mortality in immunocompromised individuals (intensive care unit patients, transplant recipients, patients with cancer, neutropenia, T-cell immune suppression (AIDS), those undergoing antibiotic therapy, placement of venous catheters and others).

The majority of invasive fungal diseases are still caused by *Candida* (9, 11) as well as *Aspergillus* and *Cryptococcus* species. The most frequently isolated species is *Candida albicans*, followed by *Candida parapsilosis, C. glabrata, C. krusei, C. tropicalis* and other *Candida* non-*albicans* strains (19).

Laboratory diagnosis relies on microscopic and culture-based methods (blood culture), and thus detection of the causative agent is delayed (13, 15).

Detection of yeasts from blood cultures is essential for microbiological diagnosis of fungaemia. The appropriate blood culture bottles must be used with specific machines. Blood cultures often remain negative even for patients dying from proven disseminated candidosis (20).

Histopathology, however, is more often not a viable option and may be contraindicated. Tissue sections should be stained using PAS, Grocott`s methenamine silver (GMS) or Gram stain as *Candida* may be missed in H&E stained sections (20).

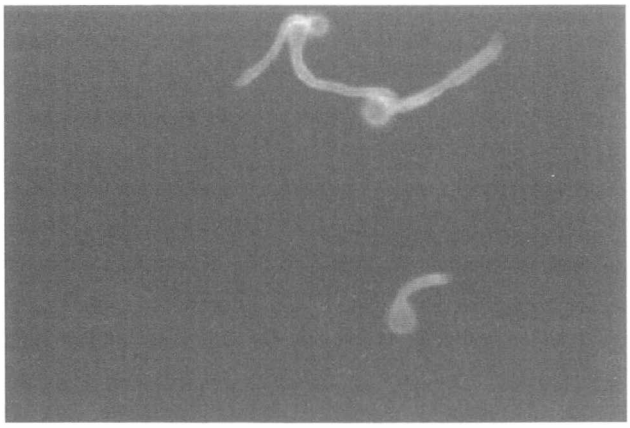
The most common serological tests for detection of *Candida* antigens and antibodies in body fluids (serum, plasma) are: indirect immunofluorescence (IIF), test for germination (germ tube), ELISA Platelia (enzyme-linked immunosorbent assay) (BioRad) and latex agglutination.

The method we use for determination of specific antibodies against *Candida* spp. is indirect immunofluorescence (IIF). In this assay a microscopic slide is prepared from culture suspension of *Candida* strain (CIP 628). The slides are fixed and stored at -20°C. The principle of the method includes 2 steps followed by visualisation on fluorescence microscope. Indirect immunofluorescence has the advantage of greater sensitivity than direct immunofluorescence.

There is also an IIF method for detection of antibodies in serum against the germ tube of *Candida albicans*. It is based on the two different phases of *C. albicans* and *C. dubliniensis* – blastoconidia (yeast) and germ-tube. Many authors agree on the importance during systemic infection as the microorganism invades the tissues, passes through the capillary walls and blocks phagocytosis. For this reason, the formation of germ tubes is considered a factor of virulence (1).

ADDRESS FOR CORRESPONDENCE:

L. Boyanova
National Centre of Infectious and Parasitic Diseases
26 Yanko Sakazov Blvd.
1504 Sofia, Bulgaria
e-mail: miraanbo@abv.bg



Candida albicans showing germ tubes. CDC / Mercy Hospital, Toledo, OH/ Dr. Brian Harrington.

ELISA (enzyme-linked immunosorbent assay) is a serological method that can be used for detection of *Candida* antigen. The immunoenzymatic sandwich microplate assay ELISA Platelia (BioRad) enables detection of the circulating antigen mannan in human serum. Mannan antigen is a polysaccharide bound to the yeast cell wall and appears to be the main biomarker for diagnosis of invasive candidosis. The assay utilizes rat monoclonal antibody directed against *Candida*. The treated samples and conjugate are added to the wells of the microplate coated with the monoclonal antibody. If the mannan antigen is present in the serum samples, a complex is formed by monoclonal antibody-mannan antigen (Ag)-monoclonal antibody / peroxydase. Mannan concentration (pg/ml) in the test samples is determined by plotting a calibration curve using 5 range points calibrators: 0 pg/ml, 62.5 pg/ml, 125 pg/ml, 250 pg/ml and 500 pg/ml. Samples with concentrations less than 62.5 pg/ml are considered "negative", samples with 62.5<C<125 – "intermediate", and samples with C> 125 – "positive" for the presence of mannan antigen (18).

The latex agglutination test is another serological method for detection of circulating antigens using sample technique. It is a qualitative test designed to detect the polysaccharide antigen mannan (*Candida* BioRad) in biological fluids. The procedure utilizes latex particles coated with monoclonal rat antibody directed against the fungal antigen. The particles react with the antigen resulting in agglutination visible to the naked eye (12). As for all immunological techniques, limitations of the test are related with the possibility of cross-reactions and should always be considered.

PCR is non-culture-based method for DNA detection that provides rapid diagnosis of infections. Amplification techniques offer increased sensitivity over traditional staining and culture methods but may give positive results in asymptomatic individuals due to colonisation (6). Real-time PCR is currently one of the fastest diagnostic tools detecting the most frequent causative agents of invasive fungal infections (8, 21).

The different methods for laboratory diagnosis of invasive candidosis have different specificity and sensitivity. Antibody titres to germ tube antigens of *Candida albicans* indicate the high specificity and sensitivity of the method introduced in routine practice. Many authors also confirm the capability of the method in the diagnosis of systemic mycoses as a prospective to distinguish between colonisation with *C. albicans* which is very important for empiric therapy in patients (4). ELISA Platelia (BioRad) tests are more sensitive and more appropriate for the analysis of a great number of serum samples, whereas latex agglutination tests (BioRad) are easier to perform in laboratory conditions (16). We must not forget also the high cost of antigen detection tests.

The detection of soluble antigens by latex particle agglutination test enables more rapid diagnosis than the conventional culture method and is designed to detect a circulating heat-sensitive *Candida* antigen (10).

The combined application of ELISA Platelia (BioRad) test is also an integral part of the clinical and laboratory patient monitoring process in order to aid therapeutic decisions. The diagnosis of invasive candidosis must be based on detection of both antibodies and circulating antigens.

Molecular methods can be applied directly to clinical materials (5). Among the advantages of molecular-based techniques are the rapid performance, enhanced sensitivity, and also detection of fungi that appear to be causing disease but cannot be cultured.

With the exception of serological methods and PCR, laboratory detection of invasive candidosis should be performed in conjunction with other diagnostic procedures such as culture methods, histological examination of biopsy samples, CT imaging and can be used to aid the diagnosis of systemic candidosis (17).

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TOXOPLASMOSIS AND PREGNANCY, DIAGNOSTIC AND THERAPEUTIC APPROACHES

R. Harizanov[a],*
N. Tsvetkova[a],
I. Kaftandjiev[a], I. Rainova[a]

[a]National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria, Department of Parasitology and Tropical Medicine

ABSTRACT

Background

Healthcare for women, especially during pregnancy, is of paramount importance to public health policies. The aim of this study is to present our experience in the management of pregnancy-related toxoplasmosis.

Methods

A group of 301 pregnant women, 9 newborn and 3 stillborn children was examined for infection with *Toxoplasma gondii*. Laboratory diagnosis was performed with ELISA IgG, IgM, IgA, ELISA IgG avidity and PCR. Data were processed using descriptive statistics and groups were compared with the Mann-Whitney test.

Results

Examination for anti-*Toxoplasma gondii* IgG showed positive results in 148 cases (seroprevalence of 49.2%). Of them, IgM antibodies were found in 67 (45.3%). The difference in IgG median values among women positive for IgG but negative for IgM, and women positive for IgG and IgM, was statistically significant. All women with positive results for specific IgM showed positive results for specific IgG. They were examined for determination of IgG avidity. Low or borderline avidity was found in 24 patients (35.8%). We conducted spiramycin therapy until delivery in pregnant women with serologic data for primary toxoplasmosis and low or borderline avidity (n=24).This group was monitored regularly with ultrasonography for pathological morphological changes in the foetus and such were not found.

Conclusions

Preventive care provides time to apply measures against mother-to-foetus transmission of several diseases, including toxoplasmosis. Tests for toxoplasmosis prior pregnancy are of utmost importance to avoid the risk of a possible congenital infection and emotional distress in pregnant women.

KEYWORDS:
toxoplasmosis, pregnancy, ELISA, IgG avidity

ADDRESS FOR CORRESPONDENCE:
Rumen Harizanov
Bulgaria, 1504 Sofia, 26 Yanko Sakazov Blvd.,
National Centre of Infectious and Parasitic Diseases, Department of Parasitology and Tropical Medicine;
email: harizanov@ncipd.org;
Phone: +35929446999; ext. 360
Fax: +35928438002

INTRODUCTION

Toxoplasmosis is a zoonotic disease caused by the protozoan pathogen *(Toxoplasma gondii)* of cosmopolitan distribution. In immunocompetent people the course of disease is usually asymptomatic or with self-limiting symptoms - lymphadenopathy, low-grade fever, fatigue. In cases of congenital toxoplasmosis or in immunosuppressed individuals (especially in AIDS patients), a life-threatening illness may develop. First human case of *Toxoplasma gondii* infection was described by Josef Janku in 1923 in infected child with hydrocephalus, microphthalmia and chorioretinitis that goes blind at 3 months old and dies at one year of age (1). Congenital toxoplasmosis occurs as a primary infection, i.e. acquired by the mother during pregnancy or immediately prior pregnancy (2-5). The rate of vertical transmission and severity of the clinical manifestations of congenital infection are related to the foetus gestational age. More serious damages to the foetus may occur during infection at the early stages of pregnancy. When the infection is at a later stage, the disease is mild or asymptomatic (6, 7).Various studies have shown that the risk of vertical transmission increases with gestational age, with highest levels being found in the third trimester (60% to 81%), while in the first trimester the risk ranges around 6% (7, 8).

