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IN SILICO DEVELOPMENT OF HIGH-RESOLUTION MLVA TYPING SCHEME FOR *ENTEROCOCCUS FAECIUM*

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ABSTRACT

Emergence of enterococci as nosocomial pathogens is frequently associated with hospital outbreaks. Vancomycin resistance is especially perturbing as it limits the possible therapeutic options. Based on vast *in silico* analysis, we introduce a new multiple-locus variable-number tandem repeat analysis (MLVA) scheme for genotyping of *Enterococcus faecium* isolates, parameters of which are comparable to these of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The scheme was tested *in silico* on all available at this time *E. faecium* genomes in NCBI Genbank. Searching for suitable variable number of tandem repeats (VNTR) loci was conducted with a set of free access applications. Ten VNTR loci were selected according to their polymorphic structure and stability using the Primer-BLAST utility of NCBI. Primers were designed to be compatible in a multiplex reaction and the method was adapted for high resolution separation techniques. As a result, a total of 60 MLVA profiles and 35 MLST profiles were generated from the analysis of 114 sequenced genomes. Minimum spanning trees were created for both MLVA and MLST in order to analyze the genetic relatedness between isolates. Hunter Gaston discriminatory index was measured for both MLVA (0,959) and MLST (0,926). Typeability was also measured for both methods (MLVA – 85.9%; MLST – 89.4%). These results suggest that the new MLVA scheme is suitable for epidemiological studies of hospital-adapted *E. faecium* isolates.

KEYWORDS:

Enterococcus faecium, MLVA, MLST, VNTR

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ABBREVIATIONS

VRE – vancomycin-resistant enterococci
PFGE – pulsed-field gel electrophoresis
MLST – multilocus sequence typing
TRs – tandem repeats
VNTR – variable number of tandem repeats
MLVA – multiple-locus variable-number tandem repeat analysis
MT – MLVA type
TRF – Tandem Repeats Finder
TRDB – Tandem Repeats Database
WGS – whole-genome sequencing
ST – sequence type
HGDI – Hunter Gaston discriminatory index
CI – confidence interval
MST – minimum spanning tree

INTRODUCTION

The genus *Enterococcus* consists of gram positive, facultatively anaerobic cocci. They are usually found in human and animal intestinal tract as part of the normal microflora. However, they also appear as important nosocomial pathogens causing infections in immunocompromised patients. *Enterococcus faecalis* and *Enterococcus faecium* are the two most common pathogens among the enterococcal species (1). Enterococci have intrinsic resistance to penicillin and cephalosporins due to expression of low affinity penicillin-binding proteins (2). *E. faecium* and *E. faecalis* have innate resistance to aminoglycosides due to reduced membrane permeability (3, 4). *E. faecium* possess an enzyme called aminoglycoside 6'acetyltransferase (AAC(6')-Ii) which confers resistance to tobramycin and kanamycin (5). Another *E. faecium*-specific enzyme is the *efmM*-encoded 16S rRNA m^5C methyltransferase which confers resistance to dibekacin, tobramycin and kanamycin (6).

The antimicrobial agents to which enterococci may possess resistance include macrolides, tetracyclines, lincosamides, chloramphenicol, vancomycin, quinolones, linezolid, quinupristin/dalfopristin. Vancomycin resistance is especially perturbing (1).

Vancomycin-resistant enterococci (VRE) are reported for the first time in the late 1980s in USA and Europe. Vancomycin resistance is usually associated with the acquisition of *vanA* or *vanB* gene cluster (7, 8). The frequency of this type of resistance is much higher for *E. faecium* than for any other enterococci. A correlation between the higher rates of acquisition of vancomycin resistance and the higher rates of infections caused by *E. faecium* was observed (9).

Most enterococci usually do not have the classic virulence factors, which is the reason they cause infections primarily in seriously ill or immunocompromised patients.

In a hospital setting enterococci can be transmitted from patients to other patients or even to the personnel. Enterococci are not necessarily causing infections. They can colonise the intestinal tract of a healthy person making him a carrier who can further transmit them. If no action is taken, this can become a prerequisite for an outbreak. Nosocomial enterococcal pathogens can be isolated from personnel's gloves, medical equipment, different surfaces and passively contaminated environment with feces and urine. Some of the risk factors for acquisition of nosocomial enterococcal infections are: severe or chronic illness; prolonged hospital stay; transplantations; urinary or vascular catheters; residence in the intensive care unit; renal insufficiency; post-antibiotic therapy; neutropenia (1).

The emergence of enterococci as a cause of hospital outbreaks defines the need for reliable and accessible method for genotyping which can easily discriminate between different strains and between pathogen and wild strains.

Pulsed-field gel electrophoresis (PFGE) is considered as the "gold standard" in molecular typing of clinically relevant isolates. This is due to its high discrimination ability, typeability and reliable epidemiological concordance it provides. It is an inexpensive method with good intra- and inter-laboratory reproducibility. There are standardised protocols and international fingerprinting database (Pulsenet) which gives the opportunity to detect and monitor the dissemination of pathogen strains among different countries. However, this method has its disadvantages. It is technically demanding and requires qualified personnel. Furthermore, fragments with lengths differing by 5% are not discriminable. Also its portability is still an issue (10).

Multilocus sequence typing (MLST) is another method for genotyping of different isolates. It is based on sequencing and its discriminatory power is comparable to that of PFGE. There is an international standardised nomenclature and free access databases (http://pubmlst.org and www.mlst.net) which contain information about the allele sequences and sequence types (ST). Thus, the results generated by this method are less likely to be ambiguous. These databases also provide an online tool (eBURST) for determining genetic relatedness between isolates. Unfortunately, this method also has its disadvantages. It is expensive, time-consuming and labor-intensive (11). Along with these, a lower discriminatory power regarding some isolates is observed which makes this method non-applicable for routine screening and monitoring of hospital outbreaks.

In 2004, J.Top et al. published detailed multiple-locus variable-number tandem repeat analysis scheme (MLVA-6) for genotyping of *E. faecium*, which was developed and tested on total of 392 isolates, from which 126 with clinical origin (blood, urine, wounds), 68 from clinical studies, 111 isolates from 25 documented hospital outbreaks, 17 from society-derived infections, and 70 isolates from different environments, animals and foods.

Searching for tandem repeats (TRs) in the genomic sequences, that were not yet completed due to the lack of reliable whole-genome sequencing (WGS) technologies at the time, is accomplished with specialised software called Tandem Repeats Finder (TRF) (12). Initially the scheme consisted of 10 loci which were selected based on the following criteria: TRs minimum length – 20 bases; conservation of TRs – (>90%); location of TRs – in non-coding regions.

Later, 4 of the loci are excluded, leaving the scheme with only 6 loci (13).

The aim of this study is to introduce a new and reliable MLVA scheme (MLVA10) for typing of *E. faecium* with parameters comparable to these of PFGE and MLST.

MATERIALS AND METHODS

Five referent genomes were randomly selected and downloaded from NCBI GenBank: 1. *E. faecium DO* (CP003583.1); *2. E. faecium Aus0004* (CP003351.1); *3. E. faecium ATCC 8459 = NRRL B-2354* (CP004063.1); *4. E. faecium Aus0085* (CP006620.1); 5. *E. faecium AUSMDU00004055* (CP027506.1). Searching for suitable loci in these genomes was conducted with a set of specialised softwares.

Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) was used first. The search of TRs was performed according to the following criteria: pattern of TRs – 1 to 100 bases; number of TRs – minimum 2; alignment parameters (match, mismatch, indel) – (2,7,7). The referent genomes were uploaded for analysis in the form of FASTA files. Subsequently we came across another web application – Polloc V (14) which was able to search for TRs in 3 genomes simultaneously. It also gave an opportunity for sequence comparison which makes selection of polymorphic loci easier. Searching criteria used were as follows: Region length – unlimited; Unit length – 3-60 bp; Copy number of TRs – 2 or more; Alignment parameters – match/mismatch/indel (-2;-7;-7); Similarity percentage – >90%.

Another web application described in publication of Lee et al. („pSTR Finder: a rapid method to discover polymorphic short tandem repeat markers from whole-genome sequences") was tested. pSTR Finder is searching for TRs with lengths 1-10bp (15). The search criteria were as follows: Alignment Score (match, mismatch, indel) – 2,7,7; Min Align Score – 50; Max Repeat Unit – 10; 5' & 3' Flanking Sequence Size – 15.

Tandem Repeats Database, described in „TRDB— The Tandem Repeats Database", was also used. Concatenation of the referent genomes was done and the search of VNTR loci was performed according to the following criteria: Alignment Parameters (match, mismatch, indels) - 2, 7, 7; Minimum Alignment Score To Report Repeat – 50.
Another software for genome processing and analysis, developed by Biomatters, used in our study, was Geneious Prime.
Primers were designed according to all criteria for primer design for each locus with Geneious Prime and Oligo7 (Table 1).
Analysis for determination and confirmation of the polymorphic structure of the VNTR was performed on all the available at this time sequenced genomes in NCBI Genbank using the web application of NCBI – Primer-BLAST.

