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PROBLEMS

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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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Papers should not have been previously published or be currently under consideration for publication.

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Named authors must fit the following three criteria:

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2. Drafting the article or revising it critically for important intellectual content; and
3. Final approval of the version to be published.

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

ACKNOWLEDGEMENTS

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

REFERENCES

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

Journal articles:

Vellinga A, Cormican M, Hanahoe B, Murphy AW. *Predictive value of antimicrobial susceptibility from previous urinary tract infection in the treatment of re-infection*. Br J Gen Pract. 2010; 60(576):511-513.

Books:

Rosa PA, Hogan D, Margolis N. *Molecular analysis of the major outer surface protein locus from a divergent Borrelia burgdorferi isolate from Europe*. In: Schutzer SE. Lyme borreliosis: Molecular and immunologic aspects. Cold Spring Harbor Laboratory Press, 1992, 95-110.

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DEVELOPMENT OF A MODIFIED MULTI-LOCUS VARIABLE NUMBER OF TANDEM REPEATS ANALYSIS FOR TYPING OF *B. PERTUSSIS* APPLICABLE TO CLINICAL SAMPLES

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ABSTRACT:
Pertussis is one of the major vaccine - preventable diseases with global distribution. In recent years the world has seen an increase in both the total number of cases and in those resulting in death of newborns. The purpose of this paper is to present the development of a new method for MLVA typing of B. pertussis applicable both to pure cultures and clinical samples. A new VNTR locus VNTR11 is described and evaluated for typing of B. pertussis. VNTR11 is characterized by seven alleles and seems to be to be highly polymorphic and hence useful for typing. In addition we have modified the method with the usage of unlabeled primers as to be applicable to agarose electrophoresis. From the 51 samples tested, 32 MLVA genotypes were registered. The results obtained by using this method show that the B. pertussis strains circulating in Bulgaria during the last decade are genetically similar but somewhat divergent from the strain used for the production of the whole cell vaccine in Bulgaria.

Key words: *B. pertussis, typing, MLVA*

INTRODUCTION

Pertussis (whooping cough) is a respiratory disease potentially fatal for infants caused by the bacterium *Bordetella pertussis*. Despite the widespread use of pertussis vaccines during the last decades pertussis has remained an endemic disease with frequent epidemic outbreaks. The typing of the circulating strains shows significant genetic differences between them and the strains used for the production of vaccines (Watanabe et al. 2002, Schouls et al. 2004, Kallonen et al. 2009, Mooi et al. 2009). Pertussis is one of the vaccine - preventable diseases with global distribution (Bamberger et al. 2008, Broutin et al. 2010, WHO 2011). In recent years the world has seen an increase in both the total number of cases and in those resulting in death of newborns (Floret 2001, Mattoo et al. 2005, Paddock et al. 2008, Bart et al. 2010, Zepp et al. 2011). The main reasons for the increase in the number of reported and laboratory confirmed cases in countries with high vaccination coverage are genetic changes in the pathogen resulting in antigenic shift. This results in an adaptation of the pathogen population and antigenic variation from circulating strains of the vaccine strains (Schouls et al. 2004, Mooi et al. 2009, Cherry 2010, Mooi 2010, WHO position paper-recommendations 2011, Zhang et al. 2011).
Multi-locus Variable number of tandem repeat analysis (MLVA) was proposed and adopted by ECDC and EUVAC.NET in 2012 as the reference method for typing strains of *B. pertussis* which can be used both for surveillance of circulating strains and investigation of outbreaks. The original method developed by Schouls (Schouls et al. 2004) described amplification of six VNTRs with fluorescently labeled primers including analysis by an automated sequencer and comparison with known standards for each VNTR.

This study describes the implementation of MLVA typing scheme for *B. pertussis* developed as a modified method with unlabeled primers. A total of six VNTR loci were targeted (MLVA6) with five previously published primer pairs (Schouls et al. 2004, Kurniawan et al. 2010, http://www.mlva.net/bpertussis/documents/Protocol_MLVA_Bpertussis.pdf,) and in addition a novel primer pair targeting the BP VNTR locus designed by us using the Tandem Repeats Finder software. The software automatically shows the nucleotides that can be used as PCR primers on both sides of the VNTR.
A universal oligonucleotide tail was added at the 5‘ ends of each of the primers. The specificity of the designed universal oligonucleotide tail was tested with the program BLAST (The Basic Local Alignment Search Tool). A universal primer with a sequence homologous to that of the tail was constructed at random so that there was no homology to the target DNA. An assessment of its specificity, i.e. lack of homology was performed using BLAST. With the addition of this universal primer a specific amplification was achieved for all the VNTR fragments simultaneously in a multiplex PCR. The purpose of this step was to increase the specificity of PCR.
We developed and introduced modified MLVA applicable for typing of both pure cultures and clinical samples from patients. Using this method we proved a genetic homology between strains circulating in Bulgaria and genetic differences between them and the vaccine strain *B. pertussis* 358 used in our country for the production of a whole cell pertussis vaccine.

OBJECTIVE

Multi-Locus Variable number of tandem repeats Analysis for typing strains of *B. pertussis* was proposed and adopted by ECDC and EUVAC.NET in 2012 as the reference method which can be used both for surveillance of circulating strains and investigation of *B. pertussis* in cases of outbreaks.
The purpose of this study was the development and validation of a modified MLVA typing method including a new VNTR marker.

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METHODS

For the development and implementation of the MLVA analysis we used: 15 *B. pertussis* strains from the collection of the Scientific Medical Academy, Moscow, USSR; DNA from 18 strains of the *B. pertussis* provided by Dr. Stefanelli from Istituto Superiore di Sanita, Rome, Italy and 18 positive for *B. pertussis* DNA from Bulgarian patients. All patient samples were strongly PCR positive demonstrating an active pertussis infection. Genomic DNA isolation from nasopharyngeal secretions and from pure cultures (on Bordet-Gengou agar) was performed by a standard phenol-chloroform method.

For the development of this typing technique the following primer sequences were used (Table 1).

At the 5‘ ends of each of the primers we added a universal oligonucleotide tail (**ATA CAC AGG TAC ATC CAG TAT GCA C**). The specificity of the designed universal oligonucleotide tail was tested with the program BLAST (The Basic Local Alignment Search Tool).

The universal primer was designed with a sequence homologous to that of the tail. Assessment of its specificity, i.e. no homology to the target DNA was made with the program BLAST (The Basic Local Alignment Search Tool).

The reference strain *B. pertussis* Tohamal was used to calculate the sizes of the resulting fragments of each VNTR locus. The VNTR size of *B. pertussis* Tohamal was: BP VNTR 1 – 383 bp, BP VNTR 3 - 135 bp, BP VNTR 5 - 143 bp, BP VNTR 6 - 274 bp, BP VNTR 8 - 232 bp, BP VNTR 11 - 365 bp. The six pairs of primers were selected so that they amplify distinct non-overlapping fragments. Each primer pair amplified a fragment with different length. The final concentration of the primers in multiplex PCR were: BP VNTR 1FR – 0,3 pmol/µl, BP VNTR 3FR – 0,17 pmol/µl, BP VNTR 5FR – 0,17 pmol/µl, BP VNTR 6FR – 0,1 pmol/µl, BP VNTR 8FR – 0,13 pmol/µl, BP VNTR 11FR – 0,3 pmol/µl, BP VNTR-Univer – 0,066 pmol/µl.

We designed the multiplex PCR analysis in two triplex reactions for each sample tested for a clear differentiation of the fragments by size. Table 2 and Table 3 show the sequence and the number of repeats in the reference strain *B. pertussis* Tohamal.

For the hybridization and amplification we used Veriti Thermal Cycler (Applied Biosystems).

The method that we have developed involves two steps (Figure 1). The first stage is multiple hybridization which aims at the hybridization of the primers to the target DNA. The second stage is multiple amplification the purpose of which is a specific amplification and polymerization of the primers.

Step I - multiple hybridization reaction:

We made a mix of primers for the first triplex: 3 µl (the final concentration of the primers in multiplex PCR were: 1 µl of 5 pmol/µl primers BP VNTR 3FR, 1 µl of 3 pmol/µl primers BP VNTR 6FR, 1 µl primers of 10 pmol/µl BP VNTR 1FR). The primer mix for the second triplex was: 3 µl (the final concentration of the primers in multiplex PCR were: 1 µl primers of 5 pmol/µl BP VNTR 5FR, 1 µl 4 pmol/µl primer BP VNTR

Table 1. Sequences of the primers used for the implementation of the modified MLVA analysis. The right 3’ site is the locus specific primer while the left 5’ site is the universal amplification tail.

BP VNTR 1F	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**cc tgg cgg cgg gag acg tgg tgg tg-3’
BP VNTR 1R	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**aa aat tgc ggc atg tgg gct gac tct ga-3’
BP VNTR 3F	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**gc ctc ggc gaa att gct gaa c-3’
BP VNTR 3R	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**gc ggg cga gga aac gcc cga gac c-3’
BP VNTR 5F	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**ga agc cgg ccc acc cga gct cca ggc tct t-3’
BP VNTR 5R	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**tg ccg ggt ttc ggc atc tcg atg gga tac g - 3’
BP VNTR 6F	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**cc aac ggc ggt ctg ctg ggt ggt c-3’
BP VNTR 6R	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**ag ggc gct ggt cac gcc acc gag gat-3’
BP VNTR 8F	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**tg ggt gtc tcc gtg ata gtg agc act tac ac-3’
BP VNTR 8R	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**ct ggc gca aaa aca gta agc ccg cac g-3’
BP VNTR 11F	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**ct cga cgc cag gga caa aac-3’
BP VNTR 11R	**5’-ATA CAC AGG TAC ATC CAG TAT GCA C**gc cag gct gga ctt gtc ctc-3’
BP VNTR -Univer	**5’-GTG CAT ACT GGA TGT ACC TGT GTA T - 3’**

Table 2. **Characteristics of the first triplex PCR used in the analysis of strain *B. pertussis* Tohamal**.

	VNTR 3	**VNTR 6**	**VNTR 1**
Size of PCR product (bp)	135 bp	274 bp	383 bp
Number of repeats	7	9	9
Repeat Length (bp)	5	9	15
Sequence of repeat	CTGGC	CGAGCCGCC	GAACCCGCCAAGCAG

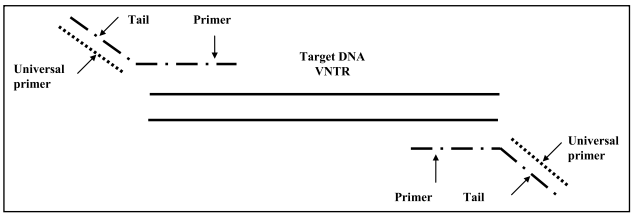
Table 3. **Characteristics of the second triplex primers used in the analysis of strain *B. pertussis* Tohamal.**

	VNTR 5	**VNTR 8**	**VNTR 11**
Size of PCR product (bp)	143 bp	232 bp	365 bp
Number of repeats	7	3	10,5
Repeat Length (bp)	6	6	12
Sequence of repeat	TGGTGC	TTTACG	CAAGCACAAGGG

8FR, 1 µl primer of 10 pmol/µl BP VNTR 11FR). To the two triplex reactions we gradually added 3 µl DMSO (5% final concentration) to improve the hybridization of the primers and to reduce secondary structures, 3 µl 10× reaction buffer with 20 mM $MgCl_2$ (Genet Bio) and 5 µl of DNA template with concentration 4 ng/µl. The best results were achieved with the following parameters for hybridization: 95º C for 10 min (for DNA denaturation); followed by 12 cycles of gradual (touch-down) decrease from 72º C to 60º C (1º C decrease per cycle for 75 sec) for a better hybridization and a final step at 60º C for 4 hours for hybridization of the specific primers to the target DNA.

Step II - multiplex amplification reaction:
The purpose of this step was the specific polymerization and amplification of the primers. This was achieved by adding a mixture of 1,5 µl dNTP (for 0.2 mM final concentration) (Thermo Fisher Scientific), 0,3 µl DNA Taq-polymerase (5U/µl, Genet Bio), 9,2 µl distilled water to each sample keeping the tubes at 60º C in the cycler. The next stage was at 72º C for 15 min to perform the polymerization initiated by the hybridized primers. After the addition of 5 µl universal primer (from 2 pmol/µl stock) without removing the tubes from the cycler the resulting final reaction volume was 30 µl. Since the universal primer was designed to be complementary to the tail of each primer specific amplification was achieved only of the targeted VNTR loci at this stage. Best results were obtained under the following amplification conditions: 30 cycles of denaturation at 94º C for 1 min, specific amplification of 60º C for 45 sec, extension at 72º C for 45 sec followed by final extension at 68º C for 20 min.

Figure 1. Modified MLVA technique scheme



The PCR products were visualized at 4% agarose gel containing ethidium bromide. For a clear and precise differatiation of VNTRs from each multiplex reaction 10 µl of the amplicons were analized on automated capillary electrophoresis system QIAxcel (Qiagen, Germany) coupled with DNA High Resolution Kit (1200). It is a highly sensitive, fast and automated method for precise separation of DNA fragments according to their size that works with unlabeled DNA. The M500 method was used for separation (10s injection, 500s separation at 5 kV). An alignment DNA marker 50-1000bp and GeneRuler 50bp DNA ladder (Fermentas, USA) were used for calibration and fragment analysis respectively. The chromatograms and band patterns were evaluated visually whereas the size of each fragment was determined by Biocalculator v3.2 software (Qiagen). The MLVA pattern of *B.pertussis* Tohama I was used as reference size standard.

RESULTS AND CONCLUSION

We have developed a modified MLVA method for typing of *B. pertussis* that can be applied to isolated DNA from both pure cultures and directly to clinical specimens from patients.
We used six pairs of primers for the analysis of the variable tandem repeats loci in the *B.pertussis* genome. Each primer pair amplified DNA fragment with different length. The method that we have developed used unlabeled primers unlike the published methods with fluorescently labeled primers. Thus the visualization of the products was carried out on agarose gel and not by using DNA sequencer. Capillary electrophoresis with 12-capillary cartridge was used for more precise estimation of the size of the fragments. The reference strain *B.pertussis* Tohama I was used as a standard with known VNTR sizes. A total of 51 samples were analized (33 DNA isolated from strains *B.pertussis* and 18 isolated DNA from nasopharyngeal secretions of patients). All six selected primer pairs were found to detect polymorphisms in the studied strains and patient DNA samples (Table 4).

Table 4. **Variations of VNTR loci.**

Variable repeat sequences	Repeat Number	Number of alleles	Allele frequency
VNTR 1 GAACCCGCCAAGCAG	6 - 9	5	6 = 15,7 %
			6,5 = 5,9 %
			8 = 66,7 %
			8,5 = 7,85 %
			9 = 3,9 %
VNTR 3 CTGGC	5 - 8	5	5 = 3,9%
			6 = 5,9 %
			6,5 = 5,9 %
			7 = 78,4 %
			8 = 5,9 %
VNTR 5 TGGTGC	5,5 - 10	5	5,5 = 2 %
			6 = 90,1 %
			7 = 3,9 %
			8 = 2 %
			10 = 2 %
VNTR 6 CGAGCCGCC	1 - 9	7	1 = 3,9 %
			5 = 49,0 %
			7 = 25,6 %
			7,5 = 9,8 %
			8 = 3,9 %
			8,5 = 3,9 %
			9 = 3,9 %
VNTR 8 TTTACG	2,5 – 4,5	5	2,5 = 5,9 %
			3 = 23,5 %
			3,5 = 64,7 %
			4 = 2 %
			4,5 = 3,9 %
VNTR 11 CAAGCACAAGGG	4,5 – 9,5	7	4,5 = 3,9 %
			6 = 3,9 %
			7 = 9,8 %
			7,5 = 23,6 %
			8 = 41,1 %
			8,5 = 9,8 %
			9,5 = 7,9 %

The data show that the variable repeat length ranged from 5 to 15 bp and their number - from 1 to 10 repetitions per locus. The most frequent allele of VNTR 1 locus (in 34 out of 51 samples / 66.7%) contained 8 repeats. The isolates with 7 repeats in VNTR 3 were prevalent - 78.4% (40 of 51 isolates). Almost all the samples (46 of 51 = 90.1%) showed 6 repeats for VNTR 5 hence this locus seems to be homogenous in Bulgarian *B. pertussis* population. In VNTR 6 almost half of the samples - 49% (25 of 51) showed 5 repeats. For locus VNTR 8 prevail the 3.5 repeat allele in 64.7% of the samples while for VNTR 11 the prevailing number of repeats was 8 (41.1%).