This study aims to present our experience in the management of pregnancy-related toxoplasmosis.

MATERIAL AND METHODS

Sera samples

The study included sera from 301 pregnant women (mean age 30 ± 4, range 16-43 years). The reason for examination was prophylaxis during pregnancy. In addition, sera from 9 newborn children were tested as well. All samples were stored at +4°C prior examination, for no longer than 1-3 days.

Tissue materials

Tissue samples from 3 stillborn children were analysed for *Toxoplasma gondii* DNA. In all cases brain tissue samples were tested and, in addition, lung material in one case and blood samples in another case were examined as well. Materials were sent to the laboratory immediately after the autopsy without preservation and DNA was extracted on the same day.

Toxoplasma gondii DNA was tested also in the amniotic fluid of 2 pregnant women (5^{th} lunar month) and in the blood and the umbilical cord of 2 newborn children. Tissue materials were sent to the laboratory without preservation and DNA was extracted on the same day.

Clinical data

Data were collected from documented medical history and from physical examinations. Patients included in the study were pregnant women who came for consultation in the Department of Parasitology and Tropical Medicine (DPTM) at the National Centre of Infectious and Parasitic Diseases (NCIPD) during the period 2014-2016. Some of them were referred to the DPTM by their observing obstetrician-gynaecologist, while others came on their own.All pregnant women with established primary toxoplasmosis were consequently consulted by a parasitologist at DPTM, where upon they received prescribed treatment and were subjected to a follow-up until birth.

Immunological methods

Sera were analysed for anti-*Toxoplasma gondii* IgG, IgM, IgA and/or IgG avidity with ELISA tests. All tests were performed and interpreted according to the manufacturer`s instructions. A quantitative ELISA method (PlateliaToxo IgG, Bio-Rad, France)was applied for detection of anti-*Toxoplasma gondii* IgG. Values above 6 IU/ml were interpreted as a positive result. Anti-*Toxoplasma gondii* IgM and IgA were tested in a semi-quantitative sandwich-type ELISA (PlateliaToxo IgM and PlateliaToxo IgA, Bio-Rad, France). The samples were considered reactive for presence of IgM or IgA antibodies to *T. gondii* when Sample Ratio (SR) was ≥ 1.00.In addition, where necessary, avidity of anti-*Toxoplasma gondii* IgG was determined (PlateliaToxo IgG Avidity, Bio-Rad, France).Results below 0.4 were interpreted as low avidity, between 0.4 and 0.5 – borderline avidity, and above 0.5 – high avidity.

PCR

Total genomic DNA was isolated from tissue materials using a commercial column-based DNeasy tissue kit (Qiagen, Germany). At least three isolations were made from each sample and the procedure was performed according to the manufacturer`s instructions. Incubation of the tissue samples with proteinase K (20 mg/ml) was held overnight and amplification was applied immediately after the extraction. Primers and PCR conditions were according to a method described by Burg et al. (9). TOXOB22/TOXOB23 primer pair for detection of B1 gene in *T. gondii* was with the following sequence: TOXOB22 (forward) 5'-AAC GGG CGA GTA GCA CCT GAG GAG A-3' and TOXOB23 (reverse) 5'- TGG GTC TAC GTC GAT GGC ATG ACA ACT-3'. The PCR mix included 1.5 mM MgCl2, 200 µM of each dNTP, 1.5 µM of each primer, 0.625 U Taq polymerase and DNA matrix 1.0 µl in 25 µl total reaction volume. Amplification was carried out in a thermocycler (GeneAmp PCR System 2700, Applied Biosystems) under the following conditions: 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, 1 min extension at 72°C, and a final extension for 15 min at72°C. Electrophoresis was performed on 1.5% agarose gel stained with ethidium bromide and the reaction was read under UV and photo-documented (Syngen, Synoptic group).

Statistical analysis

Survey data were processed with statistical software GraphPad Prism 6.0 (GraphPad Software, inc.). Descriptive statistics and Mann-Whitney test were applied. Differences of $P < 0.05$ were regarded as significant.

RESULTS

Examination for anti-*Toxoplasma gondii* IgG and IgM

All of the 301 women were tested for anti-*Toxoplasma gondii* IgG and IgM. The examination for anti-*Toxoplasma gondii* IgG showed positive results in 148 cases (seroprevalence of 49.2%). Of these, IgM antibodies were found in 67 (45.3%) of which IgA specific antibodies (Table 1) were present in 20 (6.6%).

Table 1. Serological status of the examined 301 pregnant women.

Serological status	Pregnant women (n)	Relative share	Median value of IgG (IU/ml) ± Standard deviation (SD)	Years			Interpretation
				2014	2015	2016	
IgG (-); IgM (-); IgA (-)	153	50.83%	-	37	64	52	Absence of infection
IgG (+); IgM (-); IgA (-)	81	26.93%	60.0 IU/ml ± 62.6 SD	32	24	25	Latent infection
IgG (+); IgM (+); IgA (-)	47	15.61%	191.0IU/ml ±94.14 SD	11	25	11	Primary infection
IgG (+); IgM (+); IgA (+)	20	6.63%	126.0IU/ml ±113.5 SD	7	7	6	Primary infection
Total	**301**	100%		87	120	94	

Quantitative values of anti-*Toxoplasma gondii* IgG

In the present study a quantitative ELISA test was performed for determination of anti-*Toxoplasma gondii* IgG. Positive sera (n = 148) showed median values for specific IgG of 80.56 IU/ml ± 73.59 SD. In the group of women with positive results for both anti-*Toxoplasma gondii* IgG and IgM (n = 47), and in the group positive for IgG, IgM and IgA (n = 20), the median value of IgG was 139.4 IU/ml ± 75.28 SD, whereas for the group with positive anti-*Toxoplasma gondii* IgG results and negative results for specific IgM and/or IgA (n = 81), the median value of IgG was 60.0 IU/ml ± 62.6 SD. The difference in the median values was significant (Mann-Whitney test: *P value* <0.0001).

Examination for anti-*Toxoplasma gondii* IgM

The presence of specific anti-*Toxoplasma gondii* IgM was determined in 67 pregnant women. The median value of SR for specific IgM was 2.23 ± 1.0 SD in women negative for specific IgA (n = 47), and 2.97 ± 2.2 SD in those with specific IgA (n = 20). The difference in the median values was significant (Mann-Whitney test: *P value* =0.0066).

Examination for anti-*Toxoplasma gondii* IgA

In the study148 women, of whom 67 positive for specific IgM, were examined for anti-*Toxoplasma gondii* IgA. Positive result was detected in 20 cases (42.5%). Comparison of the results with the other two specific antibodies revealed the following: 153 women were G-/M-/A-; 81wereG+/M-/A-; 47 were G+/M+/A-; and20wereG+/M+/A+.

Examination of anti-*Toxoplasma gondii* IgG avidity

All women with positive results for specific IgM showed positive results also for specific IgG. They were examined for determination of IgG avidity. Low or borderline avidity was determined in24 patients (35.8%). Of them, 12 cases were positive simultaneously for specific IgG, IgM and IgA.

PCR

All 7 cases examined for *Toxoplasma gondii* DNA revealed negative results.

Distribution of primary *Toxoplasma* infection during pregnancy

During the first trimester of pregnancy primary toxoplasmosis was detected in 22 (32.8%) women, in the second trimester in 32 (47.8%) and in the third trimester in 13 (19.4%) (Table 2).

Table 2. Distribution of primary and latent *Toxoplasma* infection during pregnancy.

Stage of toxoplasmosis	Anti-*Toxoplasma* antibodies in pregnant women	First trimester n, (%)	Second trimester n, (%)	Third trimester n, (%)
Primary infection	IgG (+); IgM (+); IgA (+)	7 (10.4%)	8 (11.9%)	5 (7.5%)
	IgG (+); IgM (+); IgA (-)	15 (22.4%)	24 (35.8%)	8 (11.9%)
Total with primary infection		22 (32.8%)	32 (47.8%)	13 (19.4%)
Latent infection	IgG (+); IgM (-); IgA (-)	10 (12.3%)	13 (16.0%)	3 (3.7%)
	IgG (+); IgM (-)	22 (27.2%)	20 (24.5%)	13 (16.0%)
Total with latent infection		32 (39.5%)	33 (40.7%)	16 (19.8%)

Therapeutic approaches

Therapeutic approaches differed depending on the immunological results and the reason for examination. Pregnant women with high avidity (n = 43) were followed with control serological examination every month of the pregnancy. In view of the possible risk of congenital infection, spiramycin therapy was conducted until deliveryin pregnant women with serological data for primary toxoplasmosis and low or borderline avidity (n = 24).This group was monitored regularly with ultrasonography performed by obstetrician-gynaecologist for pathological morphological changes in the foetus and such were not found. Following birth, part of the newborn babies (n = 9) were tested for toxoplasmosis. There was no serological evidence of congenital infection (IgM-/A-).