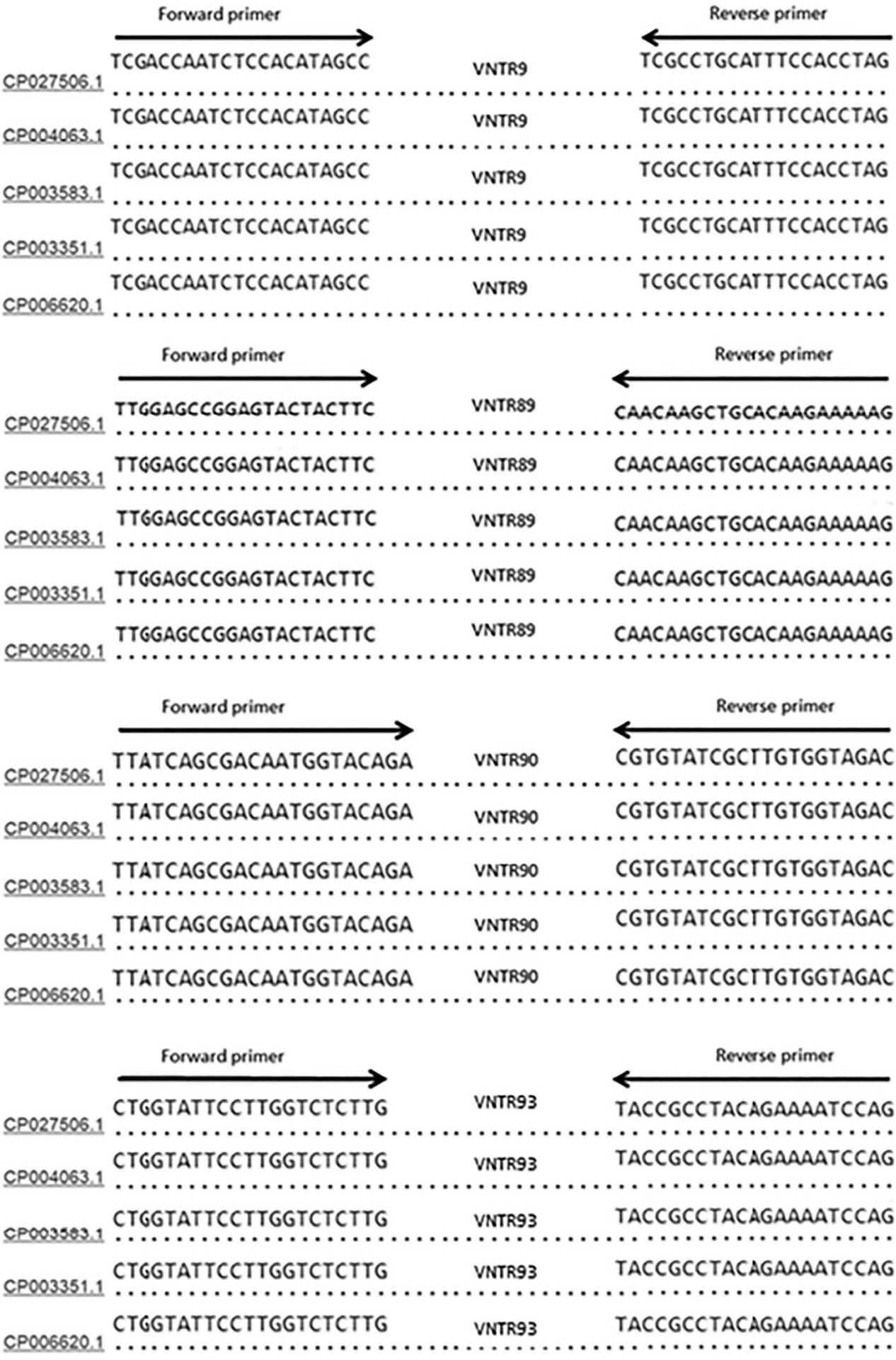
RESULTS AND DISCUSSION

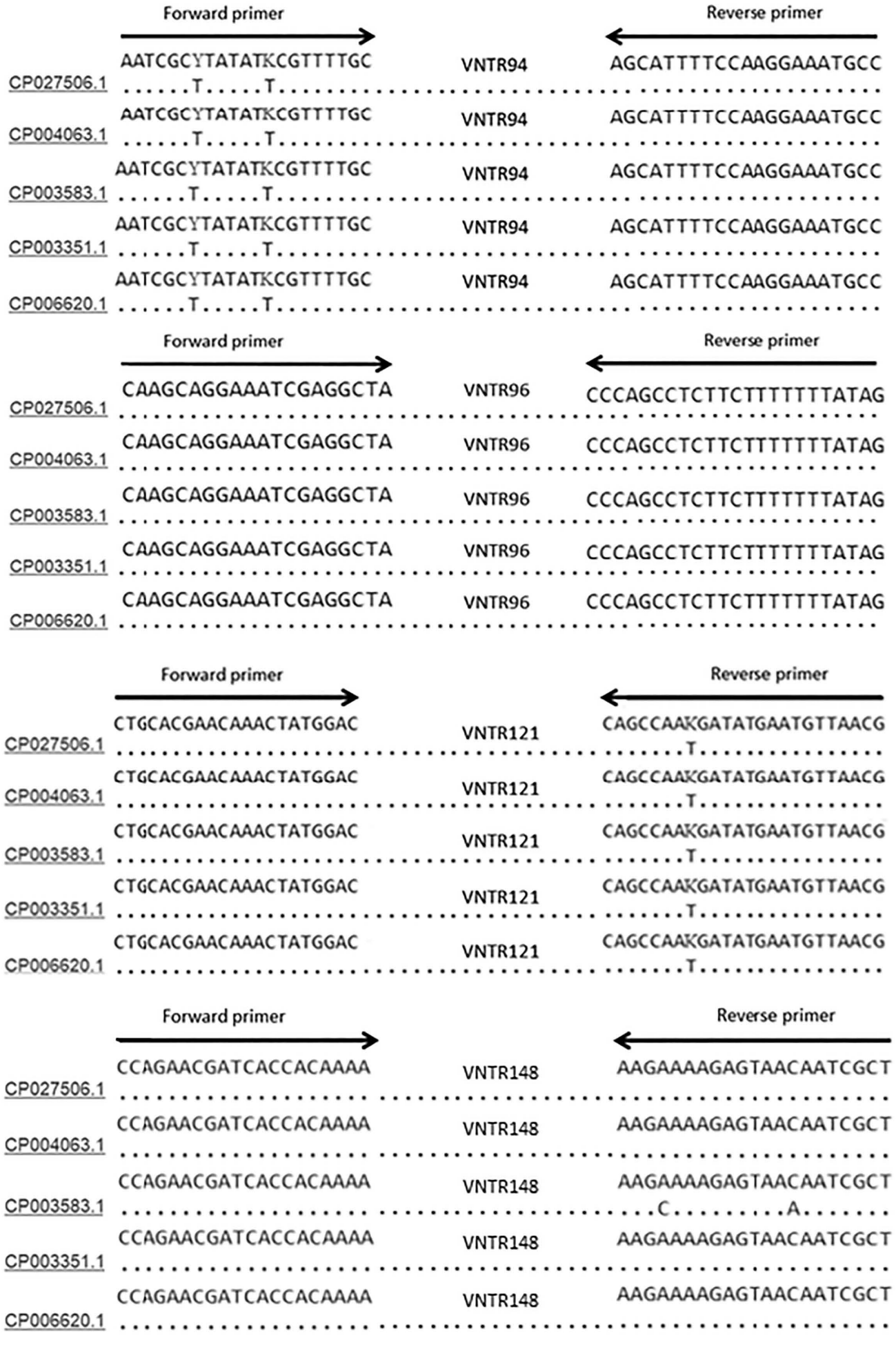
We selected 9 original loci based on their variability and stability, and designed primers for each of them. VNTR-9 from MLVA6 was included, but with new original primers and different amplicon lengths in order to use it in multiplex PCR. MLVA10 was adapted for high resolution separation techniques like capillary electrophoresis and chip-based technologies, which allow separation of fragments differing by ~5 bp.

Table 1. Primer sequences for each VNTR locus.

VNTR	Primers
VNTR9	Forward: TCGACCAATCTCCACATAGCC
	Reverse: TCGCCTGCATTTCCACCTAG
VNTR89	Forward: TTGGAGCCGGAGTACTACTTC
	Reverse: CAACAAGCTGCACAAGAAAAAG
VNTR90	Forward:TTATCAGCGACAATGGTACAGA
	Reverse: CGTGTATCGCTTGTGGTAGAC
VNTR93	Forward: CTGGTATTCCTTGGTCTCTTG
	Reverse: TACCGCCTACAGAAAATCCAG
VNTR94	Forward: AATCGCKTATATYCGTTTTGC
	Reverse: AGCATTTTCCAAGGAAATGCC
VNTR96	Forward: CAAGCAGGAAATCGAGGCTA
	Reverse: CCCAGCCTCTTCTTTTTTTATAG
VNTR121	Forward: CTGCACGAACAAACTATGGAC
	Reverse: CAGCCAAKGATATGAATGTTAACG
VNTR148	Forward: CCAGAACGATCACCACAAAA
	Reverse: AAGAAAAGAGTAACAATCGCT
VNTR152	Forward: CATCGGCTGGAATATTTCGTC
	Reverse: GCAGCTGAAAGTCTAGTTGTC
VNTR153	Forward: TTTTACGTCGTTCTGCTCT
	Reverse: AACGACGGACCTTGAAGC

It was inconvenient that TRF works only with 1 genome at a time and the data it provides cannot be filtered, which makes the process difficult and time consuming. It was also not possible to differentiate between polymorphic and non-polymorphic loci which was essential for the purposes of this study.
The web application "Polloc V", developed by Luis-Miguel Rodriguez-R and Ralf Koebnik, which was used, contains integrated TRF algorithm (12). The role of Polloc V is to arrange the loci in groups based on the rate of similarity and their flanking regions. Then the groups can be visualized with a software for genomic processing like Geneious (16).
pSTR Finder (pSTR) is developed to analyze multiple samples of genome sequences for the presence of STRs. That application is also TRF based and it is available for non-commercial use (15). The user must provide sequences as FASTA files and use one of them as referent. Additionally, the length of the 5′ and 3′ flanking regions can be specified which is useful for subsequent sequence comparison analyzes. Unfortunately, we did not achieve any success with this application. Concatenating the referent genomes was necessary for the following clustering and analysis of the potential polymorphic loci. However, processing of concatenated genomes turned out to be beyond the capabilities of this software.
This issue was resolved by using TRDB. In 1999 Benson developed TRF – the algorithm which is now the basis for most of the available softwares for tandem repeats searching. TRDB consists of 2 parts: 1. public information about TRs, their nature and location in a set of different genomes; 2. the researchers work table where the user can chose between a set of different tools for genome processing.
Initially, TRDB contain information about 22 genomes. Later, more genomes are added as sequence information became available. TRDB gives the user an opportunity to search and filter certain TRs; to cluster TRs based on sequence identity; to predict polymorphic loci based on basic mutation patterns; to construct primers with interface based on Primer3 application (17); to download and save information in different formats; to visualize dynamic histograms and scatterplots of TRs characteristics; to align and visualize TRs in a separate browser (18).
The combination of two Geneious plugins - Mauve (19) and Phobos (20) played a key role in the detection of VNTRs. Mauve was used to make multiple alignment of the five referent genomes, and the other plugin – Phobos, was used to search for VNTRs directly in the aligned genomes, which facilitated the differentiation between polymorphic and non-polymorphic loci. Primers and their templates in each referent genome are shown on Figure 1.