The investigation of variable number of tandem repeats can be successfully used to identify strains according to their MLVA profile. Each unique MLVA profile is defined as a single genotype. The profile is based on the estimated number of repeats for each locus in the following order: VNTR 1, VNTR 3, VNTR 5, VNTR 6, VNTR 8 and VNTR 11. A total of 32 MLVA types were obtained from the 51 samples analized hence the method discriminated most of the isolates into different genotypes. We obtained 24 MLVA genotypes for the DNA isolated from pure cultures (n= 33) while the patient samples (n=18) were grouped in 8 genotypes . (Tables 5 and 6).

For the estimation of the discriminatory power of the method we used the Simpson index (DI) for biodiversity. This index determines the ability of the method to differentiate closely related strains from unrelated ones, i.e. the probability two randomly selected isolates to be differentiated as separate genotypes. We used the web tool V-DICE (**V**NTR **Di**versity and **C**onfidence **E**xtractor) to calculate this indicator. (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl).

Simpson biodiversity index (DI) is calculated as the change in the number of repeats of each locus. This index varies from 0.0 to 1.0. The discriminatory ability of the typing method and the allelic diversity can be estimated using the discriminatory index of Hunter-Gaston (HGDI) (Hunter, Gaston 1988). We received the following values of the indices of Simpson and Hunter-Gaston (Table 7).

Table 5. **MLVA genotypes obtained from the analysis of DNA from cultures.**

MLVA genotypes	VNTR 1	VNTR 3	VNTR 5	VNTR 6	VNTR 8	VNTR 11
GT_1	6	7	6	5	3,5	8
GT_2	6	7	6	7,5	3,5	8
GT_3	6	7	10	5	2,5	6
GT_4	6	7	6	5	3,5	9,5
GT_5	6	7	6	7,5	3,5	7
GT_6	6	6	6	5	3,5	8,5
GT_7	6,5	6,5	6	7	3	7,5
GT_8	6,5	7	6	5	3,5	8
GT_9	6,5	7	6	7	3,5	8
GT_10	8	6	6	5	3,5	8
GT_11	8	7	6	7,5	3,5	7
GT_12	8	5	6	1	2,5	4,5
GT_13	8	6	6	7,5	3,5	7
GT_14	8	7	6	7	3,5	8,5
GT_15	8	7	6	7	3,5	7,5
GT_16	8	8	6	7	3,5	7
GT_17	8	7	6	7	3,5	8
GT_18	8	7	6	7	4,5	7,5
GT_19	8	7	6	5	3,5	8
GT_20	8	8	6	7	3,5	8
GT_21	8	7	6	5	4,5	8,5
GT_22	8,5	6,5	7	8,5	3,5	9,5
GT_23	9	7	7	9	3	9,5
GT_24	9	6,5	8	7	3	9,5

Table 6. **MLVA genotypes obtained from the analysis of DNA from patients.**

MLVA genotypes	VNTR 1	VNTR 3	VNTR 5	VNTR 6	VNTR 8	VNTR 11
GT_25	8	7	6	8	3,5	8
GT_26	8	7	6	5	3,5	8
GT_27	8	7	6	5	3,5	7,5
GT_28	8	7	5,5	5	3	7,5
GT_29	8	7	6	5	3	8
GT_30	8,5	8	6	8,5	4	8,5
GT_31	8,5	7	6	5	3,5	6
GT_32	8,5	7	6	5	3,5	8,5

Table 7. Discriminatory index.

Locus	Simpson's Diversity		Hunter-Gaston Diversity	
	Diversity Index	Confidence Interval	Diversity Index	Confidence Interval
VNTR 11	0,777	0,705 – 0,850	0,793	0,720 – 0,866
VNTR 6	0,741	0,661 – 0,822	0,756	0,675 – 0,837
VNTR 8	0,615	0,508 – 0,723	0,627	0,520 – 0,739
VNTR 1	0,551	0,404 – 0,699	0,562	0,415 – 0,710
VNTR 3	0,458	0,294 – 0,621	0,467	0,303 – 0,630
VNTR 5	0,348	0,179 – 0,516	0,355	0,186 – 0,523

The obtained values show that the VNTR 11 which is a new VNTR identified by us and VNTR 6 provide the highest discriminatory power. The confidence intervals obtained were fully comparable with those reported by other authors (Schouls et al. 2004, Amersfoorth et al. 2005, Litt et al. 2009, Kurniawan et al. 2010).
We received the following results summarized in the dendrogram (Figure 1) for the samples tested using MLVA method:

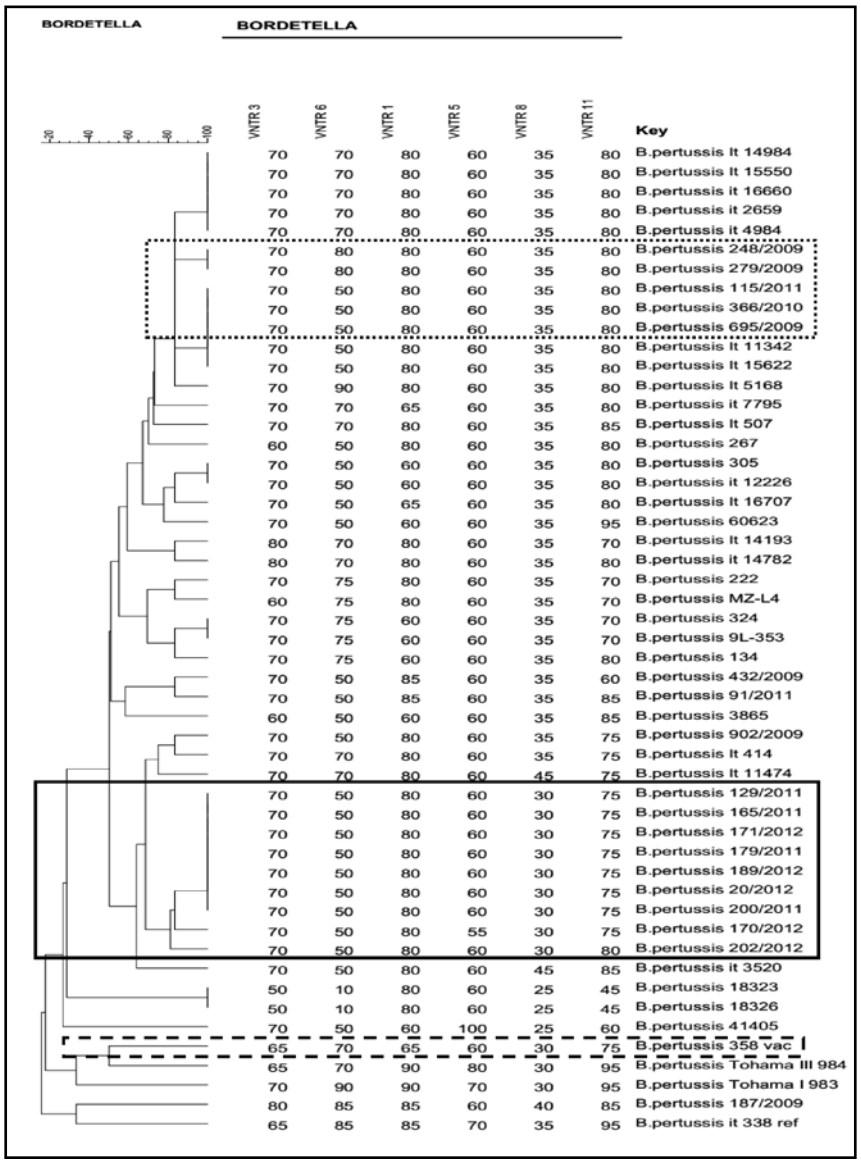


Figure1. Dendrogram of the samples included in the MLVA analysis.
(Note: BioNumerix software requires the input of integers. The number of VNTR repeats is multiplied by a factor of 10, for example 2.5 x 10 = 25)

- First patient cluster
- Second patient cluster
- Vaccine strain *B.pertussis* 358

When analyzing the results obtained by typing DNA isolated from patients we found that they are grouped predominantly in two main clusters (Figure 1). The first cluster included patients from 2009, 2010 and 2011 with similarity between 80% and 100%. In all five patients a complete identity in VNTR 1, VNTR 3, VNTR 5, VNTR 8 and VNTR 11 loci was registered with difference only in VNTR 6. The patients' data revealed that three out of the five patients (60%) had not received vaccinations for pertussis. All of them were up to 1.5 years old originated from Sofia but lacking epidemiological link. A single patient in this cluster was vaccinated with all necessary doses. We identified differences in VNTR 6. This again proves that the primer pairs VNTR 6 capture is the most discriminatory. Those data suggest that almost genetically identical strains circulated in Bulgaria during this period. Patients from 2011 and 2012 were grouped into the second large cluster. We found 100% similarity in the number of variable repeats for seven out of the nine patients (77.7%). Differences were established in VNTR 5 – in one sample 5.5 repeats were found, while in the others the repeats were 6. A single difference was found in the VNTR 11 with 8 repeats, while the others contained 7,5 repeats. Six out of the nine patients (66%) had not been immunized. All of them were at the age of 1 to 8 months and originated from Sofia or Sofia region with no epidemiological link between them. Three of the patients in this cluster were challenged with all doses of the vaccine. There is almost 100% match in the number of alleles for them in four of the six variable repeats (66.6%). The differences were found only in VNTR 5 and VNTR 11.

When comparing the data from the two clusters we have found that there is between 80% and 100% similarity in the analyzed samples. We can assume that almost genetically identical strains have circulated in our society during this four-year period. The obtained data allow us to assume the population of the pathogen *B. pertussis* in our society are homogeneous and relatively constant.

When analyzing and summarizing the results from the typing of *B. pertussis* strains we found that the most conservative and relatively constant are the variable repeats VNTR 5 and VNTR 8 (Figure 1). In these repeats there are only single variations when comparing the profiles of Russian and Italian strains. In contrast, VNTR 1, VNTR 3, VNTR 6 and VNTR 11 show the highest percentage of variability. The percentage of identity between the studied strains was 60% and more. The results showed that the MLVA profile of the studied strains is different and that our method is sufficiently discriminatory. The method groups all or most of the strains into separate branches.

The MLVA6 scheme presented here is reliable and sufficiently discriminative in order to be used successfully for typing strains *B. pertussis*. Moreover its sensitivity allows for direct typing of DNA from patient samples which is of utmost importance providing that the culture recovery of *B. pertussis* is relatively low (Loeffelholz 2012).

Using the developed method we proved that there is a significant homology among the strains circulating in the country.

We observe VNTR differences between the circulating clinical strains and the whole-cell vaccine strain B. pertussis 358 vac used until recently in Bulgaria. It can be speculatively supposed that in the country there may be antigenic changes in the clinical strains as it has been reported in other countries (Schouls et al. 2004, Kallonen et al. 2009, Mooi et al. 2009, Cherry 2010, Mooi 2010, WHO position paper-recommendations 2011, Zhang et al. 2011). However, this requires sequencing the genes of the clinical strains and comparison with the sequence of the antigens included in vaccine.

It is tempting to speculate that in addition there might be antigenic variation between the circulating strains and the vaccine strain and perhaps the genetic changes lead to antigenic shift. This can result in increased virulence of the pathogen population and in a stronger immune suppression which can lead to reduction of the effects of vaccination (Van Gent et al. 2012). With these results we confirm the trend observed in other countries with high vaccination coverage for significant genetic differences between the strains circulating in the society and the vaccine ones (Watanabe et al. 2002, Schouls et al. 2004, Kallonen et al. 2009, Mooi et al. 2009).

The process of an adaptive evolution in Bulgaria began with the start of mass immunization in 1957 with trivalent whole-cell DTP (diphtheria-tetanus-pertussis) vaccine. It is imperative to develop new vaccine strains that are genetically as close as possible to the actual circulating clinical strains as it is done annually with the production and application of the flu vaccine. By using the modified MLVA method we prove that the strains circulating in the country are VNTR different from the applied vaccine strain *B.pertussis* 358 vac. We were unable to make such comparative studies with regard to the acellular vaccine which contains purified antigens as we do not have the antigens.

The Multi-Locus Variable number of tandem repeats analysis was adopted by the ECDC and EUVAC.NET as a standardized reference method for typing strains *B.pertussis*. This method can be used for the creation of an international database for tracking the MLVA types circulating in Europe and worldwide. Each individual genotype is associated with specific local characteristics - geographic biodiversity and the type of vaccines used. This would improve the surveillance of the pertussis on a global scale and should lead to an update of the pertussis vaccines currently in use.

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RECURRENT RESPIRATORY PAPILLOMATOSIS: THE IMMUNOLOGICAL POINT OF VIEW

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ABSTRACT:
Recurrent respiratory papillomatosis (RRP) is a clinical manifestation of chronic HPV infection characterized by a significant morbidity and an unpredictable clinical course. Inefficient antiviral response in RRP is determined by impaired Th1/Th2/Th17 cytokine balance, expansion of Treg cells with inhibitory function, and viral mechanisms to avoid immune control. Bulgaria preparation "Calgevax" (BCG) modulates cellular immune mechanisms and has an antitumor activity. Preliminary results from combined surgical/ "Calgevax" treatment of RRP patients suggest that BCG restores the balance of effector and regulatory T cell subsets through changes in cytokine background.

Key Words: *Recurrent respiratory papillomatosis, HPV, Calgevax (BCG), Th17, Treg*

Abbreviations:
APC - antigen-presenting cells, DC - dendritic cells, EGFR – epidermal growth factor receptor, PBMC – peripheral blood mononuclear cells, RRP- recurrent respiratory papillomatosis, Treg – T regulatory cells.

Introduction

Recurrent respiratory papillomatosis (RRP) is a comparatively rare clinical manifestation of the widely spread human papillomavirus (HPV) infection. RRP is characterized by the recurrent growth of premalignant tumors within the upper respiratory tract. The papillomas occur most frequently in the larynx but various extralaryngeal sites (oropharynx, trachea, bronchi, lung parenchyma, and esophagus) may also be affected (1). Extension into the lower airway occurs in approximately 17% of the patients often leading to airway obstruction (2). In fact, the clinical outcome of RRP is various and rather unpredictable, ranging from spontaneous regression to malignant transformation to squamous cell carcinoma with quite poor prognosis in 3-5% of RRP patients (3-5). Two clinical forms have been outlined according to the age at onset. The juvenile form presents with multiple papillomas in children under five, equally affecting both genders. It is considered more aggressive with often dramatic presentation due to a significant airway obstruction. RRP in adults is more rare (2 per 100 000), papillomas are usually single, but with a high tendency for malignant transformation (6). Recurrence frequency is variable between patients but relatively constant within most patients. Surgical removal of the lesions can be required as frequently as every 3 to 4 weeks in severe diseases leading to more than 150 surgical procedures per lifetime (7).

More than 90% of RRP cases are caused by „low risk" HPV strains (HPV-6 and -11), the latter being responsible for more aggressive clinical forms (e.g significant airway obstruction) (7, 8). HPV genome consists of double-stranded 7900 bp DNA containing two key regions. The early region genes (E1-E8) encode regulatory proteins responsible for the viral DNA replication, transcription and transformation of host cells. E6/E7 are oncogenes inactivating the tumor suppressor proteins p53 and pRb (10, 11). HPV infects keratinocytes but the massive expression of viral genes and genome amplification occur in the terminally differentiated epithelial cells from the upper epithelial layers (12). To-date, it is unclear:

- Why and under what circumstances a highly prevalent among the human population latent infection would manifest as RRP
- What factors determine the clinical manifestation of this infection
- Why and how a low risk HPV infection may lead to a malignancy.
- How to achieve a definitive effect of surgical treatment.