DISCUSSION

Main Findings

Our study is the first of its kind in Bulgaria and encompasses a group of 301 pregnant women classified in four subgroups depending on the immune status with regard to toxoplasmosis as follows: without serological data for toxoplasmosis, with latent toxoplasmosis, with primary infection during or prior pregnancy and high IgG avidity, and with primary infection during or prior pregnancy and low or borderline IgG avidity. In order to avoid the risk of infection, the first group of pregnant women (n=153, 50.8%) was consulted on precautionary measures and monitored every month with laboratory tests for the presence of IgM-specific antibodies until the term and once after delivery. In this group we did not find cases with primary infection. Anti-*Toxoplasma* IgG antibodies were detected in 49.2% of cases. In respect to these findings, our data were similar to that of other authors who found moderate prevalence (moderate seroprevalence of 30 to 50%) in some areas of Central and Southern Europe (10-14). Low seroprevalence of specific anti-*T. gondii* antibodies ranging from 10 to 30% were found in North America, Southern Asia and Northern Europe, while high levels of seroprevalence (> 50%) were reported in some Latin American and African countries (10-12, 15).Most authors consider that the main causes of infection are related to a low social status, frequent pet contacts, poor health education and hygiene failure. In the group we surveyed, the majority of pregnant women were of good educational and social status. Some of them were cat owners but their pets were not allowed to leave the house and were not fed with raw meat or meat products. In this regard we consider that the high seroprevalence is due to gaps in

food hygiene. Because NCIPD operates the only laboratory in the country where routine studies of IgG avidity and PCR for *Toxoplasma gondii* DNA are performed, pregnant women from all over the country with established presence of IgM and/or IgA are referred to NCIPD for consultations with a parasitologist and appointment of additional studies confirming the diagnosis and specifying the stage of infection. In this sense, our study included pregnant women residing not only in the capital of Bulgaria but also in other regions of the country.

Existence of specific IgM antibodies was found in 22.2% of cases and IgA in 6.6%.In our data the proportion of pregnant women with presence of specific IgM is relatively higher than those of other authors who describe presence of specific IgM antibodies in about 2.5 - 5.3% of the cases (12, 16).This difference can be explained by the fact that a large proportion of the pregnant women studied by us, were referred to the NCIPD for confirmatory tests of already suspected or established by other laboratories primary infection, while the number of pregnant women tested with prophylactic indications only, was relatively lower.

Statistical analysis of the data showed significant differences in the mean levels of specific IgG antibodies in pregnant women with latent toxoplasmosis and those with primary infection. In this regard, we believe it is appropriate for pregnant women with high IgG values to be tested for specific IgM antibodies. To determine more precisely the stage of infection and the need of treatment, and to rule out the possibility of persistent IgM, we further investigated the samples for the presence of specific IgA and to define the IgG avidity. Of the 67 cases with primary infection, 22 (32.8%) persons were in their first trimester of pregnancy, 32 (47.8%) in the second and 13 (19.4%) in the third. All pregnant women with evidence of primary infection and low or borderline avidity of the specific IgG, were treated with spiramycin until term and they all gave birth to children without signs of infection. A comparatively small number of newborn babies was studied after birth (n=9), and serological evidence of congenital infection was not found. Treatment with pyrimethamine, sulfadiazine, and folinic acid was not applied because pyrimethamine is not commercially available in Bulgaria. *T. gondii* DNA was not detected by PCR in the three stillborn children, in the amniotic fluid of the two pregnant women and in the blood samples from the umbilical vein of two of the newborn children. The three pregnant women whose pregnancy ended up with stillbirth were not followed by us. In our study there were no cases of congenital infection or interrupted pregnancies as a consequence of such.

Strengths and Limitations

Unfortunately, in Bulgaria tests for toxoplasmosis prior pregnancy are not under regulations and are carried out at will. In a similar way, pregnant women are also being examined either as per their own wish or following the advice of the observing obstetrician-gynaecologist. This creates preconditions for eventual occurrence of intrauterine foetal infection and related complications of pregnancy (spontaneous abortion or birth of a disabled child).

Interpretation

Health care for women, especially during pregnancy, is of paramount importance to public health policies. Early care provides convenient time to apply preventive measures against mother-to- foetus transmission of several diseases, including toxoplasmosis which is with high prevalence (17). It is highly important to establish the immunological status of women before and during pregnancy (3, 5).

CONCLUSIONS

Although limited in size, our study is the first in the country to provide summarised data on the epidemiology, immunological parameters and therapeutic behaviour in primary pregnancy-related toxoplasmosis. Our data suggest that performing tests for toxoplasmosis prior pregnancy may be of critical help to avoid the risk of a possible congenital infection, as well as the psycho-emotional distress experienced by pregnant women with established primary toxoplasmosis. On the basis of our observations, we consider that spiramycin is reliable to treat pregnant women regardless of the gestational age, but the therapeutic approach should be determined individually in each case.

Contributors

Everyone of the authors processed and analysed the data; RH and NT summarised and interpreted the data and wrote the paper; RH and IK shaped the design and critically revised the manuscript; RH and IK prepared the tables; RH

shaped the final version of the article. All authors gave final approval of the manuscript version to be submitted.

Compliance with ethical standards

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Ethical approval: Not required (In our study were used data obtained during our routine clinical and laboratory work. Personal data were not used and hence approval by a local human investigations committee and obtaining informed consent from the patients was not necessary).

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APPLICATION OF SiO_2/ HYDROXYPROPYL CELLULOSE HYBRID MATERIALS DOPED WITH Zn IONS IN ZINC DEFICIENCY

D. Pencheva[1], A. Sestrimska[2], A. Dimitrova[2], L. Borisova[2], R. Karamfilova[2], N. Rangelova[2], Ts. Angelova[2], N. Georgieva[2]*

[1]Bul Bio-NCIPD, 26 Yanko Sakazov Blvd., Sofia, Bulgaria
[2]University of Chemical Technology and Metallurgy, 8 Kliment Ohridski Blvd., Sofia, Bulgaria

ABSTRACT

The presence of bactericidal properties against clinical strains of *Enterobacteriaceae* in synthesized SiO_2-hydroxypropyl cellulose hybrid materials containing Zn ions is a prerequisite for their use as a feed additive for the prevention of zinc deficiency conditions and treatment of diarrhoea in young children in developing countries.

KEYWORDS:

Hydroxypropyl cellulose, Sol-gel method, Zn ions, *Enterobacteriaceae*

INTRODUCTION

The human body acquires the necessary amount of zinc from food and water. Zinc entry into the body is characterised by rapid absorption, mainly in the small intestine, transportation in the blood plasma where it binds to albumin and globulin or passes into tissues where both zinc and cadmium are deposited (1). The body of an adult contains about 2-3 grams of zinc. Zinc is needed to perform many biological functions and has a key role in over 300 enzymes in humans. Zinc exists as part of metalloenzymes and in hormonal complexes. In biological systems it occurs in two forms: bound zinc and chemically reactive Zn^{2+}. It is a structural component of many zinc-related proteins, including molecules of the cell signalling pathway (2). Zinc takes part in the formation of ligands with organic molecules and is one of the most important microelements necessary for the vital functions of the body. Zinc has an important role in biological processes such as cell growth and division, osteogenesis, immune response, etc. At the same time, decreased concentration of zinc cations may affect essential processes in the cells. Its deficiency is often caused by pathological conditions (1, 3, 4).

In recent years, scientists have studied thoroughly zinc, zinc ions, their compounds, and effects on the body as biomarkers and antioxidants. The lethal impact upon tissue distribution, activity in some structures (cell membranes, proteins, etc.) is also examined as a perspective for pharmaceutical purposes (5). Organ systems known to be clinically affected by severe zinc deficiency conditions include the epidermis, gastrointestinal tract, central nervous system, immune system, skeletal and reproductive systems (6). The clinical manifestation of diarrhoea is known as zinc deficiency, which is a clinical feature of most cases of enteropathic acrodermatitis (6). A wide variety of immune defects (especially in compromised T-cell functions) are recognised in enteropathic acrodermatitis with a corresponding vulnerability to a wide range of viral, bacterial and fungal infections. In advanced cases, it is difficult to distinguish the effect of zinc deficiency from that of secondary protein malnutrition. However, it has been shown that immune function improves with zinc therapy, which is indicative of zinc deficiency due to the specificity and direct effect of the treatment (6). Such impact is also observed on the central nervous system functions. The use of zinc therapy in enteropathic acrodermatitis is followed by a remarkable increase in hedonic tone, motivation, vigilance and responsiveness (7). There is a correspondingly rapid decrease in irritability and anxiety. Low zinc concentration in breast milk is associated with clinical nutritional deficiency of zinc in preterm infants. A study suggests that there may be an abnormality in the uptake of zinc by the mammary gland from the plasma (8). As a result, children are assumed to be exposed to increased risk of diabetic and respiratory diseases. They have reduced physical growth and impaired neuropsychological development. Recent evidence suggests that maintaining optimal amount of zinc in the

body is perhaps the most effective, though only partial, preventive measure that can be taken to reduce morbidity in young children in developing countries (9).

There are several possible biochemical mechanisms by which zinc reduces oxidative stress in cells. Zinc has been shown to have a negative effect on the synthesis of anti-inflammatory cytokines (e.g., TNF-α and IL-1β) producing active forms of oxygen, thus metals can function in the body as antioxidants (10).

MATERIALS AND METHODS

Materials

- In this study were investigated the bactericidal properties of SiO_2/HPC/Zn hybrid materials, obtained by the sol-gel method. SiO_2 was obtained after pre-hydrolysis of tetraethyl orthosilicate. Zinc ions were included from $ZnSO_4*7H_2O$ or $Zn(NO_3)_2*6H_2O$ in different concentrations - 0, 0.5, 2.5 and 5 wt%
- Clinical isolates of *E. coli* and *Klebsiella* spp. were provided by the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance "CMAR" at NCIPD, Sofia, Bulgaria.
- Nutrient media (BulBio-NCIPD Ltd.):
  - o Soybean casein digest agar (SCA)
  - o Deoxycholate agar (DHA)
  - o Mueller-Hinton agar (MHA)
  - o MacConkey Agar (MCA)
  - o Plain agar (PA)
  - o Kligler polytropic medium
- Antimicrobial discs (BulBio-NCIPD Ltd.) used for susceptibility testing were: Cp -ciprofloxacin; Cz - ceftazidime; Am - amikacin; ST - sulfamethoxazole/trimethoprim; A - ampicillin; Cft - ceftriaxone; AS - ampicillin/ sulbactam, D - doxycycline; Tb - tobramycin; C – chloramphenicol.