Forward primer
Reverse primer
CP027506.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP004063.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP003583.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP003351.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP006620.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
Forward primer
Reverse primer
CP027506.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP004063.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP003583.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP003351.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP006620.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
Forward primer
Reverse primer
CP027506.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP004063.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP003583.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP003351.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP006620.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
Forward primer
Reverse primer
CP027506.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG
CP004063.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG
CP003583.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG
CP003351.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG
CP006620.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG


Forward primer
Reverse primer
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VNTR94
AGCATTTTCCAAGGAAATGCC
CP027506.1
CP004063.1
CP003583.1
CP003351.1
CP006620.1
Forward primer
Reverse primer
CAAGCAGGAAATCGAGGCTA
VNTR96
CCCAGCCTCTTCTTTTTTTATAG
CP027506.1
CP004063.1
CP003583.1
CP003351.1
CP006620.1
Forward primer
Reverse primer
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VNTR121
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CP003583.1
CP003351.1
CP006620.1
Forward primer
Reverse primer
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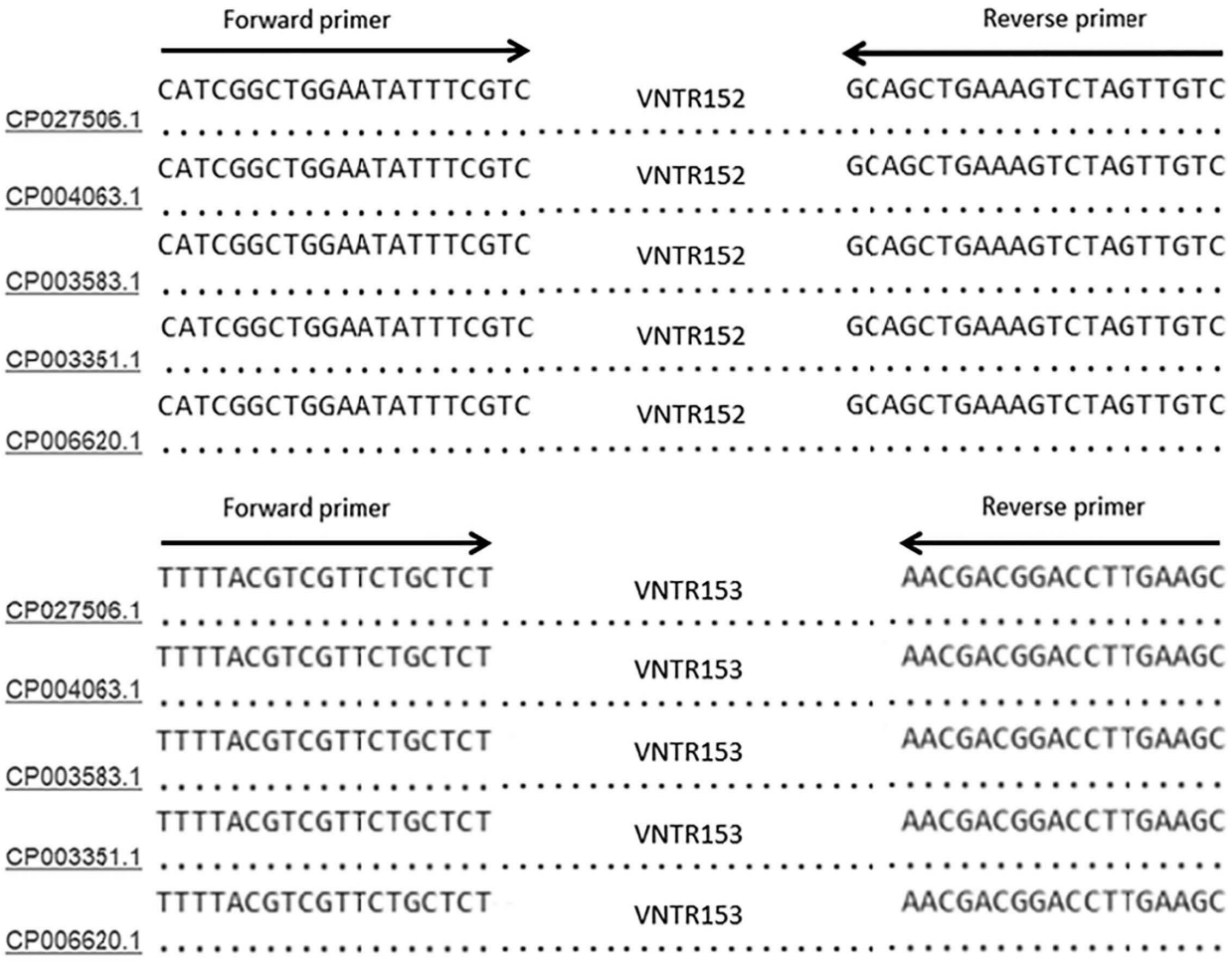

Figure 1. Used primers and the corresponding templates. Red letters correspond to ambiguous nucleotide bases (K=T/G; Y=C/T). Dots correspond to complete complementarity. At the left accession numbers of the referent genomes in NCBI Genbank are shown.

Hunter Gaston discriminatory index (HGDI) was measured for every single locus used (Table 2).

Table 2. HGDI for every locus of the new scheme.

VNTR	TR length (bp)	No. of Alleles	Fragment Length min/max (bp)	HGDI
VNTR9	121	0-2	666-911	0,509
VNTR89	33-36	2-7	298-475	0,479
VNTR90	57	6-18	331-1048	0,707
VNTR93	24	2-13	201-465	0,647
VNTR94	12	2-9	164-248	0,225
VNTR96	10	1-2,5	129-148	0,34
VNTR121	12	1-2	128-140	0,282
VNTR148	5	1-2	90-95	0,119
VNTR152	6	1-2	106-112	0,45
VNTR153	12	1-2	56-68	0,310

A total of 59 MLVA types (MTs) were generated from the *in silico* analysis of 114 isolates.

Some of the new loci showed lower discriminatory index than others. However, they were essential for discrimination between some initially indistinguishable sequence types. ST21, ST121 and ST456 were placed in one MLVA type. The addition of VNTR148 in the scheme resulted in dividing ST121 from ST21 and ST456. MLVA10 was unable to divide several variants of ST16 and ST17. ST796 and ST192 were also indistinguishable and the addition of VNTR153 solved this issue. ST5 was successfully separated from ST6 and ST1147, and also ST16 from ST282 by VNTR96. Another indistinguishable pair was ST412 and ST656. Two variants of ST412 shared the same MLVA profile with ST656. One of them was successfully separated by VNTR90, but the other remained bound to ST656. ST398 was separated from ST598 by VNTR148 and VNTR153. One variant of ST203 also remained indistinguishable from ST266.

Minimum spanning tree (MST) based on the in silico analysis was created for MLVA profiles. Eight of all the MLVA profiles contained 2 MLST profiles each (Fig.2). The opposite was also observed. Some MLST profiles (e.g. ST117, ST203) were divided and fell into different MLVA types, which is probably due to their high variability. MLVA10 failed to type 16 isolates, from which 8 were untypeable by both methods. The other 8 were successfully typed by MLST. The inability of MLVA10 to genotype some isolates is probably due to mutational events in some of the target loci

A total of 35 sequence types were acquired from the analysis in PubMLST (Public databases for molecular typing and microbial genome diversity) (21). Twelve of all the 114 isolates were not typeable by MLST. Four of these 12 were successfully typed by MLVA10. The rest 8 isolates were not typeable by both methods as mentioned above.

In order to get clearer and complete picture we analyzed the reverse option too. Minimum spanning tree based on MLST profiles was also created (Fig. 3). Sequence types which are divided into 2 or more MLVA types are pointed out by arrows. ST203, ST117 and ST17 turned out to be highly variable. ST203 is broken down into 7 MLVA types, ST117 – 10 MLVA types, ST17-7 MLVA types. These results show that MLVA10 is highly discriminatory against the variations in some of the sequence types.

With the current set of VNTR loci, MLVA10 was unable to discriminate between the following STs: ST21 and ST456; ST16 and ST17; ST6 and ST1147; ST117 and ST203; ST412 and ST656; ST117 and ST78; ST203 and ST266.

MLVA6 issues were similar – inability for discrimination of the following STs is observed: ST18, ST282, ST17, ST280 - MLVA type 1; ST65, ST16 - MLVA type 5; ST18, ST202 - MLVA type 7; ST78, ST117 - MLVA type 12; ST78, ST192, ST203, ST283 - MLVA type 159; ST279, ST314, ST307 - MLVA type 231. Table 3 summarizes the problems of the both schemes.

Table 3. Problems of MLVA6 and MLVA10

	MLVA type	Sequence type
MLVA6	1	ST18; ST282; ST17; ST280
	5	ST65; ST16
	7	ST18; ST203
	12	ST78; ST117
	159	ST78; ST192; ST203; ST283
	231	ST279; ST314; ST307
MLVA10	31	ST6; ST1147
	36	ST117; ST203
	38	ST21; ST456
	39	ST656; ST412
	41	ST203; ST266
	46	ST117; ST78
	50	ST16; ST17

ST78 and ST117 remained an issue for both the modified and the original scheme.