All these issues focus the attention on the immunopathogenetic mechanisms of RRP and its progression and the possibility of immune-based therapeutic approach.

Immune response to virus

The classical anti-viral effectors are cytotoxic CD8 T cells in association with Th1-type CD4 T cells. A number of innate cellular components are involved in the activation of virus-specfic response. The innate immune response to viral pathogens is critical in order to mobilize protective immunity. Cells of the innate immune system detect viral infection largely through germline-encoded pattern recognition receptors (PRRs). The binding of viral ligands to host sensors induces multiple signaling bioactive molecules: inflammatory proteins including type I IFN and inflammatory cytokines such as TNF-a, IL-6 and IL-12 which in turn coordinate both the innate and adaptive immune responses (13). Activated NK cells are the major anti-viral effector component of innate immunity while CD8 T cells exercise viral – specific cytotoxicity. Dendritic cells (DC) are professional antigen-presenting cells (APC) that are the key bridge between innate and adaptive immunity. They differentiate from immature actively phagocyting cells at the site of infection to potent APC efficiently activating naive CD4+ and CD8+ T-lymphocytes in the local lymph

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nodes. DC maturation and migration to lymph nodes is promoted by the cytokines (IL-1α, TNFa, IL-12 and IFN) produced by virus-infected cells (14). Activated Th lymphocytes can differentiate into Th1-, Th2-, Th17- or Treg phenotype depending on the cytokine environment (15). Th1- cells produce INF-g, TNF-a, and IL-2, and potentiate antiviral reponse by activating macrophages and inducing the proliferation and differentiation of CD8 + cytotoxic T lymphocytes that play critical roles in purging acute infections, limiting persistent infections, and conferring life-long protective immunity. IFN-g stimulates the IL-12 production by DC and macrophages while IL-12 stimulates Th1 to secrete IFN-g in a positive feedback mechanism. IFN-g increases the surface expression of MHC I class proteins so that all cell types expressing IFN-g receptors present endogenous antigens more effectively and are better targeted by cytotoxic T-lymphocytes. A reciprocal regulation exists between the basic effector cytokine types. IFN-g inhibits the production of IL-4 - a key cytokine in Th2-immune response and vice versa. In parallel to the effector CD4 and CD8 T cells, viral antigens in the presence of high IL-2 concentrations induce regulatory T cells (Treg) with non-specific inhibitory function. Their physiological role is to limit immune activation in case of high antigen burden and to promote the balanced differentiation of effector and memory antigen-sopecific T cells but may also contribute to the inefficiency of T cell effectors in the settings of persistent infection (16, Nikolova) This role is realized by combined contact-dependant and cytokine-dependant mechanisms including the regulatory cytokines IL-10 and TGFb. Thus, every stage of the anti-viral response is sensitive to signals from cytokines: initially as warning for the presence of an infection and influencing the developmental choices of the responding cells; later - other sets of cytokines support the survival of the differentiated anti-viral T cells or suppress their activities and thus favor viral persistence. A third effector cell type, Th17 (producers of IL-17A,F, IL-21, IL-22) has been described that differs from Th1 and Th2 cells both in requirements for differentiation factors and in target pathogens. Rather conflicting results about the physiopathology of Th17 subset have been reported. Th17 play a central role in host defense against a range of extracellular pathogens including bacteria, viruses and fungi. However, excess Th17 lymphocyte numbers have been associated with inflammatory autoimmune diseases. Th17 cells were shown to contribute to inflammatory disorders that afflict the respiratory tract, such as asthma and chronic obstructive pulmonary disease (COPD), (17-19). Differentiation of naive CD4+ T into Th17 is induced in the presence of IL-6 and prostaglandin E2 (PGE2) and counteracted by Th1 type cytokines. Further on, excess of IL-23 induces the differentiation of pro-inflammatory Th17 cells, secreting increased quantities of IL-17, IL-21 and Il-22 and promoting Th2 type response while the presence of IL-10 and TGF-b promotes the differentiation of non-pathological “regulatory” type of Th17. Therefore, the balance Th17 /Treg is critical for immune homeostasis and tumor pathogenesis (20, 21). More research is, however, needed to unravel the complex consequences of IL-17 production and when this is beneficial, when not and why.

Impaired antiviral immune response in RRP patients

It is estimated that 5% of the general population without evidence of RRP has detectable HPV DNA in their larynx (7). It remains unclear why a very small fraction of HPV-exposed individuals develop RRP and why still fewer develop a severe course of disease. The central question is: ‘How does the innate and adaptive immune response to HPV-6 and -11 in RRP patients differ from that of individuals who are infected with the same HPVs but never develop RRP?’

The few published studies have established no significant changes in the major lymphocyte populations (CD4 + and CD8 + T-cells, B- and NK-cells) of RRP patients (22). In addition, patients with RRP seem to have a normal immune response to other pathogens as well as to mitogenes. It is clear that patients with RRP mount an immune response that is initially manifested by the production of measurable serum antibodies to these HPV. This suggests a more subtle and probably site-specific impairment of the local immune response against HPV-6/11 infection in the airways (7). The generation of effective anti-HPV T-cell response requires inflammatory cytokines (IFN-γ, IL-2 and TNF-α) that are secreted by Th1-cells (23). Accumulating set of evidence supports the hypothesis that the effective Th1-antiviral immune response to HPV-6 and HPV-11 is disturbed as a result of polarization of the immune response to the Th2/Treg phenotype. It was shown that in vitro stimulated tumor-infiltrating lymphocytes (TILs) and peripheral blood mononuclear cells (PBMC) from RRP patients produce predominantly Th2 cytokines (IL-4, IL-5, IL-10, IL-13) after exposure to autologous papilloma tissues that inhibit IFN-g and IL-2 secretion by Th1-cells (22 - 25). Importantly, it was found that after 72h of E6 stimulation the number of IL-10 – secreting T- and non-T cells was significantly lower in RRP patients, while the number of IFN-g secreting cells was comparable to healthy controls. These findings support the hypothesis that memory Th2-like T-cells and non-T-cells likely exist and support papilloma recurrence and chronic HPV infection (21). The impaired cytokine balance correlated with disease severity (22). Constitutively increased expression Th2 cytokines has been demonstareted in CD4+ TILs from papillomas as well (22)

Significant expression of Th2 (IL-4, IL-5, TGF-β) and T-regulatory (IL-10) cytokines and low expression of type I and type II IFNs, IL-2 and IL-6 were demonstarated in T-cell containing papillomas by cytokine-specific RT-PCR (26). In contrast, the papilloma biopsies devoid of T-cells have showed a higher expression of IFN type I (IFN-α) and lack of IL-4 and IL-10 expression, indicating that T-cells rather than keratinocytes express Th2/Treg cytokines in RRP (22). This cytokine background in the absence of IFN-g can lead to polarization of naive T cells to Th2 and Treg cells (CD4 + $CD25^{high}$ $CD127^{low}$ Foxp3+) , producing IL-10 and TGF-β . Indeed, in papilomas from RRP patients 2-7 fold enrichment of Treg was established while levels of Treg cells in blood samples of patients with RRP were similar to those in blood samples from healthy controls (25). Probably, the regulatory T cells in papillomas are a major component in suppression of anti- HPV-specific Th1 immune response and are

responsible for the high rate of relapse in RRP.

Therapeutic approaches in RRP

Although not definitive, surgery remains the only currently approved therapeutic approach in RRP (28). In USA, about 15 000 surgical procedures are performed each year for patients with RRP at an estimated cost of greater than $100 million US dollars ⁄ year (22). As patients can require as many as 100 surgical procedures to maintain a patent airway this treatment is associated with significant risk of permanent laryngeal damage as well as with important emotional and social burden (29, 30). Contemporary laser microsurgery, though sparing for the patients, does not reduce the number of recidives. Several agents have been proposed as adjuvants to surgical debulking including antivirals (Ribavirin, Cidofovir, Cndole-3-carbinol), inhibitors of cyclooxygenase-3 or EGFR administered systemically or injected into the lesions. Diindolylmethane (DIM), a natural product from cruciferous vegetables in animal models has proved safe on animal models of laryngeal papilloma. However, controlled trials failed to provide sufficient evidence to draw reliable conclusions about the effectiveness of antiviral agents as adjuvant therapy in the management of RRP, (31-33). Thus, understanding the mechanism(s) by which HPV-6 and -11 polarize the immune response towards tolerance in RRP, as opposed to the development of cell-mediated immune clearance of these viruses, is critical in developing novel therapies that would prevent disease recurrence and ⁄ or reduce disease severity.

Immuno-modulating effects of Calgevax in RRP patients

Calgevax contains freeze-dried live bacteria derived from Bacillus of Calmette and Guerin (BCG) culture. It has been approved as adjuvant therapy for the treatment of malignant tumors, particularly bladder cancer and melanoma. BCG is currently considered as the most successful agent used in cancer immunotherapy. The intravesical BCG application is considered as a “golden standard” for the treatment of superficial bladder cancer (34). However, the mechanisms of BCG beneficial anti-tumor effects are not yet fully understood. It is suggested that BCG contributes to the strengthening the cell immune response in a non-specific manner by activating macrophages, cytotoxic CD8 T, and T cells with NK (CD16+CD56+CD3-) phenotype (35,36). In bladder cancer, BCG immunotherapy results in the infiltration of neutrophilis, macrophages, T-, NK- and dendritic cells combined with Th1 type cytokine secretion (33,34). High levels of IL-1, IL-2, IL-6, IL-8, IL-10, IL-12, TNFa, INF-g and GM-CSF were detected in urine samples from patients treated with BCG (35,36). This Th1 type cytokines induce both non-specific and antigen-specific cytotoxic mechanisms necessary for successful anti-tumor response. Blocking INF-g, IL-2, IL-12, and IL-18 by neutralizing antibodies significantly reduced BCG-induced macrophage cytotoxicity against bladder cancer cells (37). Experience with BCG- immunotherapy in laryngeal carcinoma is limited. *In vitro* studies have shown that BCG increases TNF-α and IL-6 secretion by macrophages during the postoperative period in laryngeal carcinoma patients. Increased TNF-α and IL-6 concentration was detected in the supernatant of PMNC cultures after treatment with BCG (38 - 41). On the other hand, a direct correlation has been established between the concentrations of IL-2, IL-6, TNF-a, IFN-g, and IL-10 detected in vitro in isolated PMNC and whole blood cultures and the clinicomorphological characteristics of the disease in patients with laryngeal cancer (42).

Despite a growing interest in immunotherapy, the effects of BCG have not been explored in RRP. To our knowledge, we have applied for the first time BCG (“Calgevax”) in combination with CO_2 laser microsurgery for systematic treatment of RRP patients following the approved scheme for melanoma. The pilot study showed that at 20 months post operation RRP patients (n=17, aged) experienced a significant reduction of relapses as compared to age- and sex-matched patients subjected solely to microsurgery (43). To explain this beneficial effect, the parameters of T cell response in Calgevax-treated patients were determined before, at 6, 12 and 20 months after the start of immune modulation. Major peripheral blood lymphocyte populations and T-cell subsets were studied by flow cytometry (FACSCanto II, BD). Percentages of effector Tc1 (CD8 + IFNγ+), Th1 (CD4+IFNγ+), and Th17 (CD4+IL-17+) T cells were determined by intracellular cytokine staining after overnight *in vitro* PHA stimulation. Production of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α in PHA-stimulated whole blood samples was quantified by flow cytometric bead array (BD CBA kit). Our results showed increased Th1, Tc1, and Th17 levels in untreated RRP patients. However, these were combined with inverted IFN-g/IL-4 and IFN-g/IL-10 and IL-10/IL-17 ratios, indicating a dysbalanced differentiation of the basic effector and regulatory subsets in the settings of persistent HPV-induced stimulation. Based on the current vision about the reciprocal regulation of Th1/Th2/Th17 and Treg subsets it could be speculated that although immune activation induces the differentiation of all effector subsets, Th2 and Th17 dominate over Th1 ***(Fig.1a)***. The resulting prevalence of Th2 cytokines inhibits IFN-g and IL-2 production leading not only to inefficient Tc1 response but also to insufficient differentiation of Treg and prevalence of IL-23 at the expense of TGF-b. Therefore, Th17 differentiate towards the proinflammatory type, futher pushing the dysbalance in favor of a Th2 type response. In addition, Treg insufficiency may result in inefficient T-cell memory as well as in depletion of antigen-specific T cell clones.

Immunotherapy with “Calgevax” significantly decreased the share of Th17 cells and increased the level of circulating Treg (at12th month), followed by normalization to control group levels. IFNγ / IL-4 and IFNγ/IL-10 ratios were restored after 20 months of “Calgevax” application. These results clearly indicate that Calgevax affects the differentiation and balance of effector and regulatory T-cell subsets by changing cytokine background ***(Fig.1b).*** As already demonstrated in other studies (38 - 41) BCG treatment induces IFNγ, IL2, and IL-6 secretion and potentiates the differentiation of Treg at the expense of proinflammatory Th17 cells. In this way “Calgevax” limits immune hyperactivation, prevents the depletion of virus-specific CD8 and CD4 T cell clones and promotes

the formation of virus-specific memory resulting in a net decrease of relapses.

Conclusion. Recent studies have indicated the importance of balanced effector/memory/ regulatory subset differentiation for a protective immune response. This balance is perturbed to a different extent in persisting viral infections. Our results in RRP patents subjected to combined surgery/immune modulation with "Calgevax" are in line with data showing that the adaptive immune response in RRP is polarized towards a Th2-like／T-regulatory phenotype. In addition, we have pinpointed the importance of the Th17 subset and its interactions with Treg for the inefficient anti-viral response. Further studies are warranted to confirm and precise the effects of "Calgevax" in RRP-patients. Specifically, it is important to study these effects at the level of DC and NK cells as innate immunity is decisive for the further polarization of adaptive immunity response. Besides, it is important to know whether the immune-modulation effects provided by Calgevax are lasting, and whether they are efficient against the malignant transformation of papillomas. Finally, it would be interesting to test the cross-effects of Calgevax against high-risk HPV strains.

Fig.1 The outcome of naïve CD4 T cell response depends on complex interactions between innate and adaptive effector and regulatory subsets, and the resulting cytokine background.
A. HPV-specific response in untreated RRP patents. The balance is polarized towards differentiation of proinflamatory Th17 and Th2 cells. In spite of increased Th1, Tc1 and Treg subsets, IFN-g secretion is dominated and suppressed by IL-10, Il-17 and IL-4.
B. Immuno-modulatory effects of Calgevax in RRP. As a result of stimulated IFN-g, IL-12, IL-2 and IL-6 secretion, the balance is restored towards a Th1/Tc1 response; induction of Treg prevents the formation of proinflammatory Th17 and maintains a balanced differentiation of memory and effector virus-specific T cells.