Methods

- Susceptibility testing was performed with the disc diffusion method (DDM), according to CLSI M2-A9 (11).
- Strains were confirmed to the genus level and belonging to the *Enterobacteriaceae* family by cultivating on different selective and differential nutrient media.
- Antibacterial activity assessment of synthesized samples of hybrid materials: for each strain were prepared 5 tubes with 0.005 g of hybrid material and varying zinc concentrations: 0%, 0.5%, 2.5%, 5%. 1 ml of sterile water for injection was added to each tube so that no material is left on the tube walls. After that 0.1 ml of standardised 10^5 to 10^6 CFUs microbial suspension prepared with patented methodology (12) was added to each tube. The experimental setting was incubated at 35°C for 18-24 hours. The remainder of the microbial suspension was incubated under the same conditions of the setting in order to serve as a positive control. On the next day each tube was plated with the surface agar method on a petri dish of plain agar. The inoculated plates were then placed under the same culture conditions for one to three days. The concentration at which bacterial growth is fully suppressed determines the minimum bactericidal concentration (MBC) of the hybrid material for the strain. This is the lowest concentration where no microbial growth is tolerated with up to 3 colony-forming units (CFUs). Microbial growth was evaluated as follows: single colonies of up to 200 counts were counted with a marker on the reverse side of the petri dish; the presence of spread microbial growth was marked as confluent.

RESULTS

Confirmatory susceptibility testing of the *E. coli* strains and interpretation according to CLSI and EUCAST yielded the following results presented in Table 1 and Table 2. The absence of a visible inhibition zone outside the diameter of the disc is indicated by a size of 6 mm.

Antibiotic susceptibility of the *E. coli* strains was interpreted also according to EUCAST guidelines since microbiological laboratories in Bulgaria are already working with the European standard (13) (Table 2).

Antibiotic susceptibility of *Klebsiella* strains was tested in an analogous way (Table 3 and Table 4).

Because antibacterial activity of the two hybrid materials was previously determined to be equivalent against clinical strains of *Salmonella* and *Shigella*, it was decided the SiO_2/HPC/$ZnSO_4$ hybrid material test to be carried out with *E. coli* strains and the hybrid material SiO_2/HPC/$Zn(NO_3)_2$ with *Klebsiella* strains.

Table 1. Antibiotic susceptibility results of the tested *E. coli* clinical strains according to CLSI 2015 (13).

Strains	AS	CLSI	C	CLSI	Cp	CLSI	Tb	CLSI	D	CLSI	Cz	CLSI
E.coli 5	6 mm	R	9 mm	R	6 mm	R	6 mm	R	15 mm	S	8 mm	R
E.coli 11	6 mm	R	9 mm	R	6 mm	R	6 mm	R	14 mm	S	6 mm	R
E.coli 13	6 mm	R	9 mm	R	6 mm	R	6 mm	R	14 mm	S	11mm	R
E.coli 800	6 mm	R	29 mm	S	6 mm	R	9 mm	R	20 mm	S	9 mm	R

Legend: S – sensitive, I – intermediate, R – resistant, Cp – ciprofloxacin, Cz – ceftazidime, Cft – ceftriaxone, AS - ampicillin/sulbactam, D – doxycycline, Tb – tobramycin, C - chloramphenicol.

Table 2. Antibiotic susceptibility results of the tested *E. coli* clinical strains according to EUCAST (14).

Strain	AS	EUCAST	C	EUCAST	Cp	EUCAST	Tb	EUCAST	D	EUCAST	Cz	EUCAST
E.coli 5	6 mm	R	9 mm	R	6 mm	R	6 mm	R	15 mm	-	8 mm	R
E.coli 11	6 mm	R	9 mm	R	6 mm	R	6 mm	R	14 mm	-	6 mm	R
E.coli 13	6 mm	R	9 mm	R	6 mm	R	6 mm	R	14 mm	-	11 mm	R
E.coli 800	6 mm	R	29 mm	S	6 mm	R	9 mm	R	20 mm	-	9 mm	R

Legend: S – sensitive, R – resistant, Cp – ciprofloxacin, Cz – ceftazidime, Am – amikacin, ST - sulfamethoxazole/trimethoprim, A – ampicillin, Cft – ceftriaxone, AS - ampicillin/sulbactam, D – doxycycline, Tb – tobramycin, C – chloramphenicol.

Table 3. Antibiotic susceptibility results of the tested *Klebsiella* clinical strains according to CLSI (13).

Klebsiella	AS	CLSI	C	CLSI	Cp	CLSI	Tb	CLSI	D	CLSI	Cz	CLSI
K.2456	6mm	R	23mm	S	6mm	R	10mm	R	15mm	S	6mm	R
Klebsiella	6mm	R	6mm	R	6mm	R	11mm	R	11mm	I	6mm	R
K.2470	6mm	R	13mm	I	6mm	R	12mm	R	6mm	R	6mm	R
K.2489	6mm	R	23mm	S	6mm	R	6mm	R	15mm	S	6mm	R
K.2438	6mm	R	25mm	S	24mm	S	6mm	R	11mm	I	18mm	I
K.2455	6mm	R	27mm	S	6mm	R	13mm	I	11mml	I	16mm	R
K.2493	6mm	R	26mm	S	29mm	S	6mm	R	18mm	S	19mm	I
K.2494	6mm	R	25mm	S	30mm	S	6mm	R	16mm	S	18mm	I

Legend: S – sensitive, I – intermediate, R – resistant, Cp – ciprofloxacin, Cz – ceftazidime, AS - ampicillin/sulbactam, D – doxycycline, Tb – tobramycin, C - chloramphenicol.

Table 4. Antibiotic susceptibility results of the tested *Klebsiella* clinical strains according to EUCAST (14).

Klebsiella	AS	EUCAST	C	EUCAST	Cp	EUCAST	Tb	EUCAST	D	EUCAST	Cz	EUCAST
K.2456	6mm	R	23mm	S	6mm	R	10mm	R	15mm	-	6mm	R
Klebsiella	6mm	R	6mm	R	6mm	R	11mm	R	11mm	-	6mm	R
K.2470	6mm	R	13mm	R	6mm	R	12mm	R	6mm	-	6mm	R
K.2489	6mm	R	23mm	S	6mm	R	6mm	R	15mm	-	6mm	R
K.2438	6mm	R	25mm	S	24mm	R	6mm	R	11mm	-	18mm	R
K.2455	6mm	R	27mm	S	6mm	R	13mm	R	11mm	-	16mm	R
K.2493	6mm	R	26mm	S	29mm	S	6mm	R	18mm	-	19mm	R
K.2494	6mm	R	25mm	S	30mm	S	6mm	R	16mm	-	18mm	R

Legend: S – sensitive, R – resistant, Cp – ciprofloxacin, Cz – ceftazidime, Am – amikacin, ST- sulfamethoxazole/trimethoprim, A – ampicillin, AS - ampicillin/sulbactam, D – doxycycline, Tb – tobramycin, C - chloramphenicol.

Results from testing the antibacterial activity of the hybrid material SiO_2/HPC/$ZnSO_4$ against four selected *E. coli* strains are presented in Table 5 and Figure 1.

Table 5. Antibacterial activity test results for SiO_2/HPC/Zn hybrid materials with different concentrations of $ZnSO_4$ against clinical strains of *E. coli.*

Clinical strains	Positive control	0%	0.5%	2.5%	5%
E.coli 5	Confluent	Confluent	Confluent	>300	0
E.coli 11	Confluent	Confluent	Confluent	>300	0
E.coli 13	Confluent	Confluent	Confluent	>300	1
E.coli 800	Confluent	Confluent	Confluent	>300	1

Figure 1: Antibacterial activity of SiO_2/HPC/Zn hybrid materials with different concentrations of $ZnSO_4$ against the *E. coli* 5 strain.

We observed the presence of bactericidal activity and determined the minimal bactericidal concentration (MBC) of the tested strain in the dilution where there was no microbial growth after inoculation with the surface agar method.

Results from testing the antibacterial activity of the hybrid material SiO_2/HPC/$Zn(NO_3)_2$ against clinical strains of *Klebsiella* spp. are presented in Table 6 and Figure 2.

Table 6: Antibacterial activity test results for SiO_2/HPC/Zn hybrid materials with different concentrations of $Zn(NO_3)_2$ against clinical strains of *Klebsiella* spp.

Klebsiella	**0%**	**0.5 %**	**2.5 %**	**5.0 %**	**+ Control**
Kl.2456	Confluent	Confluent	<300	1	Confluent
Kl.2470	Confluent	Confluent	<300	<100	Confluent
Klebsiella	Confluent	Confluent	<100	0	Confluent
Kl.2489	Confluent	Confluent	<100	<100	Confluent

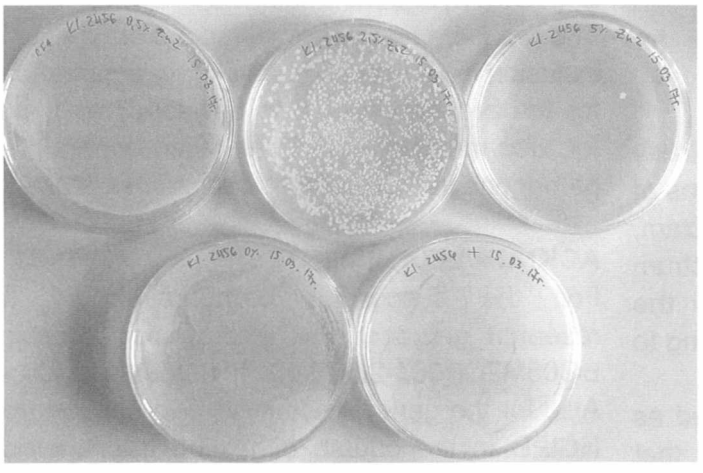

Figure 2: Antibacterial activity of SiO_2/HPC/Zn hybrid materials with different concentrations of $Zn(NO_3)_2$ against the *Klebsiella* 2456 strain.