Discriminatory power of MLVA10 and MLST based on the *in silico* analysis was measured with the web app Comparing partitions (http://www.comparingpartitions.info/?link=Home). HGDI value for MLVA10 was 0.960 with confidence intervals 95% (CI95%) from 0.932 to 0.988. HGDI value for MLST was 0,928 with CI95% from 0.902 to 0.954. In order to get accurate result, isolates untypeable by one or both the methods, were excluded.

Comparison of the typeability between MLVA10 and MLST was also made. MLVA showed 85.9% typeability and MLST – 89.4%. MLVA10 was unable to type 16 of all 114 isolates, while MLST failed to type 12 of 114 isolates.

Some of the untypeable isolates are probably wild strains which are quite divergent and their genome can differ slightly from nosocomial

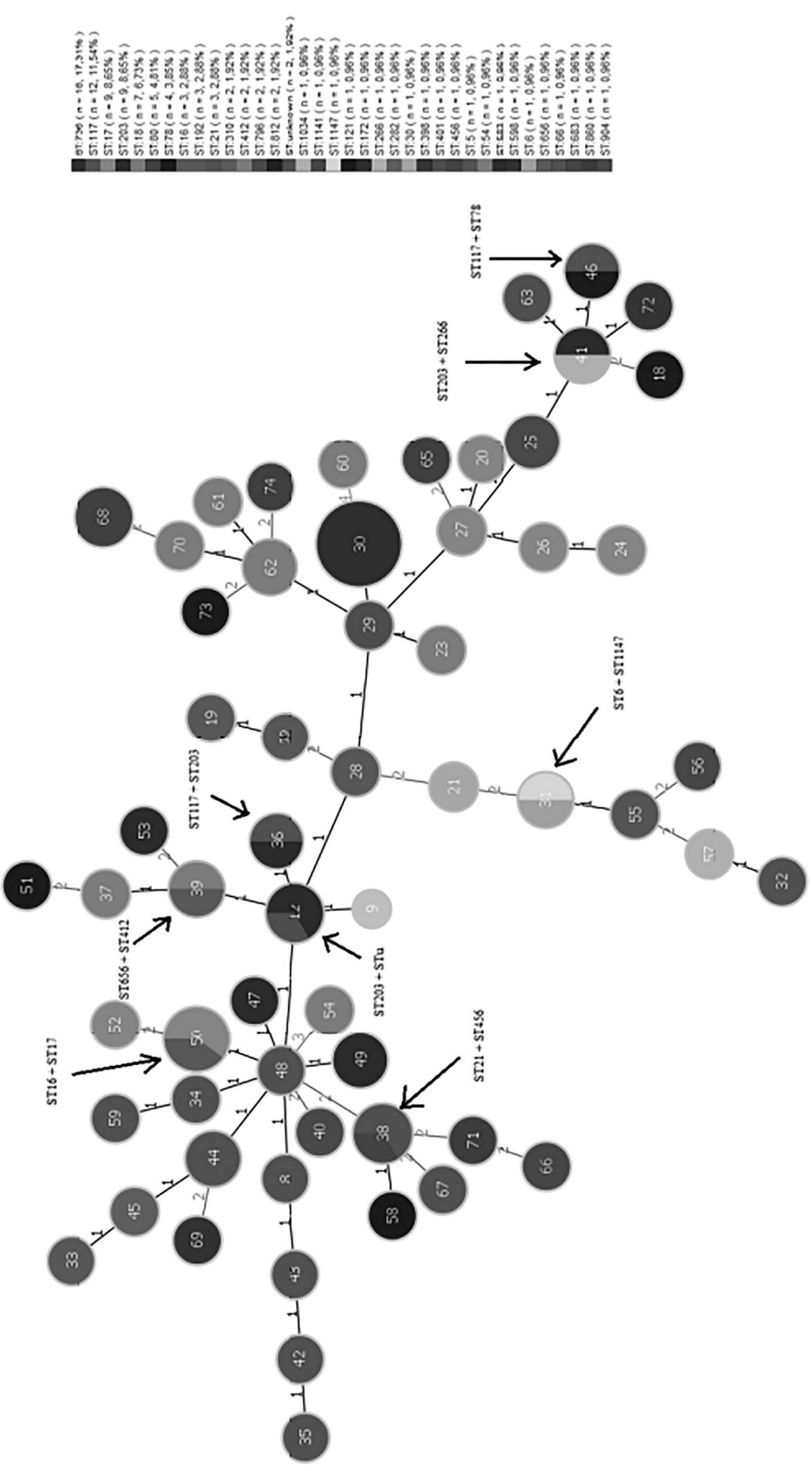

Figure 2. Minimum spanning tree (MST) based on 10 locus MLVA allele profiles of 114 *E. faecium* isolates used in *in silico* analysis. Relationship between different MLVA types is shown as line with an index of the number of loci by which they diverge. The arrows point out the MLVA types which contain more than 1 sequence types. STu corresponds to unknown sequence type. Isolates untypeable by both methods were excluded.

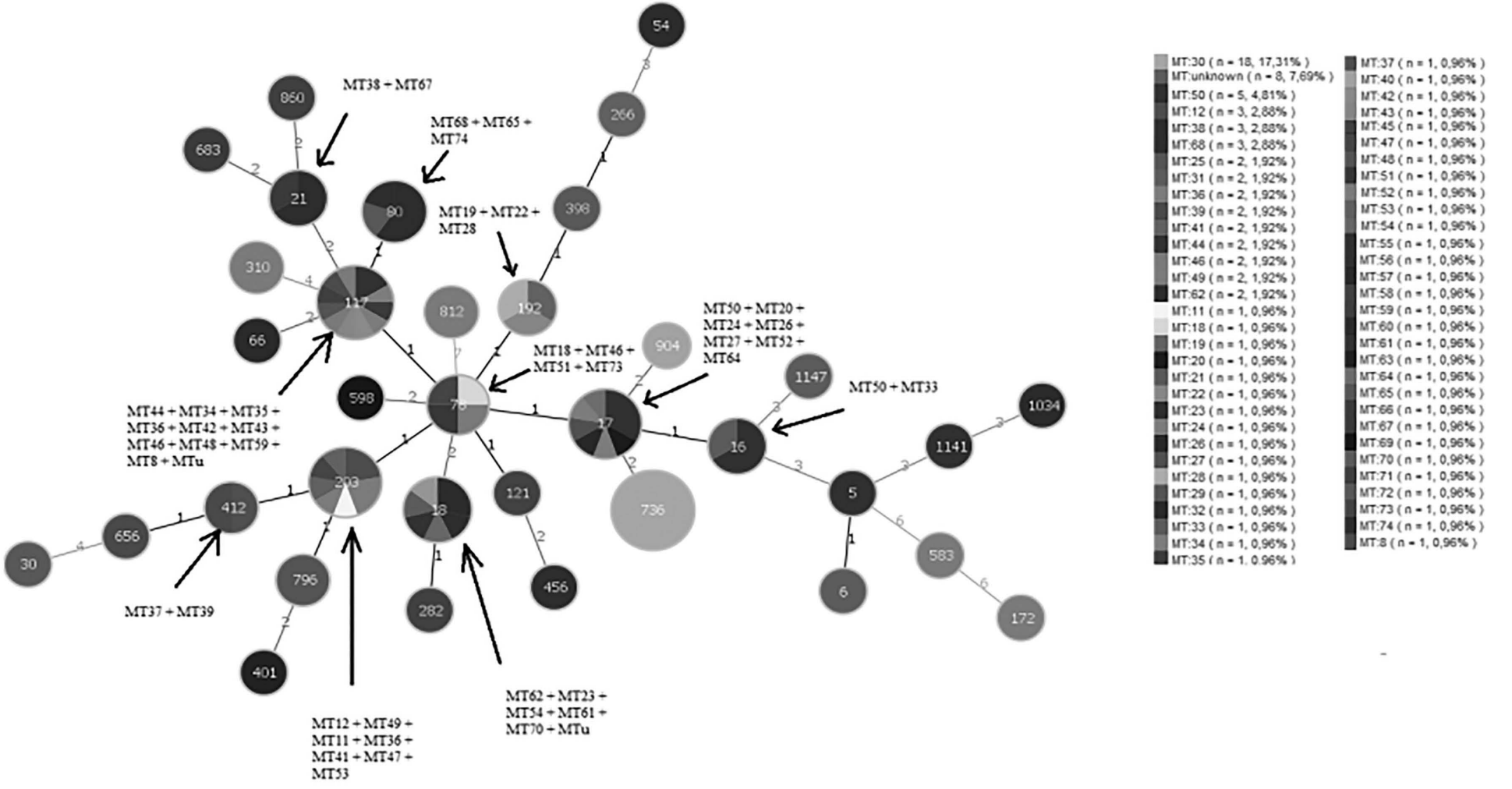

Figure 3. Minimum spanning tree (MST) based on MLST allele profiles of 114 *E. faecium* isolates used in *in silico* analysis. Relationship between different sequence types is shown as line with an index of the number of loci by which they diverge. The arrows point out the sequence types which are divided into more than 1 MLVA types. MTu corresponds to unknown MLVA type. Isolates untypeable by both methods were excluded.

strains. The inability of MLVA10 to genotype some isolates may be due to mutational events in some of the target loci.

CONCLUSIONS

Further examination and addition of more polymorphic loci compatible with the new multiplex PCR scheme can result in better discriminatory power.

MLVA shows higher discriminatory power than MLST and allows the prediction of sequence type with high accuracy.

MLVA10 is suitable for epidemiological studies of hospital-adapted isolates. Also it allows establishing of phylogenetic relatedness between geographically divergent strains.

MLVA can be performed within 4 hours (by capillary electrophoresis) per ~36 isolates, which is significantly faster than MLST and PFGE. It is cost-effective and easy to implement which makes it suitable for routine screening.