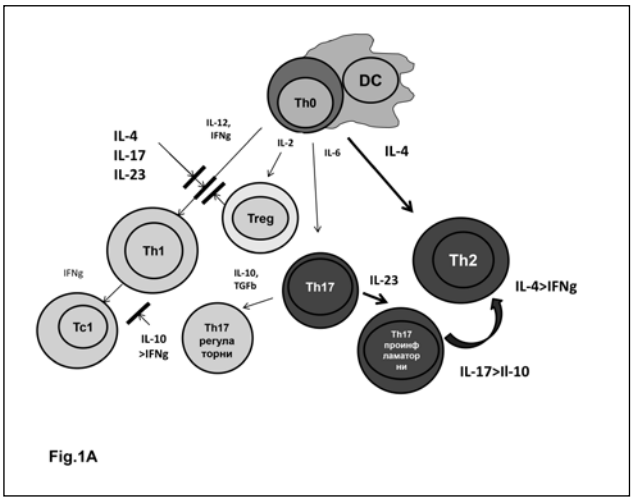

Fig.1A

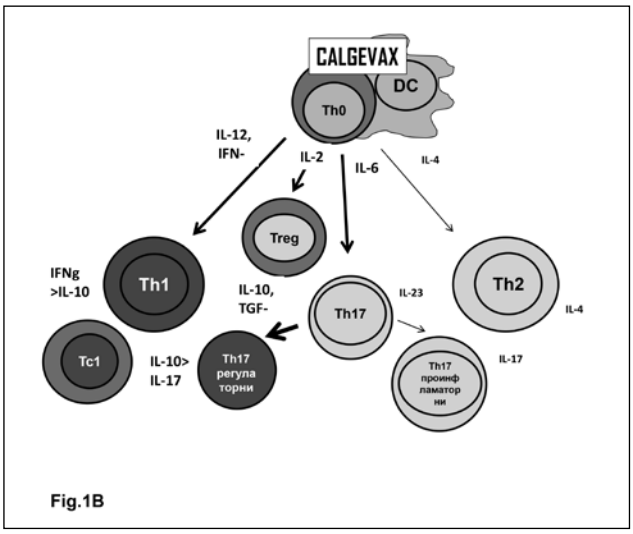

Fig.1B

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LABORATORY DIAGNOSIS OF INVASIVE FUNGAL DISEASES

Review
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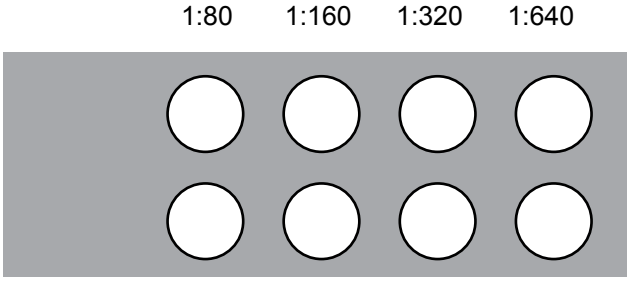
ABSTRACT:
The aim of this review is to present the common laboratory diagnostic methods for diagnosis of invasive fungal diseases. The majority of those diseases are caused by Candida and Aspergillus species but many studies show an increasing range of infection agents such as Cryptococcus, Trichosporon.
The most common serological tests for antigen and antibody-detection in body fluids (serum, plasma, BAL) are: Indirect immunofluorescense (IIF), detection of 1, 3 – β D-glucan, ELISA – Platelia (enzyme linked immunosorbent assay), latex-agglutination and PCR – molecular techniques. Our study has the aim to approve the methods for rapid diagnosis of invasive fungal diseases.

Key words: *Invasive fungal disease; Candida, Aspergillus, Cryptococcus*

Invasive fungal diseases are a leading cause of mortality in immunocompromised individuals (patients with hematological malignancies, transplant recipients, from the intensive care unit, with cancer, neutropenia, after chemotherapy, under 3 weeks of corticosteroid therapy, T-cell immune-suppressants (AIDS) and inherited severe immunodeficiency, wide spectrum antibiotic therapy, venous catheters, parenteral nutrition, renal dialysis, implanting of prothesis).
Laboratory diagnosis of invasive fungal diseases relies on microscopic and culture – based methods which delay diagnosis with 48-72 hours (20, 22). That is why rapid and reliable screening methods for early diagnosis and detection of pathogenic fungi are necessary to be used (13, 15).
The majority of invasive fungal diseases are still caused by Candida (16, 18) and Aspergillus species but recent studies indicate an increasing incidence of species such as Cryptococcus, Trichosporon, Malassezia (26).
The most common serological tests that detect antigen and antibodies in body fluid (serum, plasma, BAL) are: Indirect immunofluorescense (IIF), detection of 1, 3 – β D-glucan, ELISA – Platelia (enzyme linked immunosorbent assay), latex-agglutination and PCR – molecular techniques.
The method that we use for determination of specific antibodies is the Indirect Immunofluorescence (IIF) about Candida, Aspergillus, Cryptococcus. It is necessary to prepare microscopic slide from a cultured – suspension (10^6 - 10^7 CFU/ ml) of strain Candida (CIP 628), Aspergillus (A. fumigatus) and Cryptococcus neoformans. The slides with fixed suspension must be stored at – 20°C.
The principle of the method includes 2 steps – in the first step cover glasses have to be coated with diluted serum sample (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1: 640 and 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 – for Cryptococcus neoformans) for 30 minutes.
In the second step after washing the slides the attached antibodies are stained with fluorescein – labeled anti-human immunoglobulin antibodies and visualized with the fluorescent microscope (2, 11).



The advantages of the indirect immunofluorescence include greater sensitivity than the direct immunofluorescence.
There is also a method for detection of antibodies in a serum for a germ tube of Candida albicans by IIF. It is based on the two differences phases of C. albicans – blastoconides (yeast) and germ-tube. Many authors accept that it is important for the systemic infection because the microorganism invades the tissues, passes through the capillary walls and blocks phagocytosis. For this reason the formation of germ tubes is accepted as a virulent factor (1).
1-3 β-D- glucan (BG) detection is a diagnostic marker for invasive fungal infections. BG is a major cell wall polysaccharide component of many fungi (exception of the mucoreceous molds). Cryptococcus species and the yeast form of Blastomyces dermatitidis produce low levels of BG and BG is also a component of the Pneumocystis jirovecii cell wall.
BG testing must always be utilized with other diagnostic tests for the definitive conclusive diagnosis of invasive fungal disease (5, 6).
In our laboratory we have experience in serology antigen detection with ELISA (enzyme – linked immunosorbent assay).
ELISA – Platelia (BioRad) is an immunoenzymatic sandwich microplate assay for detection of the circulating antigen (mannan in Candida or galactomannan in Aspergillus) in human serum or plasma and in bronchoalveolar lavage fluid (BAL) – in adult and pediatric samples. Mannan and galactomannan antigen are polysaccharide non-covalently bound to the yeast cell-wall and they appear to be the main biomarkers for the diagnosis of invasive fungal disease. The rat monoclonal antibody is used which is directed against Candida or Aspergillus antigen (5, 25).
Serum, plasma or BAL –samples are heat-treated in the presence of EDTA in order to dissociate the immune complexes and to precipitate the serum proteins which could possibly interfere with the immunoassay reaction. The treated samples and the

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conjugate are added to the wells of the microplate coated with the monoclonal antibody. After incubation at 37°C the strips are washed. If there is a circulating mannan or galactomannan antigen in the human samples a complex: monoclonal antibody- anti-mannan /or galactomannan – antigen (Ag) – monoclonal antibody / peroxydase is formed. Next, the chromogen solution containing the peroxidase substrate is added and incubated at room temperature allowing the detection of any complex bound to the microplate well. The enzymatic reaction is stopped by the addition of a stopping solution.

The optical density (OD) of human samples and calibrators is determined with a spectrophotometer set at 450/620 nm wavelength.

The determination of the mannan concentration (pg/ml) in the test samples include drawing the calibration curve (using the 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 "negative"; samples with $62.5 \leq C \leq 125$ are "intermediate", and samples with $C \geq 125$ are "positive for mannan-antigen.

The presence or absence of galactomannan antigen in the test samples is determined by calculation of an index (I) for each patient specimen. The index (I) is the OD value of the specimen divided by the mean optical density of the wells containing Cut-off Control serum.

I=OD (samples)/ OD (cut-off control). Samples with $I < 0.50$ are "negative" and samples with $I \geq 0.50$ are "positive for galactomannan-antigen.

Limitations of the ELISA- procedure are:

- A negative test cannot rule out the diagnosis of invasive fungal diseases because of the very low concentration and the rapid elimination of the antigen during infection. For this reason patients with high risk of invasive diseases should be tested twice a week. Regular monitoring increases the sensitivity and early positivity of the test.
- Failure to add specimen or reagent as instructed in the procedure could result in a falsely-negative result.
- The concomitant use of antifungal therapy in some patients may result in reduced sensitivity of ELISA tests.
- Cross-reactivity of samples with Bifidobacterium, Cryptococcus, Penicillium; any cases of positive antigenemia without clinical signs should be interpreted more cautiously in this population of patients.
- There are reports for false-positive test results in patients receiving β-lactam therapy (Amoxicillin-Clavulanic acid, Piperacillin-tazobactam) or products, containing galactomannan in the presence of an alteration of the intestinal barrier, airway colonization with Aspergillus spp., colonization with Candida.

Other serological test for detection of circulating antigens using a sample technique is Latex – agglutination test (BioRad). It is a qualitative test to detect the polysaccharide antigen – mannan (Candida) and glycuronoxylomannan (Cryptococcus neoformans) in the biological fluids (serum, BAL, CSF). This procedure uses latex-particles coated with monoclonal rat antibody directed against fungal antigen. These particles react with the fungal antigen resulting in agglutination visible to the naked eye (19).

Limits of the test are associated as for all immunological techniques with the possibility of cross-reactions and should always be considered. Due to cross reaction with antibodies to galactomannan of Penicillium, positive reaction from patients infected with Penicillium marneffei or in sera, contaminated with spores from the environment could be observed (21).

Antigenic similarities between the Cryptococcus capsule and the wall of Trichosporon beigelii also can result in false-positive reactions in patients with trichosporonosis. In a case of a false result the sample can be repeated at a later time.

The non culture- based method is PCR for DNA – detection for the rapid diagnosis of infections. Amplification techniques offer increased sensitivity over traditional staining and culture methods but may give positive results in asymptomatic individuals because of colonization or subclinical infections (9, 10).

Advantages to molecularly based detection include speed, enhanced sensitivity of detection (table1), wider range of detectable organisms due to DNA sequence-based identification, detection of fungi that appear to be causing disease but can not be cultured. Molecular methods have been applied directly to clinical materials (7, 8,15). However, standardization and validation have not yet been attained for these platforms. Contaminating DNA from 12 different *Aspergillus* spp. caused a false-positive rate of 19% with sources of contamination including DNA extraction and PCR reagents (28).

The different methods for laboratory diagnosis of invasive fungal infections are with different specificity and sensitivity.

Antibody titers to antigens germ tubes of Candida albicans show high specificity and sensitivity of the method introduced in routine diagnostic laboratory practice. Many authors also confirm the ability of the method in the diagnosis of systemic mycoses because it is a future to distinguish between colonization with C. albicans in a systemic infection which is very important for empiric therapy in patients (6, 15).

Candida antibody assays have poor sensitivity and low specificity. Immunoglobulin G (Ig G) antibodies are usually positive for patients with aspergilloma and allergic bronchopulmonary aspergillosis. Quantitation of the immunoglobulin isotypes will be important to optimize the diagnostic value of the assay and to understand further the response in patients with aspergillosis (3, 12, 15).

Detection of GM (galactomannan) in serum is a feasible approach in adult neutropenic patients for the early diagnosis of invasive aspergillosis. GM – monitoring is recommended every three to four days in admitted patients. Detection of GM in BAL-fluid can be used to support the diagnosis of invasive aspergillosis and in the CSF can support the diagnosis of a fungal disease of the central nervous system. Our experience with GM – detection in pleural fluid, sputum or urine is insufficient to make some conclusion.

ELISA – Platelia (BioRad) tests are with more sensitivity and more appropriate for the analysis of a great number of serum samples but Latex – agglutination tests (BioRad) are easier to perform in laboratory conditions (23). We must not forget also the expern-

sive costs of antigen- detection tests.
The method for detection of soluble antigens by latex particle agglutination enables more rapid diagnostics than conventional culture method and is designed to detect a heat – sensitive circulating antigen of fungal species (17).

Table 1.

Range of biomarker – detection (JCM 2005; 43:2929-31)	GM	BG	PCR
Aspergillus fumigatus	+	+	+
Non-fumigatus aspergillus	+	+	+
Fusarium	-	+	+
Zygomyces	-	-	+
Candida	-	+	+
Cryptococcus	+/-	+/-	+
Penicillium	+	+	+
Paecilomyces	+	+	+

ELISA Platelia (BioRad) test combination is also an integral part of a clinical and laboratory patient monitoring process as an aid in therapeutic decisions.
A diagnosis of invasive candidiasis must be based on a combined detection of antibodies and circulating antigens.
The laboratory diagnosis of invasive fungal disease has to be used in conjunction with other diagnostic procedures such as culture technique, histological examination of biopsy samples, CT – imaging and can be used as an aid in the diagnosis of invasive fungal infection (24, 25).

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CORRELATION OF THE HEPATITIS B SURFACE ANTIGEN (HBSAG) LEVELS WITH HBV DNA CONCENTRATION IN PATIENTS WITH HBV INFECTION

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ABSTRACT:

It has been shown that hepatitis B surface antigen (HBsAg) titers correlate with serum HBV DNA levels but they vary in different phases of hepatitis B viral infection. Patients were selected according the presence of HBV DNA in the present study. Hepatitis B surface antigen was detected by ELISA and HBV DNA – by real time PCR. One of the follow up patients was HBV inactive chronic carrier with strong tendency of HBsAg seroclearance. The strong correlation of HBsAg serum titers with HBV DNA was observed for HBV mono-infection during process of HBsAg seroclearance (with low HVB DNA concentration) and in cases of acute hepatitis (with high HBV concentration). However when the levels of HBsAg in patients with medium HBV DNA concentration were evaluated a correlation was not observed. HBsAg levels were higher for HIV/HBV co-infected patients but there was not correlation with HBV DNA concentration. Data show that serum HBsAg levels correlate with HBV replication in a case of HBV mono-infection and can be used for predicting low and high serum HBV DNA levels.

Key words: *HBsAg, correlation, HBV DNA, seroclearance, HIV/HBV co-infection*

ABBREVIATIONS USED IN THIS PAPER: HBsAg - hepatitis B surface antigen, HBV – hepatitis B virus, HIV - human immunodeficiency virus, PCR – polymerase chain reaction, HCC - hepatocellular carcinoma, ELISA – enzyme linked immunosorbent assay, anti-HBe - hepatitis B e antigen, ant-HBc – antibodies against hepatitis B core antigen, CO – cut off, IU/ml – International Units per milliliter.

Hepatitis B virus (HBV) infection is an important health problem and more than 350 million suffer from a chronic form of infection. At the same time 15% to 40% of those infected with HBV develop cirrhosis, liver complications and hepatocellular carcinoma (HCC) (1). Hepatitis B virus infection was diagnosed by serological and virological markers with increased importance of HBV DNA detection. Serum HBV DNA levels are predictor of liver disease, of HCC development, of decision to administer antiviral therapy and determination of the treatment response. Hepatitis B surface antigen is important serological marker for diagnosis of acute and monitoring of chronic HBV infection. Serum HBsAg and HBV DNA levels differentiate inactive carriers from patients with active disease (2). It has been shown, that HBsAg titers correlate with serum and intrahepatic HBV DNA levels but they vary in different phases of disease (3).

The prevalence of chronic HBV infection in patients with human immunodeficiency virus (HIV) varies from 6% to 14% (4). Diagnosis of HBV co-infection is made on the basis of HBsAg positive status that determines the further diagnostics and treatment strategies. It is important to mention that the data for correlation between HBsAg and HBV DNA concentration in HIV/HBV co-infection are limited.

The relationship between the presence of HBV DNA and HBsAg levels in sera of: 1) inactive carrier; 2) patients with HBV mono-infection; and 3) patients with HIV/HBV co-infection was analyzed in this study. We evaluated the correlation between these two parameters in order to determine their importance as a marker for HBsAg seroclearance.