DISCUSSION

Antibiotic susceptibility results interpreted according to CLSI M100-S25 (Table 1) showed that all tested *E. coli* strains were resistant to ampicillin/sulbactam, ciprofloxacin, tobramycin, ceftazidime, and sensitive to doxycycline. *E. coli* 5, 11, 13 were resistant to chloramphenicol, while *E. coli* 800 was susceptible.

Interpretation following EUCAST guidelines (Table 2) showed that all *E. coli* were resistant to ampicillin/sulbactam, tobramycin and ceftazidime. With the exception of *E.coli* 800, the other three strains were also resistant to chloramphenicol. However, in the European standard there are no breakpoints available for doxycycline. Therefore, *E. coli* susceptibility test results remain without interpretation for this antibiotic.

There are many reports in published literature describing carbapenem resistance in *Klebsiella pneumoniae* (15, 16, 17, 18). Clinical strains of *E. coli* and *Klebsilella* spp. from previous studies (19, 20) were included in the current investigation of antibiotic resistance and sensitivity to synthesized hybrid materials containing silver nanoparticles. The antibiotic susceptibility results for *Klebsiella* strains interpreted according to CLSI 2015 (Table 3) are summarised as follows:

- *Klebsilella* 2456, 2489, 2438, 2455, 2493, 2494 strains were susceptible to chloramphenicol, whereas *Klebsiella* 2470 was intermediate;
- *Klebsiella* 2438, 2493, 2494 strains were the only susceptible to ciprofloxacin;
- *Klebsiella* 2456, 2470 strains were resistant to tobramycin, whereas *Klebsiella* 2455 was intermediate;

- *Klebsiella* 2456, 2489, 2493, 2494 strains were susceptible to doxycycline, and *Klebsiella*, *Klebsiella* 2438, 2455 were intermediate;
- *Klebsiella* 2438, 2493, 2494 strains were intermediate to ceftazidime, and the rest were resistant;
- All tested *Klebsiella* strains were resistant to ampicillin/sulbactam.

Interpreting the results following the European standard (Table 4) showed that:

- All tested *Klebsiella* strains with the exception of two strains - *Klebsiella* and *Klebsiella* 2470, were sensitive to chloramphenicol;
- All *Klebsiella* strains with the exception of *Klebsiella* 2438 and 2493, were resistant to ciprofloxacin;
- All tested *Klebsiella* strains were resistant to ampicillin/sulbactam, tobramycin and ceftazidime;
- There is no interpretation available for doxycycline.

When interpreting antibiotic susceptibility according to both standards, all tested strains of *Klebsiella* were resistant to ampicillin/sulbactam, which implies the production of broad-spectrum beta-lactamases. This was confirmed by the ceftazidime resistance in all strains according to EUCAST breakpoints.

E. coli and *Klebsiella* strains were identified as polyresistant to antibiotics which confirms that they were properly chosen to test the antibacterial action of hybrid materials.

The absence of bacterial growth or the presence of only 1 CFU at a zinc concentration of 5% gives a reason to consider it as the MBC for the SiO_2/HPC/$ZnSO_4$ hybrid material.

There was a slight difference in the twofold experiment, which could be explained with the powder state of the hybrid material partially remained stuck to the walls of the tube. According to the definition of bactericidal action of a substance, the results obtained for the various *Klebsiella* spp. strains were not corresponding. However, it can be assumed that bactericidal activity is present at a concentration of 5% zinc ions in the SiO_2/HPC/$Zn(NO_3)_2$ materials.

Positive controls of all strains with both hybrids showed presence of confluent growth, comparable to the plated dilutions used for negative controls with 0% zinc concentration in the hybrid material.

CONCLUSION

The polyresistant clinical strains used in this study proved to be a suitable model for inclusion in the experiment.

In summary, all negative controls with 0% zinc concentration in the hybrid material showed confluent growth comparable with the positive controls, which is indicative of a lack of bactericidal activity of the stabiliser. Thus, the bactericidal action is entirely due to the activity of the zinc ions in the material. A 5% zinc concentration in the SiO_2/HPC/$ZnSO_4$ hybrid material was found to be bactericidal against all *E. coli* strains. Although some of the *Klebsiella* strains showed greater resistance which could be attributed to the extremely thick capsule being the major virulence factor, this concentration can also be considered as bactericidal for SiO_2/HPC/$Zn(NO3)_2$ hybrid materials.

The two hybrid materials tested are prospects for inclusion in clinical trials as dietary supplements for the prevention of zinc deficiency and even for treatment of infections with opportunistic pathogens from the *Enterobacteriaceae* family.

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COMPARISON OF THE BACTERICIDAL EFFECT OF TWO SiO_2/ HYDROXYPROPYL CELLULOSE HYBRID MATERIALS, DOPED WITH Zn IONS AGAINST CLINICAL STRAINS OF *SALMONELLA* AND *SHIGELLA*

D. Pencheva[1], E. Yordanova[2], V. Susanina[2], Ts. Angelova[2], N. Rangelova[2], N. Georgieva[2]*

[1]BulBio-NCIPD, 26 Yanko Sakazov Blvd., Sofia, Bulgaria
[2]University of Chemical Technology and Metallurgy, 8 Kliment Ohridski Blvd., Sofia, Bulgaria

ABSTRACT

The current study was performed to compare the biological properties of hybrid materials composed of SiO_2/hydroxypropyl cellulose (HPC) and Zn ions. The concentration of zinc was varied between 0 – 5 wt% and derived from different precursors - zinc sulphate or zinc nitrate. Antibacterial activity of the materials was tested against selected clinical strains of *Salmonella* and *Shigella* with established resistance to two or more antibiotics. The hybrid materials doped with Zn ions showed pronounced bactericidal activity against the tested strains.

KEYWORDS:

Hydroxypropyl cellulose, Sol-gel method, Zn ions, *Salmonella*, *Shigella*

INTRODUCTION

Bio-composites with inorganic part (phase) evenly distributed in the polymer matrix (1, 2) are found in nature. Hybrid materials are a subject of study by a number of scientists because of the inclusion of organic components in an inorganic network that combines their properties. The hybrid material brings together the properties of the organic and inorganic component whereas simultaneously exhibits new properties as a result of further interaction between the two components.

Nanoparticles are a special group of materials with unique properties and extensive application in different fields (3). Investigating the physicochemical, physical and biological characteristics of these materials is of great interest to many scientists. A number of authors prove that zinc ions and nanoparticles (NPs) are non-toxic to human cells (4). This aspect of their properties allows them to be used as antibacterial agents, therapeutic agents, in surgical devices, in diagnostics, bioengineering, biodiagnostics, optoelectronics, in agriculture as surface coatings and in nanomedicine (4). On the other hand, nanoparticles have the ability to reduce bacterial growth due to membrane disruption and increase of permeability, which leads to accumulation of Zn-NPs inside the membrane and entry in the cytoplasm (5).

Cellulose ethers such as methylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, carboxymethyl cellulose, etc. have the most important industrial and practical applications. Hydroxypropyl cellulose is one of the most commonly used cellulose ethers in various fields such as pharmaceutical, cosmetic and food industry. It has an advantageous role in the synthesis of silica/cellulose hybrid materials by the sol-gel process.

In previous investigations SiO_2/HPC/Ag and SiO_2/HPC/Zn hybrid materials have been synthesized, characterised and tested as antimicrobial agents against different types of bacteria and fungi (6).

MATHERIALS AND METHODS

Materials

- In this investigation were studied the bactericidal properties of a series of SiO_2/ HPC/Zn hybrid materials obtained by the sol-gel method. $SiO_2$was obtained after pre-hydrolysis of tetraethyl orthosilicate. Zinc ions were included from $ZnSO_4*7H_2O$ or $Zn(NO_3)_2*6H_2O$ in different concentrations - 0, 0.5, 2.5 and 5 wt%
- The study was performed with selected six clinical strains of *Salmonella* spp. and *Shigella* spp. provided by reference laboratories at NCIPD, Sofia, Bulgaria.

- Nutrient media (BulBio-NCIPD Ltd.) used in the study were:
  - o Soybean casein digest agar (SCA)
  - o Deoxycholate agar (DHA)
  - o Mueller-Hinton agar (MHA)
  - o MacConkey Agar (MCA)
  - o Plain agar (PA)
  - o Kligler polytropic medium
- *Salmonella* diagnostic sera for agglutination (BulBio-NCIPD Ltd.): OC(O7,8), O7, H5, H12, O8, O20, Hz_6, Hlv, OB(O4,5), O5, Hi, Hb.
- Antimicrobial discs (BulBio-NCIPD Ltd.) used for susceptibility testing were: Cp -ciprofloxacin; Cz - ceftazidime; Am - amikacin; ST - sulfamethoxazole/trimethoprim; A - ampicillin; Cft - ceftriaxone; AS - ampicillin/ sulbactam.