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ANTIMICROBIAL SUSCEPTIBILITY OF *S. PNEUMONIAE* STRAINS ISOLATED FROM CHILDREN WITH NASOPHARYNGEAL CARRIAGE

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ABSTRACT

Streptococcus pneumoniae colonises the nasopharynx of children and could cause life-threatening diseases. As a result of the implementation of conjugate vaccines worldwide the spread of vaccine serotypes has decreased. In Bulgaria PCV10 was introduced in 2010 followed by changes in the invasive clones carrying resistance genes. The aim of our study is to determine the serotype distribution and resistance patterns of isolates from children carriers after vaccination. A total of 834 children were tested for *S. pneumoniae* and 21% showed positive culture results. All isolates were genotyped with PCR. We found that 85% of the positive samples are from children attending kindergartens and schools. The most frequent serotypes/serogroups were 6C (20%) and 24B/F (11.5%), followed by 3 (8.6%), 11A/D (8%), 35F (6.9%), 19A (6.3%), 23A (6.3%) and 15A/F (6.3%). The susceptibility to β-lactams was high and there were strains showing intermediate susceptibility to benzylpenicillin. This study found 76 (44%) MDR strains non-susceptible to at least 3 antibiotic classes and the most common resistance pattern was erythromycin-clindamycin-tetracycline.

KEYWORDS:

S. pneumoniae, resistance, pneumococcal serotypes, carriage

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INTRODUCTION

Streptococcus pneumoniae is a bacterial agent that causes severe life-threatening diseases among children such as bacteremic pneumonia, sepsis and meningitis (1, 2). Asymptomatic carriage of pneumococci is known to be a prerequisite for non-invasive and invasive pneumococcal disease (3). Children under 5 years are considered the major reservoir of infection since they are readily colonised by *S. pneumoniae* (2). Pneumococcal infections are a leading cause of hospitalisations and require common use of antibiotic treatment. Antimicrobial resistance among pneumococcal serotypes poses a serious clinical issue worldwide because it decreases the effectiveness of treatment and therefore increases the mortality risk (4).

Pneumococcal disease is successfully prevented through national programs that include mandatory pneumococcal conjugate vaccines (PCVs) for children. Vaccination of the vulnerable parts of the community prevents infections and spread of vaccine serotypes which indirectly reduces the development of resistance (5). In Bulgaria the 10-valent vaccine (PCV10) was introduced in the National Immunisation Calendar in 2010 and applied with first dose scheduled at 2 months and a booster dose at 12 months of age (6). PCV10 includes the polysaccharide of 10 serotypes (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F) conjugated with protein to induce immune response at early age (7). Pneumococcal serotypes are distinguished by the polysaccharide capsules which are genetically determined from the *cps* locus (8). The expression of a specific capsule enables the survival of pneumococci outside the host and is related to the invasive potential of different serotypes (9). Since the introduction of PCV10 in Bulgaria vaccine serotypes are not found among meningitis cases of vaccinated children that shows the effectiveness of the prevention strategy (10, 11). However, the non-vaccine serotypes are becoming a severe threat in terms of invasive potential. Serotype 3 and 19A are associated with most of the meningitis cases in our

country and these serotypes are also associated with resistance profiles (6, 10, 11).

Monitoring serotype distribution of *S. pneumoniae* and antimicrobial susceptibility patterns in pneumococcal carriers from several European countries shows decrease in antimicrobial tolerance in vaccinated cases compared to non-vaccinated (12). However serotypes not included in the vaccine also pose a problem with low susceptibility to various antimicrobials. Epidemiological research should be conducted in order to facilitate the informed choice of the primary physicians for the most effective antibiotic treatment of pneumococcal infection region-wise (13).

MATERIAL AND METHODS

Sample collection

Nasopharyngeal secretions from 834 healthy children aged 6 months to 8 years were collected and analysed in the period from February 2017 to March 2019. The children mainly resided in their own home or attended children's collectives in Sofia region. The children were vaccinated with at least one dose of PCV10. The transnasal nasopharyngeal samples were collected with flexible, sterile, dry swab in eSwab transport medium (Copan, Italy) and transported within 8 hours to the National Reference Laboratory (NRL) Molecular Microbiology at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia.

Culture and antibiotic susceptibility testing

Samples were processed within 2 hours of arrival in the laboratory and incubated on blood agar plates (Columbia CNA Agar with 5% Sheep Blood) for 24 h at 37°C in an atmosphere with elevated concentration of CO_2. The identification of *S. pneumoniae* was based on evaluation of cultural characteristics of colonies as well as microscopy – alpha-haemolytic, Gram-positive, catalase-negative cocci, sensitive to optochin and bile salts. The susceptibility of the isolates to antibiotic preparations was tested by the Bauer-Kirby disc-diffusion method, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (14). The following antibiotic discs were used: oxacillin (screening), tetracycline, erythromycin, clindamycin, vancomycin, teicoplanin, linezolid, norfloxacin, trimethoprim/sulfamethoxazole. Multidrug-resistant strains (MDR) were determined after detection of non-susceptibility to at least 3 different antibiotic classes: β-lactams (benzylpenicillin), tetracyclines, macrolides, lincosamides, glycopeptides, oxazolidinones or sulfonamides.

Initial screening with 1µg oxacillin disc was performed for the detection of β-lactam resistance. Minimum inhibitory concentration (MIC) for benzylpenicillin was tested for strains with oxacillin zone diameter less than 20 mm. MIC for benzylpenicillin was determined by microdilution in Mueller-Hinton broth + 5% lysed horse blood and 20 mg/L β-NAD (MH-F broth) according to ISO 20776-1 standard. The inoculums contained $5x10^5$ CFU/ml and were incubated in sealed panels (MIKROLATEST® MIC plates) at 35 ± 1°C for 18 ± 2h. MIC was defined as the lowest concentration of an antibiotic that completely inhibited visible growth. The exponential gradient of antibiotic in the broth was a quantitative measurement of its *in vitro* activity. Test plates for MIC determination contained 8 concentrations of benzylpenicillin. The interpretation of the MIC allowed the definition of microorganisms as sensitive, with reduced sensitivity (intermediate, low susceptibility) or resistant, using the criteria for non-meningitis isolates according to EUCAST v9.0.

DNA extraction and typing

DNA from all strains was isolated using 5% Chelex 100 with Proteinase K 20 mg/ml method as previously described (11). The detection of pneumococcal DNA was performed with conventional PCR and every reaction was checked for amplification with an internal control for the gene *cpsA*. The primers used in the reactions are described in CDC protocols grouped in 10 multiplex PCR assays for pneumococcal serotype deduction of 70 serotypes (15).

Statistical analysis

The statistical software used for calculations, including Fisher's exact test or $\chi 2$, was Statistical Package for Social Science (SPSS V22.0). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

For the period 2017-2019 all 834 samples were cultured and a total of 174 isolated strains (21% of all tested samples) were identified as *S. pneumoniae*. All *S. pneumoniae* strains were sensitive to optochin and bile salts. No statistically significant difference was found between males (n=418) and females

(n=416) and culture-positive samples (p = 0.76). This could be explained by the same colonisation density of *S. pneumoniae* for both sexes in different age groups.

Studies of pneumococcal carriage in children worldwide confirm that visiting kindergartens and nurseries is a major risk factor for colonisation. The high risk is explained by "overcrowding" in the rooms of the children's centres, bad hygiene habits of children at those age groups and risk of viral diseases. All of the indicated factors explain the high levels of colonisation reaching up to 90% in some studies (16). In this respect, we found that 85% of the positive samples are from children attending kindergartens and schools. There was a correlation between the high probability of a culture-positive result and children attending kindergartens and schools (p=0.000067). Our findings correspond with data from neighbouring countries such as Turkey, where similar levels of *S. pneumoniae* isolates were found from children carriers – 21.9% (17). In Greece children with a median age of 12 months were examined and overall 33.1% were carriers of *S. pneumoniae*; furthermore, 34.1% of children with positive results attended day centres (18). In Romania 25.25% of the examined children were found to be carriers, whereas the levels among infants were relatively lower – 16.7%, compared to 29.4% among 3- to 5-year-old children (19).

The sensitivity of *S. pneumoniae* strains to the antibiotics tested in the study was as follows: 100% to vancomycin, teicoplanin and linezolid, 96.5% to norfloxacin (screening), 93% to sulfamethoxazole/trimethoprim, 85.6% to oxacillin (screening), 58.6% to tetracycline, 50% to clindamycin, and 49% to erythromycin. MDR strains non-susceptible to at least 3 antibiotic classes were 76 in total – 44% of the isolated strains. All isolated MDR strains were erythromycin-resistant and all clindamycin-resistant strains were also resistant to erythromycin (Table 1). Genotyping defined the distribution of resistant isolates by serotypes shown in Table 1.

Table 1. Distribution of serotypes/serogroups of *S. pneumoniae* by resistance patterns.