MATERIALS AND METHODS

Patients: A total of 27 patients were included in the study. Ten were with HBV mono-infection and 17 – with HIV/HBV co-infection. The inclusion criteria are the presence of serum HBV DNA with concomitant serological test for HBsAg and specific antibodies against hepatitis B e antigen (anti-HBe). All 14 sera from HBV mono-infected patients were tested for the presence of HBsAg and antibodies against hepatitis B e antigen (anti-HBe). HIV infection was confirmed serologically in all the 17 samples from co-infected patients and HBV infection – by presence of HBsAg and HBV DNA. Sera were collected in the National Referent Laboratory of Viral hepatitis from 2012 to 2013.

Serological assays (ELISA): Detection of HBsAg was done by SURASE B-96 ELISA kit (GmbH, Germany) for qualitative detection elevated levels of HBsAg in serum or plasma according manufacture instructions. Results were reported using S/CO ratios (signal-to-cut off ratio) and HBsAg was considered positive when the S/CO ratio was greater than 1.

Detection of anti-HBe was done by HBe Ag&Ab ELISA kit (DIA.PRO, Italy) according manufacture instructions.

Determination of HBVDNA

Serum HBV viral load was determined with COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Diagnostics GmbH) with the analytical measurement range that can be directly measured on a specimen without any dilution from < 2.00E (116) + 01 IU/ml to > 1.70E + 08 IU/ml. The conversion factor between HBV copies/ml and HBV IU/ml is 5.82 copies/IU

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Table 1. Correlation between HBV DNA concentration and HBsAg levels.

HBV mono-infection (total = 14)					
Sera samples (total = 31)	HBV DNA			HBV serology	
	[IU/ml]	[copies/ml]		HBsAg (S/CO)	Anti-HBe
1.1	2.41E+01	140		+ (12.01)	+
1.2	< 2.00E+01	<116		+ (4.13)	+
1.3	< 2.00E+01	<116		negative	+
2.1	5.00E+05	2 910 000		+ (61.14)	+
2.2	8.41E+01	472		negative	+
3	2.17E+01	126		+ (2.63)	+
4	1.97E+02	1146		+ (74.79)	+
5	6.67E+02	3882		+ (74.02)	+
6	3.48E+06	20 253 600		+ (71.15)	negative
7*	1.42E+08	826 440 000		+ (76.23)	+
8	7.58E+06	43 964 000		+ (73.52)	negative
9.1	2.02E+06	11 756 400		+ (48.11)	+
9.2	< 2.00E+01	<116		negative	+
10	6.40E+03	37 248		+ (60.83)	+
HIV/HBV co-infection (total = 17)					
1	2.01E+04	116 982		+ (62.77)	NT♦
2	< 2.00E+01	< 116		+ (62.60)	NT
3	7.32E+05	4 260 240		+ (61.07)	NT
4	1.33E+08	774 060 000		+ (62.07)	NT
5	< 2.00E+01	< 116		+ (61.96)	NT
6	< 2.00E+01	< 116		+ (62.46)	NT
7	< 2.00E+01	< 116		+ (64.07)	NT
8	3.98E+04	231 636		+ (59.90)	NT
9	4.30E+03	25 026		+ (60.36)	NT
10	< 2.00E+01	< 116		+ (62.45)	NT
11	< 2.00E+01	< 116		+ (64.09)	NT
12	< 2.00E+01	< 116		+ (61.66)	NT
13	< 2.00E+01	< 116		+ (61.46)	NT
14	> 1.70E+08	> 989 400 000		+ (62.05)	NT
15	< 2.00E+01	< 116		+ (60.83)	NT
16	9.08E+06	52 845 600		+ (61.26)	NT
17	5.45E+02	3172		+ (61.02)	NT

* sera sample No7 was HBeAg negative and ant-HBc IgM positive.

♦ NT - not tested

using the WHO International Standard for HBV DNA for NAT assay testing – NIBSC 97/746.

RESULTS

Twenty seven patients were included in the study according selective criteria for the presence of HBV DNA in sera. The sera samples (respectively 1.1, 1.2 and 1.3) from patient No1 were tested for the presence of HBsAg and with quantitative PCR for HBV DNA concentration at intervals of three time points for one year. Quantification of HBV DNA was done in all the 31 sera samples and 18 (80%) were in the limit of detection of the assay (Table 1).

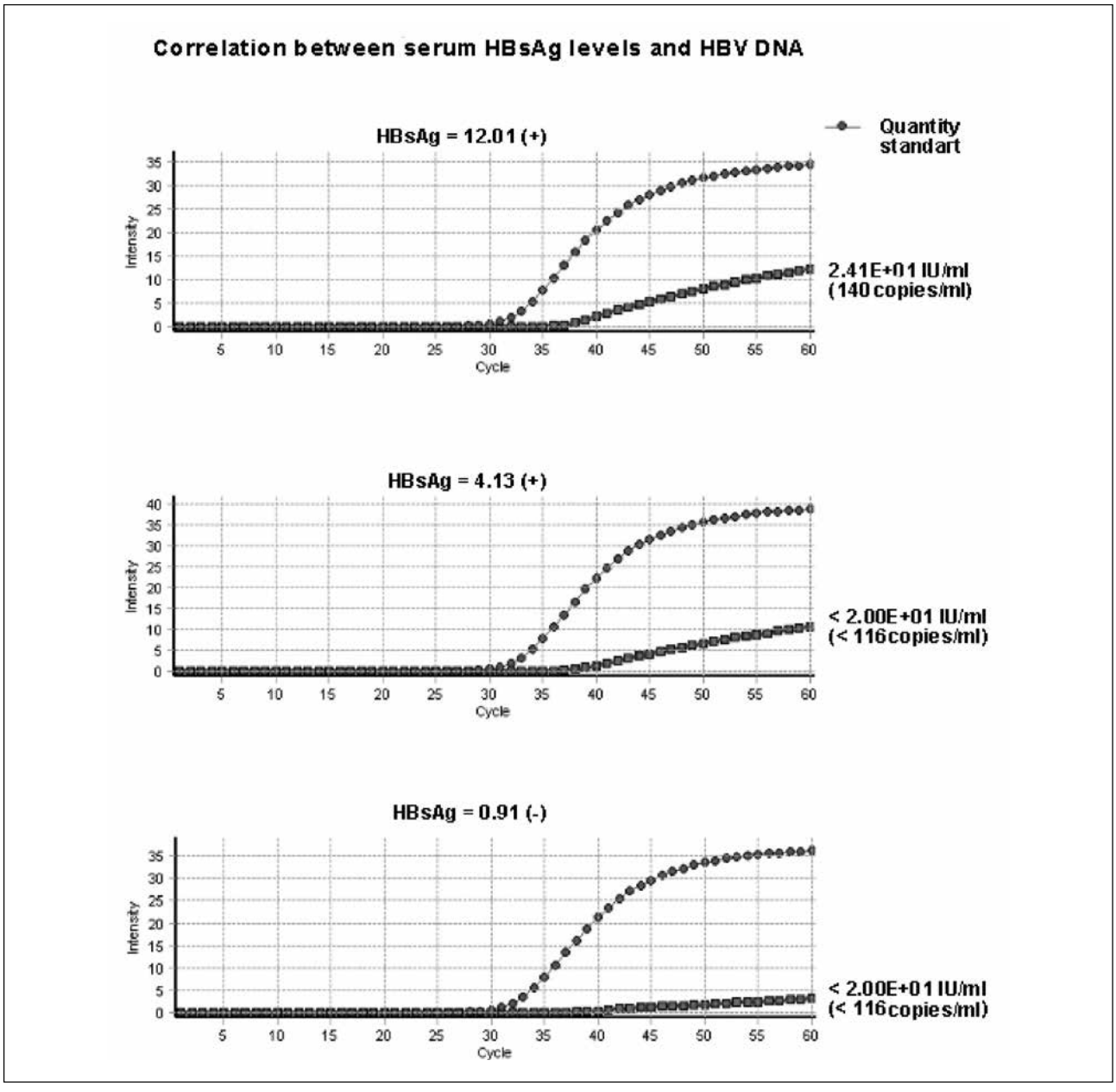
In twelve samples from 31 (39%) the concentrations were under the limit of detection and were expressed as < 2.00E+01 IU/ml (<116 copies/ml) instead of exact value. These samples were not measured as negative (result is not expressed as "target not detected") and they were accepted as positive with a very low HBV DNA concentration (Figure 1). One of the sera was with DNA concentration above the range of the assay and was expressed as greater (> 1.70E+08).

Correlation between HBsAg and HBV DNA levels in HBV mono-infected patients

Six sera samples were with HBV DNA concentration < 1000 copies/ml, two were in range > 1000 and < 5000 copies/ml, one – in range > 5000 and < 10 000 and 5 sera samples were with HBV DNA concentration > 10 000 000 copies/ml (Table 1).

When the presence of HBV DNA correlated with the serological status of the patients 11 of the 14 PCR-positive samples (78%) had HBsAg (Table 1). The correlation of 86% (12 of 14 PCR-positive)

Figure 1.



was established for the presence of anti-HBe. The value of HBsAg (S/CO) was from 2.63 to 76.23. All three HBsAg negative samples were with a very low HBV DNA concentration: two – under the limit of detection (< 116 copies/ml) and one sample with 472 copies/ml and were anti-HBe positive. The anti-HBe negative samples were with a very high HBV DNA concentration: 20 253 600 copies/ml and 43 964 000 copies/ml and with high HBsAg values, 71.15 and 73.52 respectively.

For the patient No1 who was an inactive carrier of HBV infection the correlation between HBsAg levels and concentration of HVB DNA was monitored in order to follow spontaneous HBsAg seroclearance. HBV DNA was measured at three time points and the concentration decreased from 140 copies/ml for the first samples to under the limit of detection for the second and third samples <116 copie/ml but still detectable (Figure 1). As can be seen from table 1 the correlation between DNA concentration and serum levels of HBsAg (S/CO) was 12.01 for the first sera sample (1.1), 4.13 for the second sample (1.2) and a negative value for the third (1.3).

Correlation between HBsAg and HBV DNA levels in HIV/HBV co-infected patients

In the cohort of patients with HIV/HBV co-infection 9 sera samples were with HBV DNA concentration < 1000 copies/ml as all were under the limit of detection (<116 copies/ml). One sample was with HBV DNA concentration in range > 1000 and < 5000 copies/ml, three – in range > 10 000 and < 1 000 000 and 4 sera samples were with HBV DNA concentration > 10 000 000 copies/ml (Table 1). All the patients were positive for HBsAg with values (S/CO) from 59.90 to 64.09.

DISCUSSION

The results of the present study demonstrated clearly that in the settings of HBV infection it is important to perform correct interpretation of the correlation between the main serological markers and the quantity of HBV DNA. Serum HBV DNA levels has become very important as a marker for clinical stage and prognosis of HBV infection. We established correlation between HBV DNA and serum HBsAg levels in a case of anti-HBe positivity and low DNA concentration < 1000 copies/ml. There is no correlation when HBV DNA concentration is more then 1000 IU/ml but less than 4000 IU/ml and the possible explanation is that these patients were in a low replicative phase of HBV infection. In a study of European HBsAg-positive patients it was established that in 20% of patient in the low-replicative phase of HBV infection the HBsAg levels were high even some the concentration of HBV DNA was below the detection limit of the TaqMan PCR (5). In the case of the patient No1 where we followed the process of seroclearance we detected a strong correlation between HBsAg and HBV DNA serum levels (Figure 1). We established simultaneously progressive reduction of HBV DNA and HBsAg serum levels. The presence of HBV DNA in concentration less than 20 IU/ml, (116 copies/ml) in the case of HBsAg clearance from chronic HBV carriers was established by other authors as well (6). Recently it was established that during seroclearance serum HBsAg levels correlate with intrahepatic DNA levels (7) and decline with immune clearance (8).

The significance of HBsAg quantification increases in the resent years (8). Several studies describe that the correlation between serum HBV DNA and HBsAg may vary depending of the stage of the infection (9). It is possible to quantify HBsAg concentration in IU using last generation commercial assay for detection of HBsAg – the Architect QT assay (Abbott Laboratories) and the Elecsys HBsAg II Quant assay (Roche Diagnostic) which help easy to compare with HBV DNA concentration measured in IU/ml.

In a case of HBV mono-infection patients No 2.1, 6, 7, 8, and 9.1, high serum levels of HBsAg matched with HBV DNA concentration > 2 000 000 copies/ml. A strong positive correlation between serum HBsAg and HBV-DNA was observed during prospective study of acute HBV by Jaroszewicz J. et.al. (5). Differences between these sera samples occurred in their serological status. The sample with the highest HBV load 826 440 000 copies/ml was positive for IgM antibodies against core protein of HBV (anti-HBc IgM-positive), HBeAg negative and positive for the presence of HBe antibodies. The presence of anti-HBc IgM is evidence for acute HBV infection and in combination with high HBV load and anti-HBe-positive status is an evidence for a possible infection with HBeAg defective HBV mutant that prevails in the Mediterranean area (10). This mutant has impaired HBeAg formation by an otherwise normally replicating hepatitis B virus.

In the cohort of HIV co-infected patients HBsAg values (S/CO) were high ranging from 59.90 to 64.09. Furthermore the highest value was detected in the sera with HBV DNA concentration <116 copies/ml and the lowest - in the sera with HBV DNA concentration equal of 231 636 copies/ml. The HBsAg value was 62.05 for the sera sample with the highest HBV DNA concentration (> 989 400 000 copies/ml). It was established by other authors too (11) that HBsAg levels decreased slowly despite complete suppression of HBV DNA replication during treatment. At the same time the large study from France demonstrated that HBsAg decline was influenced by HIV induced immunodeficiency (12).

Some limitations of our study need to be considered. The size of the study population is small. At the same time it is important to follow the spontaneous HBsAg clearance which occurs with a very low incidence rate (13). Our work provides evidence that serum HBsAg levels correlate with HBV DNA levels and can be used for predicting of low and high HBV replication for HBV mono-infected patients nevertheless the existing limitation.

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NEURAMINIDASE INHIBITORS SUSCEPTIBILITY TESTING OF INFLUENZA VIRUSES CIRCULATING IN BULGARIA DURING THE FIRST THREE POSTPANDEMIC FLU SEASONS

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ABSTRACT:
The aim of this study was to determine the neuraminidase inhibitors susceptibility of influenza viruses circulating during the 2010/2011, 2011/2012 and 2012/2013 flu seasons in Bulgaria.
Materials and methods: Screening of 152 influenza A(H1N1)pdm09 viruses was carried out using Real Time RT-PCR discrimination assay for detection of the H275Y oseltamivir resistance point mutation. Three influenza A(H1N1)pdm09 and 11 A(H3N2) viruses were evaluated by phenotypic method using the fluorogenic substrate MUNANA.
Results: No influenza A(H1N1)pdm09 viruses carrying a H275Y substitution were found. None of Bulgarian A(H1N1)pdm09 and A(H3N2) viruses evaluated for neuraminidase inhibitors susceptibility showed genetic or phenotypic (IC_{50}) evidence for resistance or reduced susceptibility.
Conclusion: The Real Time RT-PCR assay described in this report can be used to screen large numbers of clinical A(H1N1)pdm09 virus positive samples for resistance to oseltamivir. The article demonstrates the importance of continued influenza antiviral susceptibility monitoring in clinical specimens.

Key words: *influenza virus, resistance, mutation*

Influenza virus inhibitors are an important tool for reducing morbidity and mortality from influenza. There are two classes of antiviral drugs licensed for the treatment and chemoprophylaxis of influenza: inhibitors of the M2 ion channel - adamantanes (amantadine and rimantadine) and neuraminidase inhibitors - oseltamivir (Tamiflu), zanamivir (Relenza), peramivir (Rapiacta, Peramiflu) and laninamivir octanoate (Inavir) (the latter two drugs are licensed only in Japan and South Korea) (3). An important problem associated with the use of influenza virus inhibitors is the occurrence of resistance in influenza viruses leading to a reduction or complete loss of drug effectiveness. The resistance arises due to mutations in the genome of influenza viruses as a result of drug press or spontaneously without relationship to drugs usage. Numerous resistance-associated amino acid substitutions have been identified in the different types and subtypes influenza viruses (4, 7). Since the frequency of resistance in circulating influenza type A viruses in recent years to adamantanes is very high (> 99%) but these drugs are not effective against type B viruses the WHO does not recommend their use for treating of influenza (8). All recent influenza A and B viruses (with some rare exceptions) are sensitive to neuraminidase inhibitors that are currently the only effective drugs for treatment and prophylaxis of influenza.