Methods

- Susceptibility testing was performed with the disc diffusion method (DDM), according to CLSI M2-A9 (7).
- Strains were confirmed to the genus level by cultivating on different selective and differential nutrient media.
- Serotyping of *Salmonella* spp. and *Shigella* spp. strains was performed with slide agglutination reaction (Gruber) with known diagnostic agglutinating sera.
- Antibacterial activity assessment of synthesized samples of hybrid materials: for each strain were prepared 5 tubes with 0.005 g of hybrid material and varying zinc concentrations: 0%, 0.5%, 2.5%, 5%. 1 ml of sterile water for injection was added to each tube so that no material is left on the tube walls. After that 0.1 ml of standardised 10^5 to 10^6 CFUs microbial suspension prepared with patented methodology (12) was added to each tube. The experimental setting was incubated at 35°C for 18-24 hours. The remainder of the microbial suspension was incubated under the same conditions of the setting in order to serve as a positive control. On the next day each tube was plated with the surface agar method on a petri dish of plain agar. The inoculated plates were then placed under the same culture conditions for one to three days. The concentration at which bacterial growth is fully suppressed determines the minimum bactericidal concentration (MBC) of the hybrid material for the strain. This is the lowest concentration where no microbial growth is tolerated with up to 3 colony-forming units (CFUs). Microbial growth was evaluated as follows: single colonies of up to 200 counts were counted with a marker on the reverse side of the petri dish; the presence of spread microbial growth was marked as confluent.
- A method for assessing the reduction of bacterial inoculum was developed based on literature data (9) and BDS EN ISO 11133: 2014 standard (10) used in qualifying nutrient selectivity. When assessing the action of disinfectants, a reduction in the biological load with 5 logarithms (9) is required in a starting microbial inoculum of 10^5 to 10^6 CFUs. In this study was used the same density of bacterial suspension and the MBC was determined from the concentration where there is no bacterial growth or up to 3 CFUs of the respective strain. Petri dishes plated with the surface agar method and incubated for 24 hours at 35°C were counted for microbial growth on the following day and the number of single colonies formed (CFU/ml) was calculated with the following formula:

$$\mathbf{R_R = D_O - D_S}$$

R_R – degree of reduction expressed as a decimal log of the degree difference

D_O – initial concentration of bacterial load 10^5-10^6 CFUs

D_S – reported number of colonies on the agar medium, expressed in base 10.

RESULTS

Following culture confirmation to the genus level, *Salmonella* serotyping was performed by slide agglutination with polyvalent OA-OE serum. There were clearly visible large agglutinates with clarification of the liquid and confirmation of the O-serogroup of each strain. Following H-agglutination and determining the H-phase, the Kaufman-White scheme was used to assign the antigenic serotype formula to each *Salmonella* strain. The confirmed O- and H-agglutinins are shown in bold in Table 1.

Table 1. Serotype confirmation results for *Salmonella* strains with Gruber agglutination.

Serogroup	Serotype	Antigenic formula		
		O-antigens	1 H-antigen phase	2 H-antigen phase
B	*S.* Typhimurium 47	1, 4, **[5]** ,12	**i**	1 ,2
C 1	*S.* Infantis 6	6, **7**, 14	r	**1 ,5**
C 2-3	*S.* Corvallis 1888	**8, 20**	z_4 , z_{23}	**z_6**
B	*S.* Brandenburg 854	4, **[5]** ,12	**l ,v**	e ,n , z_{15}
B	*S.* Paratyphi B 176	1, 4, **[5]** ,12	**b**	1 ,2
B	*S.* Brandenburg 891	4, [5] ,12	**l , v**	e,n,z_{15}

Confirmatory antibiotic susceptibility test results are presented in Table 2. The absence of a visible inhibition zone outside the diameter of the disc is indicated by a size of 6 mm.

Table 2. Inhibition zone diameters obtained in the antibiotic susceptibility testing of the strains.

Strains	Inhibition zones (mm)						
	Cp	Cz	Am	ST	A	Cft	AS
S. Typhimurium 47	32	26	20	6	6	6	-
S. Infantis 6	29	26	20	6	28	30	-
Shigella 34	22	17	18	6	6	6	14
Shigella 35	27	17	14	6	6	6	15
Shigella 38	21	13	16	6	6	6	13
Shigella 39	24	-	15	6	6	6	15
Shigella 40	24	16	19	6	6	6	14

Legend: Cp – ciprofloxacin, Cz – ceftazidime, Am – amikacin, ST - sulfamethoxazole/trimethoprim, A – ampicillin, Cft – ceftriaxone, AS - ampicillin/sulbactam.

Interpretation of antibiotic susceptibility test results was performed according to CLSI M100-S17 (11) (Table 3).

Table3. Antibiotic susceptibility interpretation of the tested strains.

Strains	Antibiotic susceptibility interpretation	
	S	R
Salmonella Typhimurium 47	Cp, Cz, Am	ST, A, Cft
Salmonella Infantis 6	A, Cft, Cp, Cz, Am	ST
Shigella 34	Cp, Cz, Am, AS,	A, Cft, ST
Shigella 35	Cp, Cz, Am, AS,	A, Cft, ST
Shigella 38	Cp, Cz, Am, AS,	A, Cft, ST
Shigella 39	Cp, Cz, Am, AS,	A, Cft, ST
Shigella 40	Cp, Cz, Am, AS,	A, Cft, ST

Legend: S-sensitive, R-resistant.

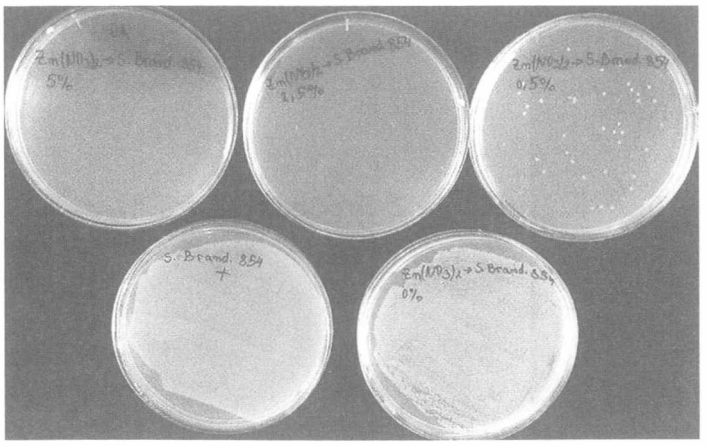

Figure 1: Antibacterial activity of SiO_2/HPC/$Zn(NO_3)_2$ hybrid materials with different zinc concentrations against *Salmonella* Brandenburg 854.

Table4. Antibacterial activity test results for SiO_2/HPC/Zn hybrid materials with different concentrations of $Zn(NO_3)_2$ against *Salmonella* strains.

Strains	CFU				
	0%	0.5%	2.5%	5%	Control
S. Typhimurium 47	Confluent	0	0	0	Confluent
S. Infantis 6	Confluent	13	0	0	Confluent
S. Corvallis 1888	Confluent	6	0	0	Confluent
S. Brandenburg 854	Confluent	35	0	0	Confluent
S. Paratyphi B 176	Confluent	3	0	0	Confluent
S. Brandenburg 891	Confluent	4	0	0	Confluent

Test results data for determining the bactericidal activity of the hybrid materials against *Shigella* strains are presented in Table 5 and demonstrated in Figure 2.

Table5. Antibacterial activity test results for SiO_2/HPC/Zn hybrid materials with different concentrations of $Zn(NO_3)_2$ against *Shigella* strains.

Strains	CFU				
	0%	0.5%	2.5%	5%	Positive control
Shigella 34	Confluent	5	0	0	Confluent
Shigella 35	Confluent	1	0	0	Confluent
Shigella 38	Confluent	0	0	0	Confluent
Shigella 39	Confluent	0	0	0	Confluent
Shigella 40	Confluent	1	0	0	Confluent
Shigella 41	Confluent	0	0	0	Confluent

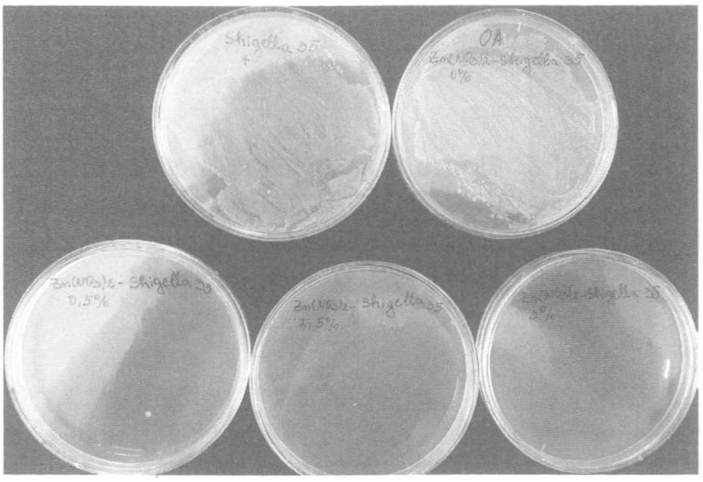

Figure 2: Antibacterial activity of SiO_2/HPC/$Zn(NO_3)_2$ hybrid materials against Shigella 35.

The same *Shigella* and *Salmonella* clinical strains were tested for determining the antibacterial activity of SiO_2/HPC/$ZnSO_4$ hybrid materials. Test results for *Shigella* strains are presented in Table 6 and demonstrated in Figure 3.

Table 6. Antibacterial activity test results for SiO_2/HPC/Zn hybrid materials with different concentration of $ZnSO_4$.