Serotype/ serogroup	Number of isolates	PEN	ERY	CLI	TET	SXT	MDR
6C	35	1	30	28	27	4	29
24B/F	20	2	18	18	14	1	16
3	15	1	4	4	2	-	2
11A/D	14	-	5	5	4	-	3
35F	12	-	-	-	1	-	-
19A	11	4	5	5	4	-	5
23A	11	1	8	8	6	1	6
15A/F	11	-	6	6	6	3	6
15B/C	9	-	1	1	2	1	1
23B	5	-	1	1	1	-	-
35B	5	3	4	4	-	-	3
6A	4	-	3	3	1	2	2
18A	4	-	-	-	-	-	-
4	3	-	1	1	1	-	1
10F/C	3	-	-	-	-	-	-
21	3	-	-	-	2	-	-
33F/A	3	-	2	2	1	-	1
10A	2	-	-	-	-	-	-
5	1	-	-	-	-	-	-
17F	1	-	-	-	-	-	-
19F	1	-	-	-	-	-	-
31	1	1	1	1	-	-	1
Total number (%)	174 (100)	13 (7.5)	89 (51)	87 (50)	72 (41.4)	12 (7)	76 (44)

* PEN – benzylpenicillin (penicillin G), ERY – erythromycin, CLI – clindamycin, TET – tetracycline, SXT – sulfamethoxazole/trimethoprim

The sensitivity to β-lactams was tested according to the chart presented in EUCASTv9. The initial screening with oxacillin showed 14.4% non-susceptibility, followed by MIC testing for benzylpenicillin. High-level penicillin resistance was not found in the study, however intermediate susceptibility was determined (MIC50, 0.06 to 2 mg/L) for 13 strains (7.5% of all isolates) (Fig. 1). In the period 1991-1993, before the introduction of PCV10 in Bulgaria, resistance to penicillin in clinical isolates was 24.3%. Moreover, 40% of strains isolated from asymptomatic carriers among children were penicillin-resistant and more than half of all isolates were multidrug-resistant (20). The situation with *S. pneumoniae* resistance appears to be stable in Europe, with few countries reporting increasing or decreasing trends during the period 2015-2018. There are large inter-country variations among European countries, for example, penicillin-non-susceptibility of non-invasive pneumococcal isolates varies from 1.7% in Norway to 83% in Romania. Due to the geographical diversity of the resistance found in *S. pneumoniae* strains which depends on the local antimicrobial policy, there is a need of epidemiological studies in each region (21). A study from 9 European countries including Austria, Belgium, Croatia, France, Hungary, Spain, Sweden, the Netherlands and the United Kingdom, examined nasal swabs from 200 healthy persons older than 4 years (except for UK) with no history of antibiotic therapy or hospitalisation in the previous 3 months. A large variation was found in the serotype distribution among the participating countries, as well as difference in antimicrobial resistance including multidrug resistance. The highest rate of resistance to ceftazidime and penicillin was observed among strains from serotype 14. Serotype 14 was the most frequent serotype showing resistance to penicillin, followed by serotype 19A and 15A. This might be due to differences in the use of antimicrobial agents in the participating countries (12). High rates of resistance to macrolides and penicillin were observed in Romania, especially in serotypes covered by PCV13. Antibiotic resistance rates among nasopharyngeal isolates in Hungary were reported as 22% for erythromycin and 17% for clindamycin and tetracycline; 21% had intermediate resistance to penicillin. Nasopharyngeal isolates in Ukraine showed resistance to ciprofloxacin (100%), trimethoprim/sulfamethoxazole (48%), erythromycin (33%), azithromycin (33%), amoxicillin/clavulanic acid (R and I, 33%), penicillin (20%) and cefuroxime (12%). Between 60% and 80% of isolates from invasive disease cases in Poland were susceptible to penicillin across the different age groups (22).

β-lactam antibiotics are the first choice in the treatment of pneumococcal disease. Other widely used alternatives are macrolides because of the good tolerance and wide range of action (23). The widespread use of macrolides limits their effectiveness (24). For 2017 Bulgaria was ranked among the European countries with the highest rate of macrolide resistance in *S. pneumoniae* strains. For most countries, macrolide non-susceptibility has been more frequent than penicillin non-susceptibility (25). Another class of antibiotics to which pneumococci were reported as resistant are the tetracyclines. A frequent resistotype found among carriers in China was erythromycin-clindamycin-tetracycline that differs from the clinical resistotypes. In our study we found a high level of resistance to erythromycin with the most frequent resistotype in 85.5% of MDR strains being the erythromycin-clindamycin-tetracycline which corresponds to the results observed in China (26).

CONCLUSION

Attending kindergartens and other children's centres was found as the major risk factor for *S. pneumoniae* colonisation. Children asymptomatically carry pneumococci susceptible to β-lactams which could be associated with the estimated high vaccine coverage in Bulgaria. The results show high rates of macrolide resistance among isolates from carriers and co-resistance to lincosamides which corresponds to data reported for invasive pneumococci in our country. MDR strains were represented with a specific resistotype found also in other studies on *S. pneumoniae* isolates from carriers.

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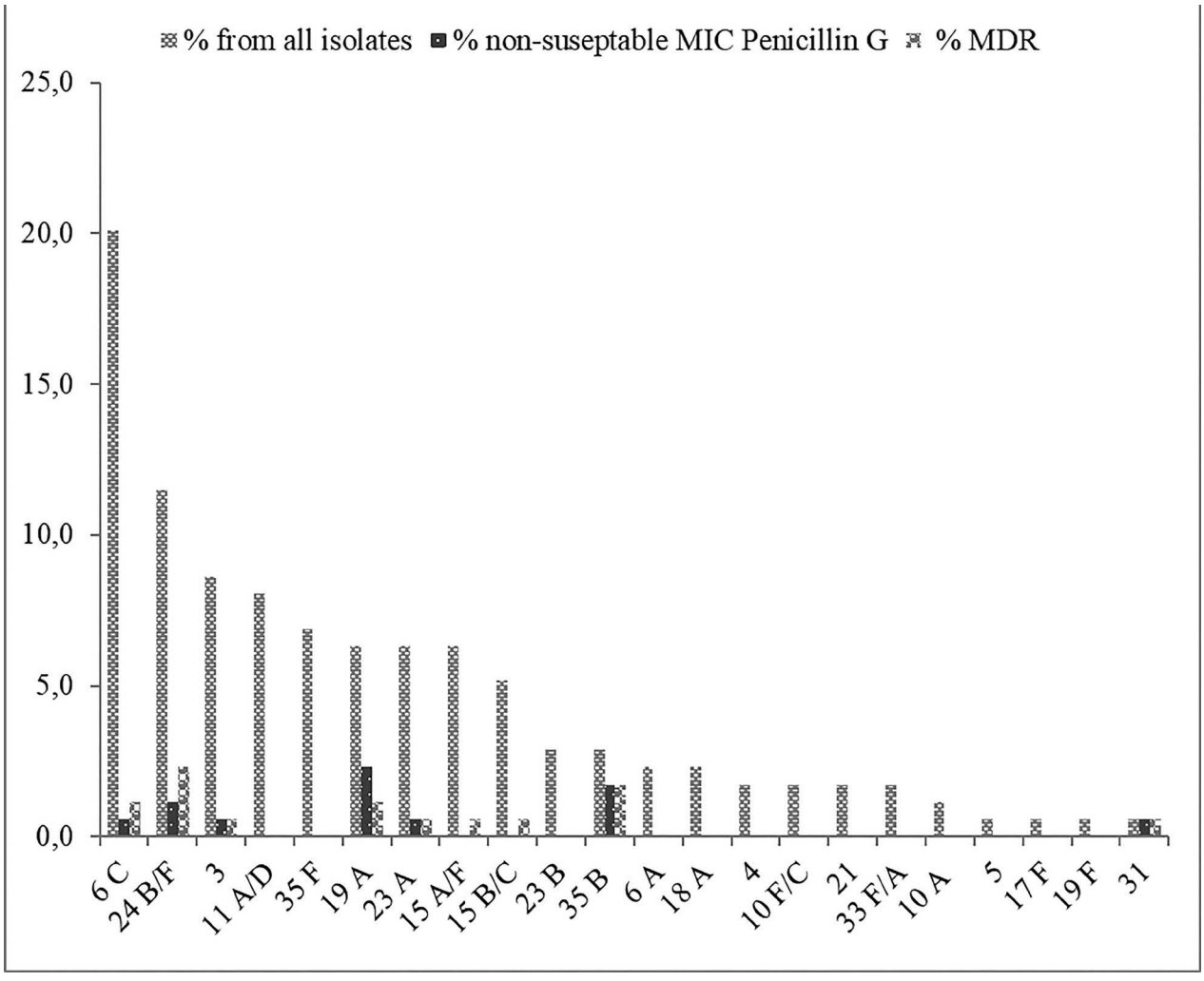

Figure 1.

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ISONIAZID-MONORESISTANT TUBERCULOSIS IN BULGARIA

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ABSTRACT

Background. Isoniazid is a common drug in the treatment regimens for TB infection. Mono-isoniazid resistance reduces the probability of a successful treatment outcome and increases the risk of acquiring additional drug resistance.

Material and methods. For the period 2015-2016 a total of 36 TB cases were confirmed in NRL TB, Sofia, as isoniazid-monoresistant *Mycobacterium tuberculosis* complex. Minimum inhibitory concentration testing for isoniazid was conducted with BACTEC MGIT 960 System in the following concentrations of the drug: 0.1 μg/ml, 0.15μg/ml, 0.2μg/ml, 0.3μg/ml, 0.4μg/ml. Molecular testing was performed with GenoType® MTBDR*plus* in order to detect the most common mutations associated with resistance to isoniazid.

Results. Only 25% of the tested *M. tuberculosis* complex isolates with phenotypic isoniazid monoresistance had the S315T1 mutation in *katG*; all isolates were with MIC over 0.4 μg/ml. C15T in the promoter region of *inhA* was detected in 22.22% of cases and only 1 of them showed MIC below 0.4 μg/ml. No mutations were detected in nearly half of the cases (n=19, 52.78%) and most of these isolates were with lower MIC values (n=12).

Conclusion. The rapid testing with GenoType® MTBDR*plus* can be used as a screening procedure indicating whether further examination of isoniazid MIC is relevant in resistant *M. tuberculosis* and whether higher doses could be considered.

KEYWORDS:

tuberculosis, isoniazid, resistance

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INTRODUCTION

Isoniazid (isonicotinylhydrazide) is a common drug in the treatment regimens for active and latent tuberculosis (TB) infection. It is estimated that the highest burden of isoniazid resistance is in the Eastern European region of the World Health Organisation (WHO) – nearly half of the TB cases, while outside the region one in seven incident TB cases has resistance to isoniazid (1).