Because influenza viruses carrying resistant mutations can spread rapidly and displace fully sensitive viruses WHO encourages to monitor the susceptibility of circulating influenza viruses. For this purpose various methods have been used - phenotypic (functional) and genotyping. Phenotypic assays (fluorescent and chemiluminescent) measure the concentration of drug required to inhibit neuraminidase activity of influenza virus by 50% (ie inhibitory concentration, IC_{50}). These methods evaluate impact of multiple mutations - known and unknown. Phenotypic analysis is widely accepted as the "gold standard" for detecting influenza virus drug resistance, however, the assays are lengthy, labor-intensive, require viral culture and expensive equipment and reagents. Genotyping assays are fast, highly sensitive and can be done directly on clinical specimens. Among them Sanger sequencing and pyrosequencing identify the presence of numerous known and unknown mutations associated with resistance. Genotyping assays based on PCR are very sensitive, lower cost assays which, however, allow detection of a single known mutation (5). PCR genotyping assays have been used mainly to detect a point mutation (cytosine to thymine) at position 823 of the neuraminidase N1 gene that results in histidine to tyrosine substitution at position 275 (H275Y) in the neuraminidase active site of A(H1N1) viruses. H275Y is the most usual mutation conferring high-level resistance to oseltamivir, but not to zanamivir. Virtually all oseltamivir-resistant influenza A(H1N1) viruses, including pandemic, contain this mutation.

Aim of this study was to determine the neuraminidase inhibitors susceptibility of influenza viruses circulating over the past three epidemic seasons in Bulgaria.

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MATERIALS AND METHODS:

Neuraminidase inhibitors susceptibility of influenza viruses circulating in Bulgaria during the 2010/2011, 2011/2012 and 2012/2013 flu seasons was determined. Detection and typing / subtyping of influenza viruses were carried out in the NRL „Influenza and ARD" by the method Real Time RT-PCR using specific primers and probes received from CDC Atlanta, USA. Isolation of influenza viruses was performed using embryonated hen's eggs and Madin-Darby canine kidney cells (MDCK-SIAT). Detection of single point mutation in the neuraminidase gene that results in H275Y amino acid substitution was conducted by a modified method Real Time RT-PCR described by Van der Vriest et al (6). In this assay two probes differing in position 823 of the neuraminidase gene were simultaneously used: the first probe contains a cytosine at position 823 and is labeled with FAM (275H) while the second probe contains thymine in the same position and is labeled with HEX (275Y). Real-Time RT-PCR was carried out using a SuperScript III Platinum One Step RT-PCR System (Invitrogen). Conditions of the RT-PCR were as follows: RT reaction at 50^0 for 30 min, initial denaturation at 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 20 s and primer annealing at 60°C for 1 min with subsequent

Tabl. 1. Results of Real Time RT-PCR discrimination assay for detection of the H275Y oseltamivir resistance mutation in A(H1N1)pdm09 viruses.

Season of detection	Number of A(H1N1) pdm09 viruses tested	Number (%) of influenza viruses shown a positive signal with 275H probe	Number (%) of influenza viruses shown a positive signal with the 275Y probe
2010/2011	100	100 (100%)	0
2012/2013	52	52 (100%)	0

Tabl. 2. Neuraminidase inhibitor susceptibility of influenza viruses circulating during the 2010/2011 and 2011/2012 seasons in Bulgaria determined by phenotypic fluorescence-based method

Influenza virus isolates	Type/ subtype	IC_{50} for oseltamivir (nM)	Susceptibility to oseltamivir	IC_{50} for zanamivir (nM)	Susceptibility to zanamivir
A/Bulgaria/26/2011	A(H1N1)pdm09	0,58	sensitive	0,53	sensitive
A/Bulgaria/31/2011	A(H1N1)pdm09	1,63	sensitive	1,31	sensitive
A/Bulgaria/57/2011	A(H1N1)pdm09	0,85	sensitive	1,19	sensitive
A/Bulgaria/41/2012	A(H3N2)	0.81	sensitive	0.54	sensitive
A/Bulgaria/42/2012	A(H3N2)	1.21	sensitive	0.71	sensitive
A/Bulgaria/43/2012	A(H3N2)	1.24	sensitive	0.78	sensitive
A/Bulgaria/182/2012	A(H3N2)	1.86	sensitive	1.3	sensitive
A/Bulgaria/217/2012	A(H3N2)	1.06	sensitive	0.68	sensitive
A/Bulgaria/218/2012	A(H3N2)	1.25	sensitive	1.19	sensitive
A/Bulgaria/280/2012	A(H3N2)	0.98	sensitive	0.98	sensitive
A/Bulgaria/311/2012	A(H3N2)	0.9	sensitive	0.84	sensitive
A/Bulgaria/312/2012	A(H3N2)	1.1	sensitive	1.35	sensitive
A/Bulgaria/313/2012	A(H3N2)	1.54	sensitive	1.25	sensitive
A/Bulgaria/314/2012	A(H3N2)	1.07	sensitive	1.45	sensitive

detection of fluorescence (6). A total of 152 clinical samples positive for pandemic influenza virus were tested using this method to determine the presence / absence of the H275Y mutation. Influenza viruses A/Denmark/524/2009 (sensitive, 275H) and A/Denmark/528/2009 (resistant, 275Y) received from World Health Organization Collaborating Centre (WHO-CC) in London were used as positive controls. Neuraminidase activity and drugs susceptibility of influenza viruses were determined in the WHO-CC, London by phenotypic fluorescence-based assay using the substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA). This assay when performed in the presence of inhibitors allows the concentration of drug required to inhibit viral neuraminidase activity by 50% (IC_{50}) to be determined (2). Viruses are considered as resistant to an inhibitor if the IC_{50} is > 100 nM. If the IC_{50} is 2-100 nM viruses have reduced susceptibility. In sensitive viruses IC_{50} is 0-2 nM. Sanger sequencing of the haemagglutinin and neuraminidase genes of Bulgarian A(H1N1) pdm09 and A(H3N2) isolates proved in the 2010/2011 and 2011/2012 seasons was carried out in WHO-CC, London.

RESULTS AND DISCUSSION:

100 and 52 influenza A(H1N1)pdm09 virus positive clinical samples (nasopharyngeal swaps) collected in different regions of the country during the 2010/2011 and 2012/2013 flu seasons respectively were screened for the H275Y substitution by Real Time RT-PCR. In the 2011/2012 epidemic season there was no A(H1N1)pdm09 virus detected in Bulgaria. All samples tested showed positive fluorescent signals with the FAM dye that correspond to the oseltamivir sensitive genotype (275H). No signals were observed with the HEX dye that indicates the oseltamivir-resistant genotype (275Y) (Table 1).

This study demonstrated the application of the Real-Time PCR method in detecting the H275Y mutation in viral neuraminidase that confers resistance to oseltamivir. The method correctly identified the oseltamivir-sensitive (H275) and oseltamivir-resistant (275Y) genotypes of influenza A(H1N1)pdm09 viruses.

By phenotypic method using the fluorogenic substrate MUNANA three Bulgarian A (H1N1)pdm09 virus isolates, detected in the 2010/2011 season, and 11 A(H3N2) virus isolates from 2011/2012 season were investigated. In Table 2 the inhibitory concentration (IC_{50}) of neuraminidase inhibitors oseltamivir and zanamivir for Bulgarian influenza viruses is shown. Among A(H1N1)pdm09 viruses IC_{50} ranging of 0.58 to 1.63 for oseltamivir and 0.53 to 1.19 for zanamivir. Among A(H3N2) viruses IC_{50} ranging from 0.81 to 1.86 for oseltamivir and 0.54 to 1.45 for zanamivir. All Bulgarian A(H1N1) pdm09 and A(H3N2) viruses proven during the 2010/2011 and 2011/2012 seasons were sensitive to oseltamivir and zanamivir.

Sequence analysis of the haemagglutinin and neuraminidase genes of five Bulgarian influenza viruses detected during the 2010/2011 and 2011/2012 seasons did not reveal the presence of mutations implicated in resistance to neuraminidase inhibitors (7).

Our research showed that the tested influenza viruses circulating in the country over the past three influenza seasons were sensitive to neuraminidase inhibitors oseltamivir and zanamivir. The surveillance of neuraminidase inhibitors susceptibility of influenza viruses is essential and recommended by WHO due to the likelihood of occurrence and rapid spread of resistant viruses with better replicative capacity and ease of transmissibility which could displace briefly sensitive viruses and become predominant. For example, before 2007 the resistance of seasonal A(H1N1) viruses to oseltamivir was very low (0.5-1%). In the 2007/2008 season in Europe proportion of resistant virus sharply increased: from 2% in Spain to 67.3% in Norway (average 25%). Resistant viruses spread very rapidly: in 2008/2009 they were 95% and in 2009/2010 - 100% (1). For now, the frequency of resistance to oseltamivir in pandemic A(H1N1)pdm09 viruses which completely replaced the seasonal influenza viruses of the same subtype is very low. Since the beginning of pandemic in February 2009 until the summer of 2011 605 oseltamivir resistant A(H1N1) pdm09 viruses, carriers of the H275Y mutation were identified in the world. Global incidence of oseltamivir-resistant pandemic virus is around 1%. Resistance to zanamivir is extremely rare. For now, zanamivir is the only one licensed drug for treatment of patients infected with oseltamivir-resistant viruses. Seasonal influenza A(H3N2) and B viruses are sensitive to neuraminidase inhibitors (4).

CONCLUSION:

Due to the extreme variability of influenza viruses, the problem of resistance to existing antiviral drugs is very important. It is conducting ongoing monitoring of neuraminidase inhibitors susceptibility on national and global levels as well as developing of new substances with antiviral activity to expand the range of products available, effective for treatment and prophylaxis of influenza.

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ANTIFUNGAL SUSCEPTIBILITY TESTING OF MEDICALLY IMPORTANT YEASTS

Review

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ABSTRACT:
The aim of this review is to present the new approaches in antifungal susceptibility testing of medically important yeasts. The most commonly used standardized reference methods for yeasts are CLSI M27-A3 broth dilution method and similar to it EUCAST method and CLSI M44-A2 disk diffusion method. The alternative agar based approaches for yeasts are Neo-Sensitabs tablets and E-test. Etest is easy to use in the routine setting and have been recently incorporated into clinical laboratory practice and allows determination of minimal inhibitory concentration of the most commonly used antifungal agents. Molecular analyses of antifungal drug resistance are focused mainly on the different mechanisms of azole resistance of Candida spp.

Key words: antifungal agent, susceptibility, fungal infection

The introduction of modern patient management technologies and therapies in the last 20 years has resulted in significant rise in number of chemically induced immunosuppressed patients who are highly susceptible to severe fungal infections. The number of patients with immune dysfunction has increased dramatically because of AIDS epidemic, raised bone marrow transplants (BMTs) and solid organ transplants and intensive chemotherapy. Health care-associated fungal infections are also emerging as significant problems. Fungal infections increase the risk of patient mortality and raise both the length of stay and cost associated with hospitalization (1).

With the increase of fungal infections there has been a significant rise in the use of antifungals for both systemic and localized fungal infections (11, 12). This has lead to the introduction of an important new class of antifungal agents (the echinocandins), expansion of the spectrum of an established class of agents through chemical modification (the triazoles) and the development of novel method for delivering established agents (lipid-based formulations of amphotericin B). It is not surprising that the expanded use of antifungal drugs has accelerated the development of resistance to these compounds (2). Fungal infections are very important public health problem and the increase of antifungal resistance has induced the pharmaceutical companies to develop new agents with either a broader spectrum or different targets of activity. Currently, there have been approved thirteen agents for the treatment of systemic fungal diseases: polyene macrolide antibiotics conventional amphotericin B (Fungizone; Apothecon) and its three lipid formulations; flucytosine (5-fluorocytosine) (Ancobon); Valeant Pharmaceuticals), a synthetic pyrimidine; the azoles ketoconazole (Nizoral; Ortho-McNeil-Janssen Pharmaceuticals), fluconazole (Diflucan; Pfizer), itraconazole (Sporanox; Ortho-McNeil-Janssen Pharmaceuticals), voriconazole (Vfend, Pfizer), and posaconazole (Noxafil; Schering-Plough); and the echinocandins caspofungin (Cancidas; Merck), micafungin (Mycamine; Astellas Pharma Fujisawa Healthcare), and anidulafungin (Eraxis in the United Stades, Ecalta in Europe; Pfizer).

The significant increase of antifungal drug resistance mechanisms has made the in vitro susceptibility testing very important tool for the adequate choice of the appropriate antifungal therapy. In vitro susceptibility tests: 1) provide a reliable measure of the relative activities of antifungal agents, 2) correlate with in vivo activity and predict the likely outcome of therapy, 3) provide a means with which to monitor the development of resistance among a normally susceptible population of organisms, and 4) predict the therapeutic potential of newly discovered investigational agents (3). The reference tests for susceptibility testing are the broth microdilutions assays devised by the Clinical and Laboratory Standards Institute (CLSI) and by the European Committee on Antibiotic Susceptibility Testing (EUCAST). These reference methods are robust and reproducible; however, they remain time consuming and poorly suited for the routine clinical laboratory settings. In order to overcome these limitations have been developed many commercially available methods, such as the E-test, Sensititer Yeast One or disk diffusion methods that are easy to use in routine practice. These methods have been recently incorporated into routine clinical laboratory practice and thus generate a considerable amount of antifungal MIC data from clinical fungal isolates. In our country the monitoring of antifungal drug susceptibility is performed by the National Reference Laboratory of Mycology that uses standardized methods to test clinical isolates received from collaborating clinical laboratories. The reference laboratory thus collect invaluable data for the monitoring of susceptibility trends on national and international scales. However, there is a need to develop antifungal susceptibility monitoring at a local or regional scale. This could also improve patient care and generate significant cost reductions of yeast infections, their morbidity, and the costly protracted treatments required (13).

M27-A3, Standardized Broth Dilution Method for Yeasts

The development of the CLSI reference method M27-A3 document (approved standard, 3rd ed.)(4) has improved the reproducibility of in vitro antifungal susceptibility data and facilitated the establishment of interpretive breakpoints for the triazoles fluconazole, itraconazole, and voriconazole and the echinocandins anidulafungin, caspofungin, micafungin versus *Candida spp*. (5) (Table 1). Based on historical data and the pharmacokinetics of flucytosine interpretive

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breakpoints for flucytosine and *Candida spp*. also have been established. Most M27-A amphotericin B MICs (minimal inhibitory concentrations) are within a very narrow range (0.25 to 1 μg/ml)(4) precluding a clear discrimination between susceptible and potentially resistant isolates.

The main differences between the previous version (M27-A2/S2) and M27-A3/S3 are the inclusion of quality control (QC) parameters for several new agents, additional breakpoints recommendations and the reading of amphotericin B and fluconazole MICs after 24 hours of incubation. There should be considered some warnings about the applicability of fluconazole breakpoints with *Candida glabrata* isolates and the suggestion that if the MIC is ≤32μg/ml with this species patients should be treated with a maximum-dosage of fluconazole.

This method can be performed both as macrodilution or as microdilution but the broth microdilution test is the most widely used technique for antifungal susceptibility testing. The standard medium recommended by this method is the RPMI 1640 broth medium with L-glutamin and a pH indicator and without sodium bicarbonate. RPMI medium with 2% dextrose and yeast nitrogen base broth may enhance the growth of yeasts facilitating the determination of MICs. 2% dextrose could falsely elevate the MICs at 48h and produce lower MICs at 24h. The EUCAST method includes the use of this modified RPMI medium (6).