Strains	CFU				
	0%	0.5%	2.5%	5%	Positive control
Shigella 34	Confluent	13	0	0	Confluent
Shigella 35	Confluent	0	0	0	Confluent
Shigella 38	Confluent	0	0	0	Confluent
Shigella 39	Confluent	0	0	0	Confluent
Shigella 40	Confluent	1	0	0	Confluent
Shigella 41	Confluent	30	0	0	Confluent

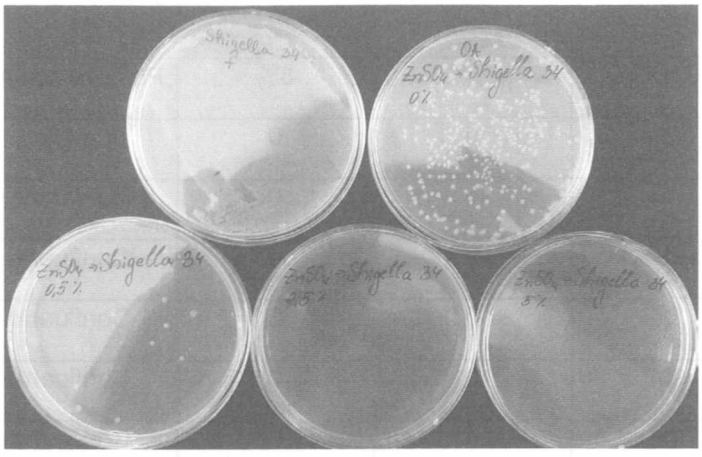

Figure 3: Antibacterial activity of SiO_2/HPC/$ZnSO_4$ hybrid materials against *Shigella* 34. The standardised suspension used in this assay served as a positive control and demonstrated apparent confluent growth.

Data for determining the bactericidal action of the hybrid material containing zinc sulphate as a precursor against *Salmonella* strains is presented in Table 7 and demonstrated in Figure 4.

Table 7. Antibacterial activity test results for SiO_2/HPC/Zn hybrid materials with different concentrations of $ZnSO_4$.

Strains	CFU				
	0%	0.5%	2.5%	5%	Positive control
S. Typhimurium 47	Confluent	124	0	0	Confluent
S. Infantis 6	Confluent	54	0	0	Confluent
S. Corvallis 1888	Confluent	4	0	0	Confluent
S. Brandenburg 854	Confluent	22	0	0	Confluent
S. Paratyphi B 176	Confluent	16	0	0	Confluent
S. Brandenburg 891	Confluent	139	0	0	Confluent

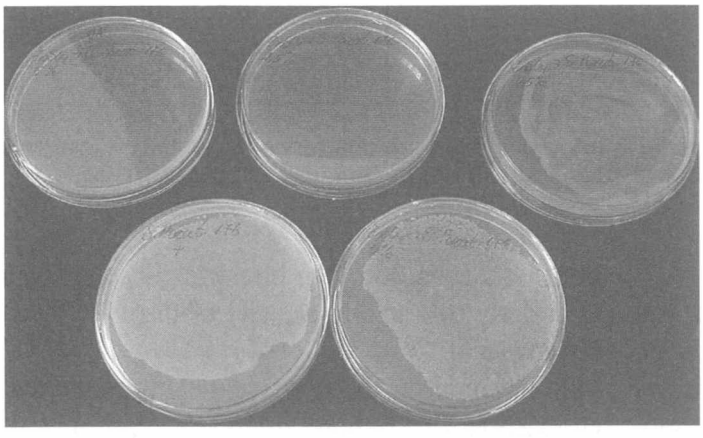

Figure 4. Antibacterial activity of SiO_2/HPC/$ZnSO_4$ hybrid materials against *Salmonella* Paratyphi B 176. The standardised suspension used in this assay served as a positive control.

The calculated results for degree of reduction of the inoculum for *Salmonella* strains are presented in Table 8.

Table 8. Degree of reduction in *Salmonella* strains cultivated in the presence of SiO_2/HPC/$Zn(NO_3)_2$ hybrid materials with different zinc concentrations.

Strains $D_O=10^5$-10^6	SiO_2/HPC/Zn hybrid materials with different concentrations of $Zn(NO_3)_2$								Positive control	
	0%		0.5%		2.5%		5%			
	Ds	R_R	Ds	R_R	Ds	R_R	Ds	R_R	Ds	R_R
S. Typhimurium 47	10^5-10^6	0	10^0	5-6	0	6	0	6	10^5-10^6	0
S. Infantis 6	10^5-10^6	0	10^1-10^2	4	0	6	0	6	10^5-10^6	0
S. Corvallis 1888	10^5-10^6	0	10^0-10^1	5	0	6	0	6	10^5-10^6	0
S. Brandenburg 854	10^5-10^6	0	10^2-10^3	3	0	6	0	6	10^5-10^6	0
S. Paratyphi B 176	10^5-10^6	0	10^0-10^1	5	0	6	0	6	10^5-10^6	0
S. Brandenburg 891	10^5-10^6	0	10^0-10^1	5	0	6	0	6	10^5-10^6	0

Legend: R_R – degree of reduction expressed as a decimal log of the degree difference; D_O –initial concentration of bacterial load 10^5-10^6 CFUs; D_S – reported number of colonies on the agar medium expressed in base 10.

The calculated results for degree of reduction of the inoculum for *Shigella* strains are presented in Table 9.

Table 9. Degree of reduction in *Shigella* strains cultivated in the presence of SiO_2/HPC/Zn $(NO_3)_2$ hybrid materials with different zinc concentrations.

Strains $D_O=10^5$-10^6	SiO_2/HPC/Zn hybrid materials with different concentrations of $Zn(NO_3)_2$								Positive control	
	0%		0.5%		2.5%		5%			
	Ds	R_R	Ds	R_R	Ds	R_R	Ds	R_R	Ds	R_R
Shigella 34	10^5-10^6	0	10^0-10^1	5	10^0	6	10^0	6	10^5-10^6	0
Shigella 35	10^5-10^6	0	10^0-10^1	5	10^0	6	10^0	6	10^5-10^6	0
Shigella 38	10^5-10^6	0	10^0	5-6	10^0	6	10^0	6	10^5-10^6	0
Shigella 39	10^5-10^6	0	10^0	5-6	10^0	6	10^0	6	10^5-10^6	0
Shigella 40	10^5-10^6	0	10^0-10^1	5	10^0	6	10^0	6	10^5-10^6	0
Shigella 41	10^5-10^6	0	10^0	5-6	10^0	6	10^0	6	10^5-10^6	0

Legend: R_R – degree of reduction expressed as a decimal log of the degree difference; D_O – initial concentration of bacterial load 10^5-10^6 CFU; D_S – reported number of colonies on the agar medium, expressed in base 10.

DISCUSSION

All *Salmonella* strains were identified as resistant to sulfamethoxazole/trimethoprim, and *Salmonella* Typhimurium 47 was found to be resistant to ampicillin and ceftriaxone. Antibiotic susceptibility testing of *Shigella* strains (Table 3) showed resistance to ampicillin, ceftriaxone and sulfamethoxazole/trimethoprim in all of them. These results confirm the alarming resistance levels observed in Gram-negative microorganisms (13, 14, 15) and such polyresistant strains are increasingly isolated from clinical specimens. The strains used in this study were thus recognised as an appropriate model for testing the antimicrobial activity of hybrid materials (16, 17).

It is assumed that 99.9% of the microorganisms have to be killed in order to establish the presence of bactericidal activity (12). For this purpose, we determined the minimal bactericidal concentration (MBC) as the dilution where no microbial growth is observed after plating the test strain with the surface agar method. All positive controls showed confluent growth comparable to that from all plated dilutions serving as negative controls with 0% zinc concentration in the hybrid material. This is indicative of the fact that the stabiliser does not have bactericidal effect which is entirely due to the activity of the zinc ions in the material.

The antibacterial activity assessment of SiO_2/HPC/$Zn(NO_3)_2$ hybrid materials revealed the presence of bactericidal activity against the tested strains. One *Salmonella* strain was fully inhibited at all zinc concentrations and three strains showed almost complete suppression at 0.5% zinc concentration. However, the other two *Salmonella* strains tested with SiO_2/HPC/$Zn(NO_3)_2$ hybrid materials were determined with MBC at 2.5% zinc concentration (Table 4, Figure 1). For *Shigella* strains single colonies were present at 0.5% zinc concentration, with bactericidal activity exhibited at concentrations of 2.5% and 5% (Table 5, Figure 2).

Bactericidal activity was found also in the assessment of antibacterial activity of SiO_2/HPC/$ZnSO_4$ hybrid materials. 0.5% zinc concentration was determined as the MBC against four *Shigella* strains (No. 35, 38, 39 and 40) and 2.5% for the other two strains (Table 6, Figure 3). All *Salmonella* strains tested with SiO_2/HPC/$ZnSO_4$ hybrid materials had equal value of the MBC, namely 2.5% zinc content (Table 7, Figure 4).

The new method used in this study to evaluate the biocidal properties of the hybrid materials with degree of reduction of the bacterial inoculum, identified them as good biocides at zinc concentration greater than 0.5% (Table 8 and Table 9).

The insolubility in water due to the powder state of the hybrid material, part of which stuck to the walls of the tube, posed some difficulties in conducting the assay. However, it can be noted that at 2.5% zinc concentration, irrespective of the type of precursor used - zinc nitrate or zinc sulphate, bactericidal activity was observed with 5 mg of the material dispersed in 1 ml water for injection.