Isoniazid performs its activity only in metabolically active bacterial cells. It requires intracellular activation by the KatG peroxidase (encoded by the *katG* gene) in order to inhibit the mycolic acid synthesis via the NADH-dependent enoyl-acyl carrier protein reductase (ACP) encoded by *inhA* (2). The most common molecular mechanisms of isoniazid resistance are related to mutations in *katG*, *inhA* or its promoter. The most frequent mutation in *katG* is S315T1 (Ser → Thr) which results in insufficient activation of the drug and is associated with a high level of resistance (MIC> 1 μg/ml). Mutations in the *inhA* gene or its promoter region lead to InhA overexpression and are most commonly associated with low level of resistance (MIC <1 μg/ml). The most frequent mutation in the promoter is at position -15C/T (Cys→Thr) (2).

The level of phenotypic isoniazid resistance cannot be predicted only from results of testing with GenoType® MTBDR*plus* because mutations outside the tested regions can elevate minimum inhibitory concentration (MIC) values.

Mono-isoniazid resistance reduces the probability of a successful treatment outcome and increases the risk of acquiring additional drug resistance (1).

The aim of this retrospective study was to describe isoniazid-monoresistant clinical strains isolated

in Bulgaria in the period 2015-2016 and further tested in the National Reference Laboratory for Tuberculosis (NRL TB) at the National Centre of Infectious and Parasitic Diseases (NCIPD).

MATERIAL AND METHODS

Patients and clinical isolates: 36 TB cases were confirmed in NRL TB as isoniazid-monoresistant *Mycobacterium tuberculosis* complex. Polyresistant or multidrug-resistant strains were not in the scope of the study. All TB cases were mapped according to the official address registration and each patient was represented by a single strain.

Identification of the isolates as *M. tuberculosis* complex was carried out by BD MGIT™ TBc Identification Test.

Drug susceptibility testing was performed with BACTEC MGIT 960 System to first-line drugs as follows: rifampin (RMP) – 1.0 μg/ml, isoniazid (INH) – 0.1 μg/ml, ethambutol (EMB) – 5.0 μg/ml and streptomycin (STR) – 1.0 μg/ml. Minimum inhibitory concentration testing for isoniazid was conducted in the same conditions with the following concentrations: 0.1 μg/ml (the official critical concentration of the drug), 0.15μg/ml, 0.2μg/ml, 0.3μg/ml, 0.4μg/ml.

Molecular testing was performed with GenoType®MTBDR*plus* in order to detect the most common mutations associated with resistance to isoniazid.

RESULTS

As isoniazid-monoresistant were identified 36 clinical isolates of *M. tuberculosis*; 21 of them were isolated in 2015 and 15 in 2016. Most of the patients were male – 80.56% (n=29) and 19.44% (n=7) were female. In 1 of the cases there was a co-infection with HIV. Ten of the patients were previously treated for tuberculosis and the other 26 were new cases.

Figure 1. Distribution of 36 TB cases with phenotypic isoniazid monoresistance, Bulgaria 2015-2016.

The distribution of the isoniazid-monoresistant TB cases in the country showed higher occurrence in the boundary districts, mainly at the seaside – 38.9% (n=14) of the cases were from Dobrich, Varna and Burgas (Fig. 1). Two of the patients were prisoners – one in Varna, the other one in Sofia.

Table 1. GenoType® MTBDR*plus* results of 36 TB cases with phenotypic isoniazid monoresistance, Bulgaria, 2015-2016.

Mutation		Frequency	
katG	***inhA***	**n**	**%**
S315T1		9	25
S315T2		0	0
	C15T	8	22.22
	A16G	0	0
	T8C	0	0
	T8A	0	0
S315T1	C15T	0	0
WT1	WT 1-2	19	52.78

Among the 36 *M. tuberculosis* isolates with phenotypic isoniazid monoresistance 25% (n=9) had the S315T1 mutation in *katG*. C15T in the promoter region of *inhA* was found in 22.22% of the tested strains. In 52.78% (n=19) no mutations were detected with GenoType® MTBDR*plus*. Co-occurrence of S315T1 and C15T was not found among the tested strains (Table 1).

As expected, all strains harbouring the *katG* S315T1 mutation showed MIC over 0.4 µg/ml. Most of the strains with C15T mutation in the promoter of *inhA* had MIC over 0.4 µg/ml (n=7) and only 1 had MIC below 0.4 µg/ml (Table 2). The majority of strains with mutations in other genes (those without detected mutations using the molecular assay) showed lower MIC values (n=12) and one-third of them were with MIC>0.4 µg/ml.

Table 2. Isoniazid MIC values and detection of mutations with GenoType® MTBDR*plus* in 36 TB cases with phenotypic isoniazid monoresistance, Bulgaria, 2015-2016.

n	%	INH MIC	Detected mutation, n	
22	61.1	> 0.4µg/ml	S315T1	9
			C15T	7
			no mutation detected	6
2	5.6	0.2µg/ml	C15T	1
			no mutation detected	1
11	30.5	0.15µg/ml	no mutation detected	11
1	2.8	not valid	no mutation detected	1

DISCUSSION

This retrospective study on isoniazid-monoresistant clinical strains isolated in Bulgaria for the period 2015-2016 has some noteworthy findings. Out of 131 cases resistant to any anti-TB drug 27.48% (n=36) were confirmed in NRL TB as isoniazid-monoresistant (3, 4). Most of the patients were newly diagnosed. The distribution of cases was mainly in the boundary districts.

Although the mutation S315T1 in *katG* is well-known as the leading cause of isoniazid resistance in 42-95% of the cases globally, in our study it was detected in only 25% of the examined strains (5). When comparing its frequency in Bulgarian isolates with different level of resistance we found that S315T1 occurs in 50% of the XDR-TB and is insignificantly represented in MDR with fluoroquinolone resistance (Table 3).

The mutation C15T in the promoter region of *inhA* was detected in only 22.22% of the tested strains although it is very common among MDR-TB strains in Bulgaria (6, 7, 8, 9) (Table 3).

As expected, co-occurrence of S315T1 and C15T was not detected among the isoniazid-monoresistant strains. So far, we have found these mutations simultaniously only in multidrug-resistant Beijing strains (7, 8, 9).

The most unexpected result of the study was that in 52.77% of the tested isolates no mutations were detected using GenoType® MTBDR*plus*. The resistance can be explained with mutations in many other loci or genes (*furA-katG, fabG1-inhA, ahpC-oxyR* intergenic region, *efpA, fadE24, iniA, iniB, iniC, kasA, nat, ndh)* (5, 10).

Table 3. Frequency of S315T1 and C15T mutations in Bulgarian clinical isolates of *Mycobacterium tuberculosis* with different types of resistance.

TB resistance	S315T1 %	C15T %	Sensitive by GenoType® MTBDR*plus*	Clinical isolates, n
Mono-isoniazid resistance	25	22.22	52.78	36
MDR (7)	27.92	50.45	19.82	222
MDR with additional fluoroquinolone resistance (8)	11.54	80.77	0	26
XDR-TB (9)	50	55.56	5.55	18

The rapid testing with GenoType® MTBDR*plus* can exclude the possibility of a therapeutic effect of isoniazid in high doses in each TB case (if the presence of S315T1 in *katG* is detected). In all other cases isoniazid MIC testing in resistant *M. tuberculosis* strains is suitable and higher doses could be considered (16 - 20 mg/kg body weight per day) (11).

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MOLECULAR EPIDEMIOLOGICAL ANALYSIS OF THE TRANSMISSION CLUSTERS OF THE HIV-1 CIRCULATING RECOMBINANT FORMS CRF01_AE AND CRF02_AG IN BULGARIA

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ABSTRACT

Background

The purpose of this study was to analyse the underlying HIV transmission clusters of individuals from different vulnerable groups infected with the recombinant forms of HIV-1 – CRF01_AE and CRF02_AG, between 1986 and 2011 using sequencing and phylogenetic analysis.

Material and methods

Blood samples from randomly selected 242 individuals diagnosed with HIV-1 CRF01_AE and CRF02_AG in Bulgaria were analysed. HIV-1 *pol* gene fragment was sequenced using the ViroSeq HIV-1 Genotyping Test (Abbott) and/or TruGene DNA Sequencing System. The phylogenetic tree reconstruction was performed with the IQ-TREE program. Identification of the phylogenetic clusters was performed using the ClusterPicker program with two parameters to identify recent and distant infection.

Results

Two main local independent epidemics confined to different geographical regions of the country were caused by HIV-1 CRF01_AE and CRF02_AG. The various viral strains circulate predominantly in two major separate regions – CRF01_AE in Sofia and CRF02_AG in Plovdiv. Most of the individuals infected with viral strains analysed in this study were people who inject drugs (PWID) or heterosexuals and only a few were men who have sex with men. The phylogenetic analysis revealed transmission clusters in both recombinant forms, few in number when confined within a short period of time and multiple clusters over an extended timeframe.

Conclusions

The introduction and rapid spread of two different strains of HIV-1 into geographically distant groups of PWID triggered local epidemic outbreaks. The phylogenetic analysis indicated the accelerated transmission of HIV, which is a characteristic of the spread through injection practices. Our study demonstrated that transmission cluster monitoring is important for better understanding of the development of epidemic and could be used as a tool for the identification of risk indicator populations.

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INTRODUCTION

HIV-1 is a result of cross-species transition from SIV in chimpanzees to HIV in humans (1, 2). HIV-1 has several major groups: M, H, O and P. Group M is the most significant for the current pandemic and contains several phylogenetically distinct subtypes (A, B, C, D, F, G, H, J and K), circulating recombinant forms (CRFs) and numerous unique recombinant forms (URFs) (3).

HIV-1 subtypes and CRFs are unevenly distributed in the world. This phenomenon is the result of different founder effects followed by local spread of a specific subtype within certain socioeconomic

environment and circulation within specific vulnerable groups (3, 4).

Subtype B is dominant in North America and Western Europe, and subtype A – in some countries of Eastern Europe and Central Asia, including Russia (3). Subtype C is the most abundant HIV-1 subtype in the world and is prevalent mostly in South and Eastern Africa and Southeast Asia (3). CRFs and URFs are widely distributed in Africa and in countries where different subtypes co-circulate (3, 5).