There are some modifications of CLSI reference methods that offer promise as alternative approaches that may better serve clinical laboratory needs. Some of those modifications use **spectrophotometric** automated plate reading and yield MIC values that are more accurate and objective than those by other tests. A very essential step is the agitation of microtiter plates by use of a microdilution tray shaker before MIC determination in order to obtain homogeneous cell suspensions in the wells and hence a more precise MIC determination. A method similar to CLSI M27-A3 for broth microdilution testing of yeasts has been developed under the auspices of the Subcommittee on Antifungal Susceptibility Testing of the EUCAST (7). Unlike M27-A3 this method employs flat-bottom microtiter plates, a higher inoculum (10^5 cells/ml) and RPMI medium supplemented with additional glucose (2%) in order to encourage rapid yeast growth and facilitate early reading (24h) of endpoints. EUCAST method has a good intralaboratory reproducibility and there is a good agreement between this method and CLSI microdilution method but CLSI breakpoints should not be used to interpret EUCAST MICs, the latter MICs are consistently lower than reference values and there is a risk of falsely susceptible results. The two methods are moving closer together with the introduction of 24 h incubation time of the CLSI method (19).

Colorimetric indicators or fluorescent dyes can also be used to facilitate determination of MIC endpoints. Commercial (Sensititre YeastOne and Fungitest) procedures have been adapted for antifungal susceptibility testing (8). There is a good correlation between the results received with these procedures and the CLSI M27-A3 and also this method minimizes the trailing effect of azoles.

Vitek 2 Yeast Susceptibility testing is developed by bio-Mérieux and it is based on a commercial test system with spectrophotometric analysis. These system exhibited excellent agreement with the reference broth microdilution method for detecting resistance with overall categorical agreement of 97.5% for both fluconazole and voriconazole.

Standardized Disk Diffusion Method for Yeasts (M44-A2 document)

Agar disk diffusion testing is a simple, flexible, and cost-effective method which serve as an alternative to broth dilution testing. This method is developed for testing Candida spp. with caspofungin (5μg disks), fluconazole (25μg disks), voriconazole (1μg disks) and posaconazole(5μg disks) but there are interpretive criteria only for the first tree of the mentioned

Table 1. Interpretive MIC breakpoints and corresponding zone diameters for in vitro susceptibility testing of Candida species with CLSI-recommended methods

Antifungal agent	MIC breakpoint, in μg/ml[b] (zone diameter, in mm[c])				
	Susceptible	Susceptible (dose dependent)	Intermediate	Resistant	Nonsusceptible
Fluconazole[d]	≤8 (≥19)	16-32 (15-18)	-	≥64 (≤14)	-
Voriconazole	≤1 (≥17)	2 (14-16)	-	≥4 (≤13)	-
Itraconazole	≤0.125 (NA)	0.25-0.5 (NA)	-	≥1 (NA)	-
Flucytosine	≤4 (NA)	-	8-16 (NA)	≥32 (NA)	-
Anidulafungin[e]	≤2	-	-	-	>2
Caspofungin[e]	≤2.0 (≥11)	-	-	-	>2 (≤11)
Micafungin[e]	≤2	-	-	-	>2

[b]Method performed as described in CLSI document M27-A3.
[c]Method performed as described in CLSI document M44-A2; NA, not available
[d]Fluconazole breakpoints apply to both 24- and 48-h readings. Isolates of Candida krusei are assumed to be intrinsically resistant to fluconazole. The results of fluconazole susceptibility testing of this species (zone diameter and MIC) should not be interpreted using this scale.
[e]There is no Resistant category assigned to the echinocandins; isolates with MICs higher than 2.0 μg/ml may be described as nonsusceptible.

antifungals. A very significant advantage of M44-A2 is that results can be obtained after 20 to 24 h of incubation. Since the introduction of this method there is an extensive worldwide testing of voriconazole and fluconazole as part of a global survey.

The alternative agar based approaches for yeasts are NeoSensitabs tablets and E-test. There are commercially available E-test strips for amphotericin B, fluconazole, flucytosine, ketoconazole, itraconazole, posaconazole, voriconazole, caspofungin, micafungin and anidulafunin. When a clear zone of inhibition is seen the MIC can easily be read where the zone of inhibition intersects the strip and false susceptibility has not been reported. The E-test may be useful in testing yeasts suspected of being potentially resistant to Amphotericine B (10, 18). Amphotericin B MICs of ≥0.38 µg/ml determined by Etest for *Candida spp*. have been associated with therapeutic failure in patients treated with amphotericin B for candidemia but there are suggestions that factors other than MIC may have a greater impact on the outcome of invasive candidiasis (9).

Etest and disk diffusion methods are easy to use in the routine setting and have been recently incorporated into clinical laboratory practice and thus generate a considerable amount of antifungal MIC data from clinical fungal isolates. These two methods are successfully conducted in the Reference Laboratory of Mycology and enable the collection of MICs data for medically important yeast isolates sent from different medical centers all over the country. All this enables the conduction of regional population-based candidemia surveillance and assay of antifungal agent resistance.

In order to be useful clinically in vitro susceptibility testing should reliably predict the in vivo response to therapy in human infection. There are factors related to the drug, the host immune response, current underlying diseases, the infecting organism and the interactions of the organism with both the host and the therapeutic agent which have more value than the MIC as predictors of clinical outciome.

Molecular analyss of antifungal drug resistance has focused mainly on *C. albicans* but studies have been done on *C. glabrata* and *C. krusei* as well as *C. neoformans*. A very important new approach is the introduction of molecular tools in the identification and typing of clinically significant *Candida spp.* which is conducted prior antifungal susceptibility testing of the strains (14, 15). The molecular analysis of antifungal drug resistance in *C. albicans* is complicated by the clonal nature of this diploid yeast, thus it is important to determine the relatedness of a susceptible isolate and resistant isolate before comparing them by molecular analysis (16, 17). Azole resistance in *Candida spp*. can be result of the following mechanisms: modification of the quality or quantity of the target enzymes, reduced access of the drug to the target or some combination of these mechanisms. Thus point mutations in the gene (ERG11) encoding the target enzyme, lanosterol 14-α demethylase leads to an altered target with decreased affinity for azoles. Overexpression of ERG11 results in overproduction of the target enzyme leading to the need of higher concentrations of the drug within the cell to inactivate all the target enzyme molecules. When there is an up-regulation of genes encoding for multidrug efflux pumps it will result in more active efflux of the azole antifungal agents out of the cell. Up-regulation of genes encoding the major facilitator type efflux pump (MDR) leads to fluconazole resistance and up-regulation of genes encoding the adenosine triphosphate (ATP)-binding cassette transporters (CDR) leads to resistance to multiple azoles.

Unlike resistance in parasite and mammalian systems drug resistance in the fungi is not normally associated with gene amplification. Fungi in general have elaborate systems for the regulation of gene transcription and thus gene amplification is not necessary to alter gene expression. Unlike prokaryotes studies with fungi have not yet identified any plasmids or other episomes containing resistance markers and also there is no documented situation in which the resistance markers from one isolate were transferred to another isolate. Given the multiple molecular mechanisms of resistance and the real possibility of more existing a simple molecular test for resistance would be difficult to develop.

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OPPORTUNISTIC AND COINFECTIONS IN HIV POSITIVE PATIENTS AT THE CLINIC OF INFECTIOUS DISEASES, "ST.GEORGE" UNIVERSITY HOSPITAL, PLOVDIV

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ABSTRACT:
Objective: to be determined the type and frequency of the opportunistic and coinfections in HIV positive patients as well as their connection with the degree of the immune deficiency.
Methods: the study included 170 patients with verified HIV infection- 146 men and 24 women. They were seperated in 3 groups: stage A- 16; stage B- 104; stage C- 50. The used methods are clinical observation, laboratory and microbiological analysis. In all patients HIV was confirmed by present diagnostic methods (ELISA, Western-Blot, PCR, flow cytometry).
Results: The opportunistic infections in HIV positive patients were: candidiasis - 32 (4- stage A; 8- stage B; 20- stage C); pneumocystis pneumonia- 14 patients (stage C); tuberculosis – 8 (1 – stage B; 7- stage C); HHV8 – 2 patients- both of them in stage C; 1 patient with salmonella sepsis; 1 patient with toxoplasma meningitis.
Coinfections with hepatotropic viruses: 110 patients (64.7%) infected with hepatitis C ; 23 patients infected with hepatitis B; 15 patients were positive for both hepatitis B and C. Intravenous drug users in these groups were 68%; 65.21% and 80% respectively.
Conclusions: The most common opportunistic infections are candidiasis (18.8%) and pneumocystis pneumonia (8.2%). The most common coinfection is hepatitis C. Coinfections with hepatotropic viruses correlate with the mechanism of transmission. The intravenous drug users are at a very high risk.
Key words: HIV, AIDS, opportunistic infections, coinfections.

Introduction: About 42 million people are infected with HIV worldwide. Each year the newly infected add up to approximately 1 million. The cases of HIV/AIDS in Europe continue to increase every year and the disease acquires more and more importance for public health. During 2010 the newly registered cases in Europe were more than 27 000 which presents an incidence rate of 5.7/100 000 (1). For 2009 the registered cases in Europe were 53 427 – they were reported from 49 of all 53 countries (2). During 2008 the new cases of HIV/AIDS in Europe were 51 600 (3) which is an increase in comparison with 2007 when they were 48 892 (4). In Bulgaria the total number of HIV positive population according to the Ministry of health till the end of August 2012 was more than 1500. Four hundred of them are already diagnosed with AIDS.
Emerging opportunistic infections (and their treatment) in patients with HIV is of major importance for the patients' clinical condition and the outcome of the disease (5, 6). The **aim** of the study is to be determined the type and frequency of the opportunistic infections and the coinfections in HIV positive patients as well as their relation to the degree of the immune deficiency.

Materials and methods:
The study includes 170 patients with verified HIV infection - 146 men and 24 women. They are separated in 3 groups - stage A - 16; stage B - 104; stage C - 50. The methods used are clinical observation, laboratory and microbiological analysis. In all patients HIV was confirmed by contemporary diagnostic methods (ELISA, Western-Blot, PCR, flow cytometry).
According to the mechanism of infection the patients are separated in 3 groups: intravenous drug users - 51%; heterosexual intercourse - 40 %; men, who have sex with men (MSM) - 9 %.

RESULTS

The age distribution of the patients is shown on Figure 1. Most of them (50%) are older than 50 years.
Before the widespread use of potent combination antiretroviral therapy (cART) opportunistic infections were the principle cause of morbidity and mortality in this population. The widespread use of cART starting in the mid-1990s has had the most profound influence on reducing opportunistic-related mortality as well as improving the quality of life in HIV-infected persons. Despite the availability of ART in industrialized countries, opportunistic infections continue to cause considerable morbidity and mortality because many patients are unaware of their HIV infection, certain patients are aware of their HIV infection but do not undergo ART because of psychosocial or economic factors; certain patients are prescribed ART but fail to attain adequate virologic and immunologic response because of factors related to adherence, pharmacokinetics, or unexplained biologic factors.
The most common opportunistic infection among the HIV-positive population at the clinic is candidiasis - 32 patients (4 - stage A, 8 - stage B, 20 - stage C); pneumocystic pneumonia - 14 patients (stage C); tuberculosis - 8 patients (1-stage B, 7 - stage C); HHV8 - Sarcoma Kaposi - 2 patients in stage C, 1 patient with salmonellosis and 1 patient with toxoplasmic meningitis - both in stage C (Figure 2). The vast majority of the patients have more than one opportunistic infection which complicates the therapeutic effect.
According to the coinfections with hepatotropic viruses, HIV+ patients are separated in three groups -

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Figure 1: Age distribution of the patients