CONCLUSION

After performing the disc diffusion method, we found resistance to some antibiotics in the clinical strains of *Salmonella* and *Shigella* used in this study. It can be concluded that the extent of inhibition of the test strains with load 10^5-10^6 CFUs depends directly on the concentration of zinc ions contained in 0.005 g hybrid material dispersed in 1 ml water for injection and incubated for 18-24 hours. Based on the method for determining the antibacterial activity of SiO_2/HPC/$Zn(NO_3)_2$ and SiO_2/HPC/$ZnSO_4$ hybrid materials with varying amounts of zinc, we can summarise that both materials have bactericidal activity. At 0.5% zinc concentration in the material, microbial growth was still observed in most *Salmonella* and some *Shigella* strains. However, at 2.5% there were no colonies present with both types of hybrid materials and was established strong bactericidal activity. Therefore, 2.5% zinc concentration can be assigned as the MBC for both materials.

It is noteworthy that the number of single colonies observed for *Shigella* strains was significantly lower than *Salmonella* counts, indicating that regardless of the zinc precursor used, the hybrid materials have a more pronounced potential to inhibit *Shigella* strains. By performing the in-process reduction method we confirmed the biocidal properties of 0.005 g hybrid material with zinc concentrations of 2.5% and 5% against inoculum prepared from clinical strains of *Shigella* and *Salmonella*.

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BULGARIAN GPs AND OBGs ATTITUDE TOWARDS INFLUENZA VACCINATION DURING PREGNANCY

A. Galev

SCMEH – MMA, Sofia

ABSTRACT

Vaccination is considered the best tool for prevention of seasonal influenza distribution. Even though pregnant women are regarded as a high-risk group during the active flu season, small percentage of general practitioners (GPs) and obstetrician-gynaecologists (OBGs) advise on vaccination which is yet available in the list of recommended vaccines. The reason for vaccine refusal both by pregnant women and medical professionals is not fully clarified. The goal of this study is to define the attitude of GPs and OBGs towards influenza vaccination in pregnant women as well as to define a mode of action to increase the vaccine coverage.

KEYWORDS:

seasonal flu, vaccination, pregnancy

INTRODUCTION

Seasonal influenza is a severe viral disease that affects millions all over the world and tens of thousands die (8). Besides high morbidity registered during the epidemic season, it leads to high mortality due to underlying conditions or related complications (2, 6, 7, 14, 15, 19). The prevention of virus distribution in human population (9, 10, 11)via isolation or disease treatment meets countless problems and the used methods (12,15)are often proven ineffective. The main preventive measure is vaccination of the risk population, including pregnant women.

ADDRESS FOR CORRESPONDENCE:

Andrei Galev,
Scientifically applied Center for Military Epidemiology and Hygiene,
Military Medical Academy
Sofia, 3 St. Georgi Sofiyski str.
e-mail andrey_galev@abv.bg

Despite of legislation changes and newly issued recommendations, vaccine coverage in high-risk groups is still low. There are a number of explanations that vary from personal to financial or organisational matters. The goal of this study is to define the Bulgarian GPs and OBGs` attitude towards pregnant women influenza vaccination – knowledge, recommendations, style of work, physician-patient interaction. Introduction of modern medical practices in individual patient care is a cornerstone of modern healthcare. The problem identification allows to be evolved more effective tools and practices for increasing vaccine coverage among pregnant women.

MATERIAL AND METHODS

An inquiry with ten questions was distributed to GPs and OBGs. One hundred and twenty GPs and 63 OBGs returned a reply. Data were developed with statistical package IBM SPSS Statistics 23.0. $P<0.05$ was considered as the level of significance that rejects the zero hypothesis. Methods for data statistical development included descriptive analysis to present the studied indices distribution in tabular mode, graphic analysis to visualise the achieved results and the precise Fisher test and χ^2test to check the presence of a link between category variables.

RESULTS

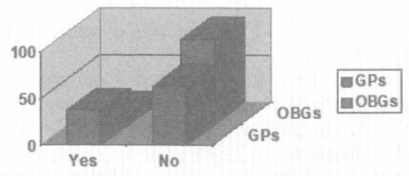
The median age of participating GPs was 53years ranging between 38-79 years with28 (23%) men and 92 (76%) women. The predominant age in the male group (14) was 50-59 years and the largest female age group (54) was under 59 years old. OBGs respondents included 33% male and 67% female participants. The predominant age in the male group was 50-59 years old (47%) and 40% of women were below 39 years of age. Most of the GPs run practices with 500 to 1999 patients (59%), while the OBGs predominantly cover no more than 100 women (63%). The majority of practices are located in cities or downtowns (69%) while only 4% are located in villages.

Thirty seven percent of GPs recommend flu vaccination to pregnant patients. The majority of them do so during the active flu season (27%) and 13% recommend vaccination before pregnancy. No vaccine is recommended during the second trimester. Only 15% of OBGs recommend flu vaccination to pregnant women. Half of them

offer vaccination to all female patients during the active season, and the other half only to patients with chronic and underlying conditions (Table 1).

Table 1. GPs and OBGs distribution on flu vaccine recomendation

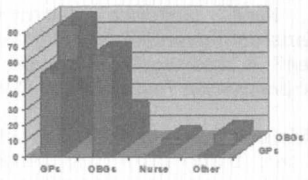
- GPs flu vaccine recommendation
  - Yes – 45 (37.5%)
  - No – 75 (62.5%)
- OBGs flu vaccine recommendation
  - Yes – 10 (15.9%)
  - No – 53 (84.1%)



The majority of GPs (65%) consider OBG who checks the pregnancy progress, to be the person who should recommend influenza vaccination. Participating OBGs consider the GP as the person who should recommend and apply the vaccine to pregnant women (76%) (Table 2).

Table 2. GPs and OBGs distribution on person to promote vaccination

- GPs opinion:
  - GPs – 53.3%
  - OBGs – 65.8%
  - Nurse – 0.0%
  - Other – 0.0%
- OBGs opinion:
  - GPs – 76.2%
  - OBGs – 19.0%
  - Nurse – 3.2%
  - Other – 6.3%

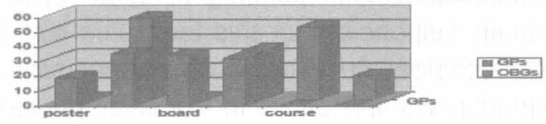


The collected data shows that OBGs do not think the flu vaccine is either safe for the mother or beneficial to the baby (89%). In evaluating the reasons for pregnant women flu vaccination refusal,76% of participants point out the worry about foetal damage caused by vaccination, while 46% underline the insufficient information on the risks to the foetus in case of maternal flu (lower birth weight, premature birth, stillbirth). Financial worries have the least impact (5%).

According to 62.5% of GPs participating in the survey, the best way to provide information on influenza vaccination is media involvement, followed by distribution of booklets (35.8%). OBGs express different opinions where 55% point out that the most adequate strategy would be to provide posters and booklets with information addressing pregnant women and flu vaccine, followed by individual and group courses for students and practitioners (49%). Media campaigns are suggested by 33%, and 16% believe such campaigns should not be organised (Table 3).

Table 3. GPs and OBGs distribution on way to promote vaccination

- GPs how to promote vaccination
  - Poster - 23 (19.2%)
  - Booklets – 43 (35.8%)
  - Boards – 42 (35%)
  - Media – 75 (62.5%)
  - Course – 0 (0%)
  - No action 0 (0%)
- OBGs how to promote vaccination
  - Poster – 0 (0%)
  - Booklets – 35 (55.6%)
  - Boards – 0 (0%)
  - Media – 21 (33.3%)
  - Course – 31 (49.2%)
  - No action – 10 (15.9%)



Vaccination of pregnant women in the practice of the majority GPs (80%)is under 2%. Only one GP or 0.8% has achieved more than 50%coverage. There is no significant relationship between gender of the physician and attitude towards flu vaccination. Comparative analysis of the data shows that 80% of physicians recommending vaccination are below 49 years of age. Older physicians are more sceptical towards vaccination and tend not to recommend it to their patients.

DISCUSSION

Influenza vaccine coverage in Bulgaria is very low, approximately 3%,and thus unlikely to affect significantly circulation of various types/ subtypes/lineages of influenza viruses (9, 11). Flu vaccination is recommended for pregnant women during the active flu season(1, 2)and the vaccine coverage remains low (7, 14, 16, 18). Despite the available evidence of the vaccine safety as well as the possible favourable effect on the mother and foetus, vaccination coverage in the target group remains below 10% (14, 20) with the exception of the USA - 42-44% (17, 18), Canada - 16% (13) and Great Britain - 44.9% (3). Results from our study show even lower vaccination coverage – vaccinated pregnant women during the active influenza season are about 2%.

According to Steelfisher et al. the pregnant woman has to believe that the vaccine is very safe and beneficial to the baby in order to accept vaccination (4, 5, 16, 17), but the main role to mediate this process should be carried out by GPs. Blanchard et al. indicate that direct recommendation from GP or other medical professional is the main motivation for vaccination acceptance (4). Our data shows that GPs tend not to recommend the vaccination and OBGs are even more reluctant mainly due to the doctrine that pregnant women should not receive any medicine unless there is a life-threatening risk.

There is no agreement between GPs and OBGs about who should endorse and conduct flu vaccination, with pointing at each other as the main responsible for this task. The fact that OBGs consider flu vaccine as neither safe for the mother nor beneficial to the baby reveals a significant gap in knowledge. There is also a split of opinions between GPs and OBGs on the ways to provide vaccine information such as media campaigns, posters and booklets distribution. The common ground between them seems to be that the person responsible should be someone else.

Vaccine coverage and motivation behind vaccine refusal or acceptance based on ethnicity, age, education or income, still remain unclear.

The physician's recommendation is the basic behavioural motivator, however it should be flexible and tailored according to the situation (pandemic, epidemic or sporadic) and patient, and knowledge should be further enhanced. In order to be effective, the conducted measures must target not only patient and physician, but also identified organisational and logistical problems.

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