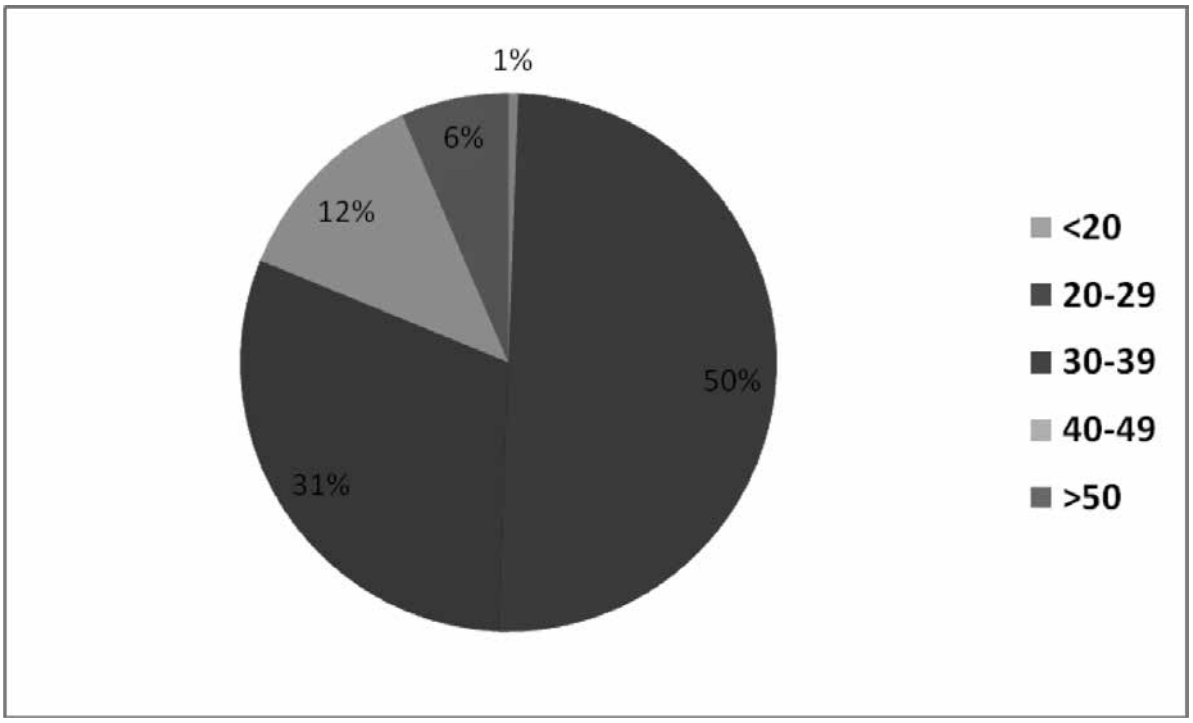


Figure 2: Frequency of HIV-related opportunistic infections

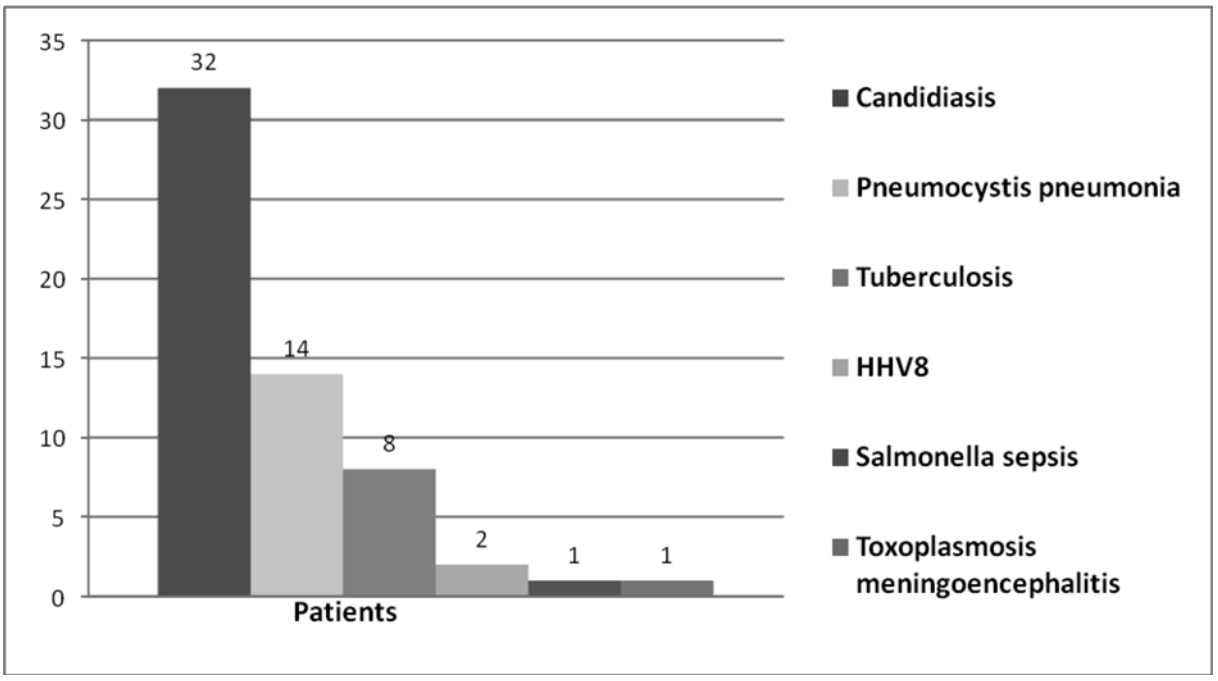
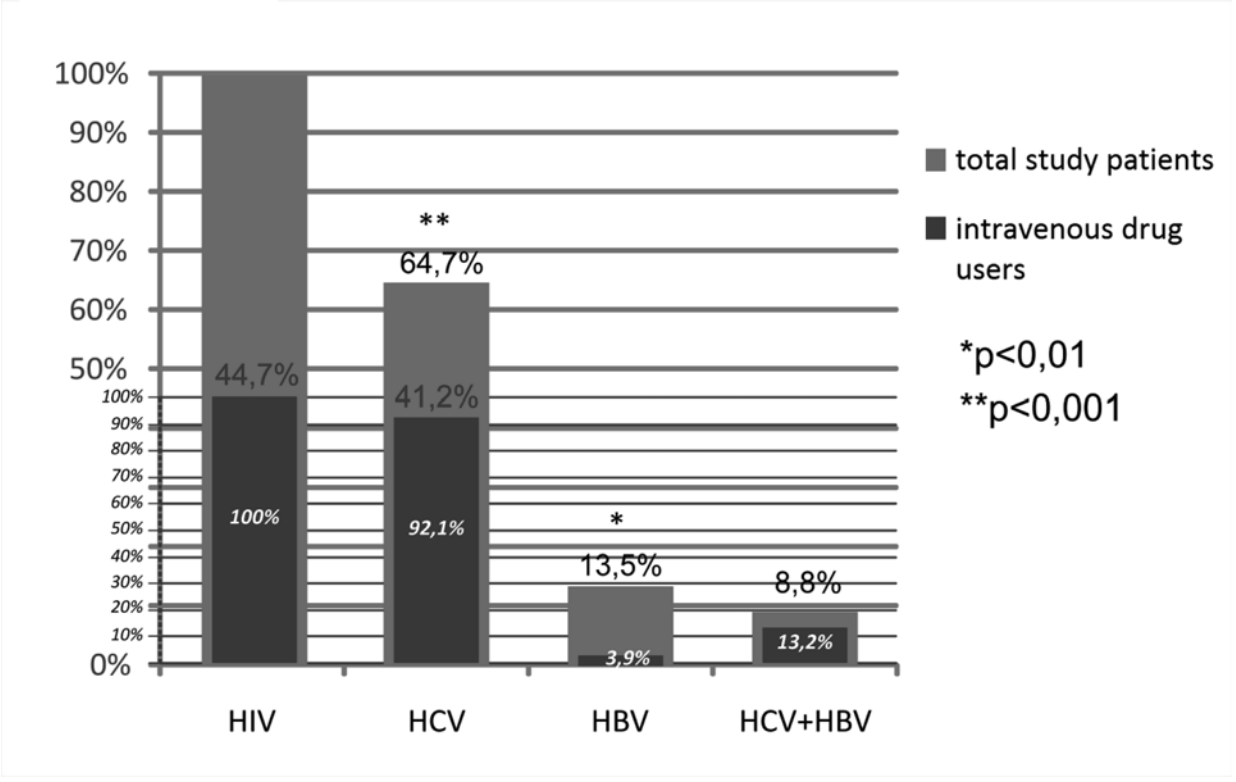


Figure 3: Coinfections with hepatotropic viruses among HIV-positive patients, total study patients and intravenous drug users



HCV+; HBV+; HCV+HBV (Figure 3). The results: 92.1 % of the intravenous drug users group are HCV+ compared to 64.7% HCV+ patients among the total study patients: there is a statistical significance between the groups by confidence interval 5%. The same result is present by the HBV+ group. About the HCV+HBV group the results are not statistically significant.

CONCLUSIONS

The most common opportunistic infections in our HIV-positive patients were candidiasis (18.8%), pneumocystis pneumonia (8.2%) and tuberculosis (4.7%). The most common coinfection was hepatitis C. Coinfections with hepatotropic viruses correlate with the mechanism of transmission. The intravenous drug users are at a very high risk.

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HEPATITIS A IN PLOVDIV REGION, 2007-2012

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ABSTRACT:
Introduction: Viral hepatitis A is an infectious disease showing one of the highest levels of incidence rate in Bulgaria. In the mid nineties of the previous century several highly effective vaccines for protection against the disease were licensed. Many countries have introduced these vaccines as a routine practice and thus managed to achieve significant decrease in the incidence. This vaccine is not yet included in the immunization calendar of Bulgaria and this is the reason why the morbidity of hepatitis A in Bulgaria has the highest rate compared to other European Union member countries. **The aim** of the study is to conduct a clinical and epidemiological analysis of hepatitis A cases in Plovdiv region for the period 2007-2012.
Materials and methods: A total of 2 427 patients with hepatitis A were studied in the research. In all cases the diagnosis was confirmed with a positive test for anti-HAV IgM. The complex method of epidemiological research, routine clinical, biochemical and laboratory methods have been used. **Results:** For the period 2007-2012, 2 427 confirmed cases of hepatitis A occurred. The incidence rate during the years ranged from 2.98/100 000 to 173.58/100 000. The average yearly incidence for the entire period was 57.77/100 000, the lowest rate was in 2009, and the highest – in 2011. The highest percentage of cases and the highest incidence were observed for the age group 5-9 years (297.24/100 000). During the research period 17 epidemic outbreaks were registered, respectively: 2007 – 5; 2010 – 4; 2011 – 4 and 2012 - 4. Clinically the course of the disease remained predominantly mild or moderate. Relative increase of cases in a moderate course was established as compared to these in the mild forms. **Conclusions:** 1.Viral hepatitis A maintains its epidemic endemic occurrence in Bulgaria. 2. The incidence of hepatitis A in Plovdiv region (as well as in Bulgaria) remains still at a high level. 3. We find appropriate the introduction of a routine immunization against the disease.

Key words: hepatitis A, incidence rate, immune prophylaxis

Introduction: Hepatitis A is a contagious disease holding one of the highest levels of morbidity in Bulgaria (1, 2). In the mid-90s of the last century several highly effective vaccines against the disease were licensed. A number of countries (Australia, China, Belarus, Italy, Spain, USA, Israel, Bahrain) introduced them as a routine, which led to significant reductions in morbidity (3-6). This vaccine is not yet included in the immunization calendar in Bulgaria which is why the incidence of HAV in this country is the highest compared to other countries of the European Union (7). **The aim** of this study is to perform clinical-epidemiological analysis of the cases of hepatitis A in Plovdiv region for the period 2007-2012.

Materials and methods: The study included 2 427 patients with hepatitis A from Plovdiv region, Bulgaria. Plovdiv region is the second biggest region of Bulgaria with a population of about 700 000 (the population of Bulgaria is about 7 million people). The diagnosis was confirmed with a positive test for anti-HAV IgM in all of the studied cases. A complex method of epidemiological research, routine clinical, biochemical and laboratory methods have been used. The incidence rate for the whole population and by age groups has been examined.

Results:
For the period (2007-2012) 2427 proven cases of HAV have occurred. Incidence over the years ranged from 2.98/100, 000 to 173.58/100 000 (Table 1). The average annual incidence for the entire period is 57.77/100, 000, it had the lowest level in 2009, and the highest - in 2011.
The largest proportion of cases and the highest incidence occurred in the **age group** 5-9 years (297.24/100 000) – Table 2.
The analysis of seasonality (Diagram 1) showed that the incidence of HAV in Plovdiv region had its highest levels in January and February. In 2007, the reason for this was the „tail“ of the 2006 epidemic which affected more than 3000 people. The remaining years were characterized by increase in morbidity still in the last months of the preceding year (October to December) with a peak in January-February.
During the study period 17 outbreaks were recorded, respectively: 2007-5, 2010-4, 2011-4 and 2012-4.

Clinical course of viral hepatitis A for surveyed period
An analysis of 400 patients during the period done in accordance with generally accepted observations gave us grounds to conclude rapid improvement in subjective health in patients since the onset of jaundice. However, in 249 (62.5%) of the patients in the first 1-3 days, some symptoms were observed that we present in Table. 3, compared with those of foreign authors from the 70‘s, 80‘s and 90‘s of the XX century.
Our concepts on severity of HAV manifestation is that the most important indicator of HAV severity is the degree of intoxication and it is determined by the existence of at least two factors:

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Table 1: Incidence rate (per 100 000) of hepatitis A in Plovdiv region and Bulgaria for the period 2007-2012

		2007	2008	2009	2010	2011	2012	average annually
Plovdiv region	N: of cases	429	30	21	285	1218	383	404
	Incidence	69,40	4,25	2,98	40,20	173,58	56,25	57,77
Bulgaria	N: of cases	2800	908	1064	2350	5888	4919	2988
	incidence	36,28	11,88	15,99	31,07	74,46	67,13	38,55

Table 2: Incidence rate by age (per 100 000) of hepatitis A in Plovdiv region, 2007-2012

	2007	2008	2009	2010	2011	2012	average annually
0-4	105,49	9,80	2,4	232,62	442,18	74,86	144,56
5-9	291,42	6,76	6,6	221,25	1099,76	157,64	297,24
10-19	154,17	4,71	2,96	64,17	198,07	127,78	91,93
20-29	84,08	5,47	1,9	16,56	142,29	76,67	54,49
30-39	64,84	2,72	8,41	16,03	147,71	64,26	50,66
40-49	48,9	6,19	4,17	13,37	123,34	61,62	42,93
50-59	26,78	2,08	1,02	9,25	77,57	23,96	23,44
>60	5,8	1,17	0	3,1	15,49	5,07	5,1
Total	69,40	4,25	2,98	40,20	173,58	56,25	57,77

Diagram 1: Relative share of the cases with hepatitis A by months

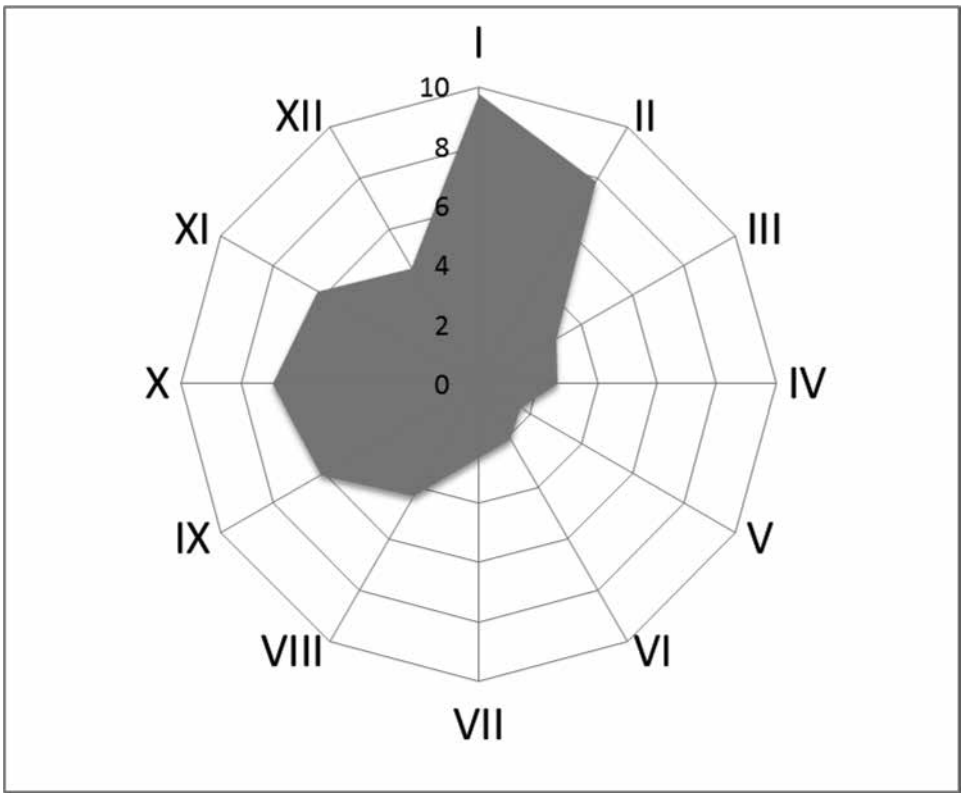

Table 3: "Score" system for determining the clinical forms

Symptoms	Our 2007-2012	Bluger	Gust и Feinstone	Ilieva P. (adults)	Ilieva P. (children)
adynamia	82.85%	82.0%	77.0%	82,16%	79,27%
decreased appetite	74.7%	75.4%	77.0%	87,36%	79,2%
nausea	49.8%	50.0%	90.0%	89,03%	84,84
stomach ache	45.78%	46.7%	87.0%		12,6%
fever	33.7%	42.6%	37.0%	58,8%	64,0%
headache	13.25%	12.3%	75.0%	18,96%	21,8%
vomitus	12.5%	13.1%	7.0%		
catarrh of URT	11.65%	4.9%	71.0%	16,66%	
diarrhea	5.6%	5.7%	10.0%		
itching	2.4%	18.0%	25.0%		
			42.0%		

Table 4: Clinical forms

Sex/number	Light form	Moderate form	Severe form
Male/110	21	72	17
Female/120	29	78	13
Total/230	50/21.75%	150/65.21%	30/13.04%

1. The tactic for treatment depends on the degree of expression of this symptom.
2. The development of acute liver failure manifests itself with all signs of increased toxicity. Hyperbilirubinaemia is regarded as an additional criterion.

According to the intoxication, criteria upon Bluger for the clinical forms of HAV, adopted by us too, are: light, moderate and severe.

We expanded the criteria and specified the indices trying to be as objective as possible introducing a „score" system which gives a point for each sign – Table 4. The maximum number of points of the "score" system is 24. Cases of up to 8 points are defined as mild, from 9-14 as moderate, and from 15 to 24 – as severe.

Moderate forms of the disease were prevalent on average. Transaminase activity (ALT) in them reached 4000 IU/L and at the height of the disease ALT level remained at a level above 700 IU/L. Upon hospital discharge a significant decrease was measured without achieving full normalization.

Discussion: Hepatitis A is still a widespread disease in some countries and retains its social significance. Globally there are an estimated 1.4 million cases every year. Hepatitis A is associated with poor sanitation and lack of safe water. Epidemics can be explosive in growth and cause significant economic losses. Improved sanitation and the hepatitis A vaccine are the most effective ways to combat the disease (8). Risk groups for hepatitis A are also health care workers and these from daycare centers (9, 10). The implementation of the vaccine may eliminate r**egional, ethnic, and racial differences in the incidence of the disease.** The incidence of hepatitis A in adults in USA states with immunization has decreased significantly suggesting a strong herd-immunity effect associated with immunization (11).

Conclusions: 1. In Bulgaria, hepatitis A retains its endemic-epidemic spread. 2. Incidence of hepatitis A in Plovdiv region (and the whole country) remains at a high level. 3. We consider it appropriate to introduce routine vaccination against the disease.

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