In Bulgaria, multiple HIV-1 subtypes and recombinant forms have been introduced from different countries of the world. Subsequently, the introduced strains were disseminated unequally among individuals from different transmission groups, including heterosexuals (HET), men who have sex with men (MSM) and people who inject drugs (PWID) (4, 6, 7, 8). Due to random events, certain HIV-1 strains have a chance of spreading sharply after being introduced into vulnerable groups forming expanding transmission clusters representing local outbreaks. Such events have been observed since 2005 when two different CRFs (CRF01_AE and CRF02_AG) were independently introduced and rapidly disseminated among two geographically distinct subgroups of PWID, resulting in local HIV-1outbreaks (4, 9).

Subtype B is the most widespread in Bulgaria but found in less than half of the HIV-1+ individuals in the country. The remaining over 50% of the introduced strains are non-B subtypes, CRFs and URFs, of which CRF01_AE and CRF02_AG are the most prevalent. Although various subtypes were initially introduced in the country, subtype B, CRF01_AE and CRF02_AG are the three major HIV-1 strains in the country. HIV-1 CRF01_AE and CRF02_AG have been found to be the most prevalent among the most vulnerable groups, such as PWID. In addition, according to our epidemiological data, vulnerable individuals represent a significant proportion of the current HIV+ population in Bulgaria and since 2005 there has been a sharp increase in the incidence of HIV among PWIDs, leading to an outbreak with significant involvement of the CRF01_AE and CRF02_AG viruses (4, 10, 11).

The purpose of this national representative study was to analyse the underlying HIV-1 transmission clusters of the two most widespread recombinant forms of HIV-1 CRF01_AE and CRF02_AG in Bulgaria using the most up-to-date methods for phylogenetic analysis. Using the ClusterPicker program, we analysed the transmission clusters of the CRF01_AE and CRF02_AG and the participation of different transmission groups in the accelerated spread of these viruses in Bulgaria.

MATERIAL AND METHODS

Study design and specimen preparation

Blood samples from all individuals diagnosed with HIV-1 CRF01_AE and CRF02_AG between 1986 and 2011 were analysed during a clinical follow-up at the National Reference Confirmatory Laboratory of HIV in Sofia, Bulgaria. Plasma samples were linked to epidemiological data through an anonymous numerical code according to the established ethical standards of Bulgaria as previously described (7).

Phylogenetic analysis and cluster identification

The HIV-1 *pol* gene fragment was sequenced using the ViroSeq HIV-1 Genotyping Test (Abbott) and/or TruGene DNA Sequencing System (Siemens Healthcare) and either the Applied Biosystems 3130xl genetic analyser or an OpenGene DNA sequencing system following the manufacturer's protocol (4).

HIV-1 CRF01_AE and CRF02_AG of the analysed sequences was determined using the automated subtype identification tool COMET v2.2 (12) and REGA HIV-1 subtyping tool version 3.0 (13).

Sequence alignments were performed using the MUSCLE algorithm implemented in AliView version 1.23 (14, 15). Additional quality control of the subtype purity and possible presence of gaps in the sequence was performed. After the clean-up and preliminary quality analysis, the complete dataset contained 141 CRF01_AE sequences comprised of 901 nucleotides in length and 101 CRF02_AG sequences comprised of 918 nucleotides in length.

The phylogenetic tree reconstruction was performed with IQ-TREE v1.6.11 program and was built through the construction of an initial parsimony tree by the phylogenetic likelihood library and search for the best model among 88 DNA models included in the program. The best-fit model was found to be: TPM3 + F + I + G4 according to the Bayesian information criterion (16, 17, 18). Verification of the topology of the phylogenetic tree was performed with generating 1000 samples for ultrafast bootstrap (18). The phylogenetic tree was rooted with midpoint root and was used for further analysis of the phylogenetic clusters. The tree was visualised using FigTree v1.4.4.

Identification of phylogenetic clusters was performed using the ClusterPicker program at a genetic distance of 0.5% and 1.5% corresponding to 0.005 and 0.015 nucleotide substitutions/site, respectively.

RESULTS

Study population demographics

In this study, we generated and analysed 242 HIV-1 *pol* gene sequences from the second and third most prevalent HIV strains in Bulgaria – CRF01_AE and CRF02_AG (Table 1). Men were 74.4% and women 25.6%, most of them in the age group 20-29. According to the permanent address, patients were focused in three major regions: Sofia, Plovdiv and the town of Peshtera. Sofia and Plovdiv were the dominant regions with almost identical proportion of infected individuals (38.4% and 37.6%, respectively). It is curious that completely different viral strains circulated in these two major regions: CRF01_AE in Sofia and CRF02_AG in Plovdiv. In addition, although Peshtera is close to Plovdiv, CRF01_AE that is typical for the more remote region of Sofia has been distributed in Peshtera.

Table 1. Study population.

Characteristics	CRF01_AE		CRF02_AG		Total	
	(n)	(%)	(n)	(%)	(n)	(%)
Total	141	100.0	101	100.0	242	100.0
Men	96	68.1	84	83.2	180	74.4
Women	45	31.9	17	16.8	62	25.6
Age (years)						
≤19	16	11.3	14	13.9	30	12.4
20-29	69	48.9	58	57.4	127	52.5
30-39	41	29.1	18	17.8	59	24.4
40-49	12	8.5	6	5.9	18	7.4
≥50	3	2.1	5	5.0	8	3.3
Region						
Permanent address – Sofia	83	58.9	10	9.9	93	38.4
Permanent address – Plovdiv	8	5.7	83	82.2	91	37.6
Permanent address – Peshtera	18	12.8	0	0.0	18	7.4
Permanent address – other	32	22.7	8	7.9	40	16.5
Transmission category						
HET	63	44.7	23	22.8	86	35.5
MSM	2	1.4	0	0.0	2	0.8
MSM+PWID	3	2.1	1	1.0	4	1.7
PWID	69	48.9	77	76.2	146	60.3
MTCT	4	2.8	0	0.0	4	1.7

HET – heterosexuals; MSM – men who have sex with men; PWID – people who inject drugs; MTCT – mother-to-child transmission.

Epidemiological data in combination with results from the phylogenetic analysis, reviewed that the HIV-1 strain CRF02_AG was highly concentrated in the region of Plovdiv and only 17.8% of these viruses were dispersed outside this region. In contrast, CRF01_AE was more evenly distributed in the country including Sofia (58.9%), Peshtera (12.8%) and other regions (28.4%) – Fig. 1 and Table 1.

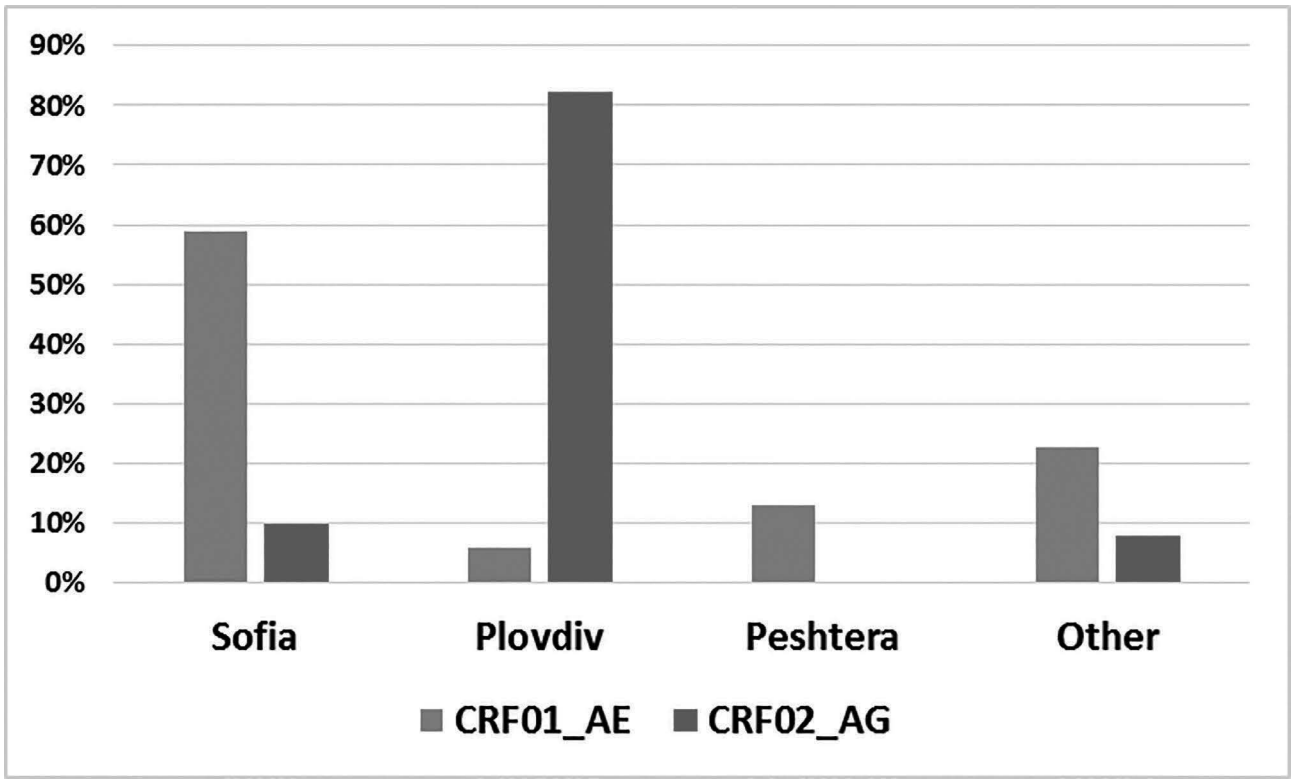

Figure 1. Distribution of CRF01_AE and CRF02_AG in different regions of the country.

There was also a significant difference in the spread of CRF01_AE and CRF02_AG among different transmission groups of the population. CRF01_AE was more evenly distributed between PWID (48.9%) and HET (47.7%), whereas CRF02_AG was found predominantly in PWID (76.2%), and much less in HET (22.8%) – Fig. 2 and Table 1.

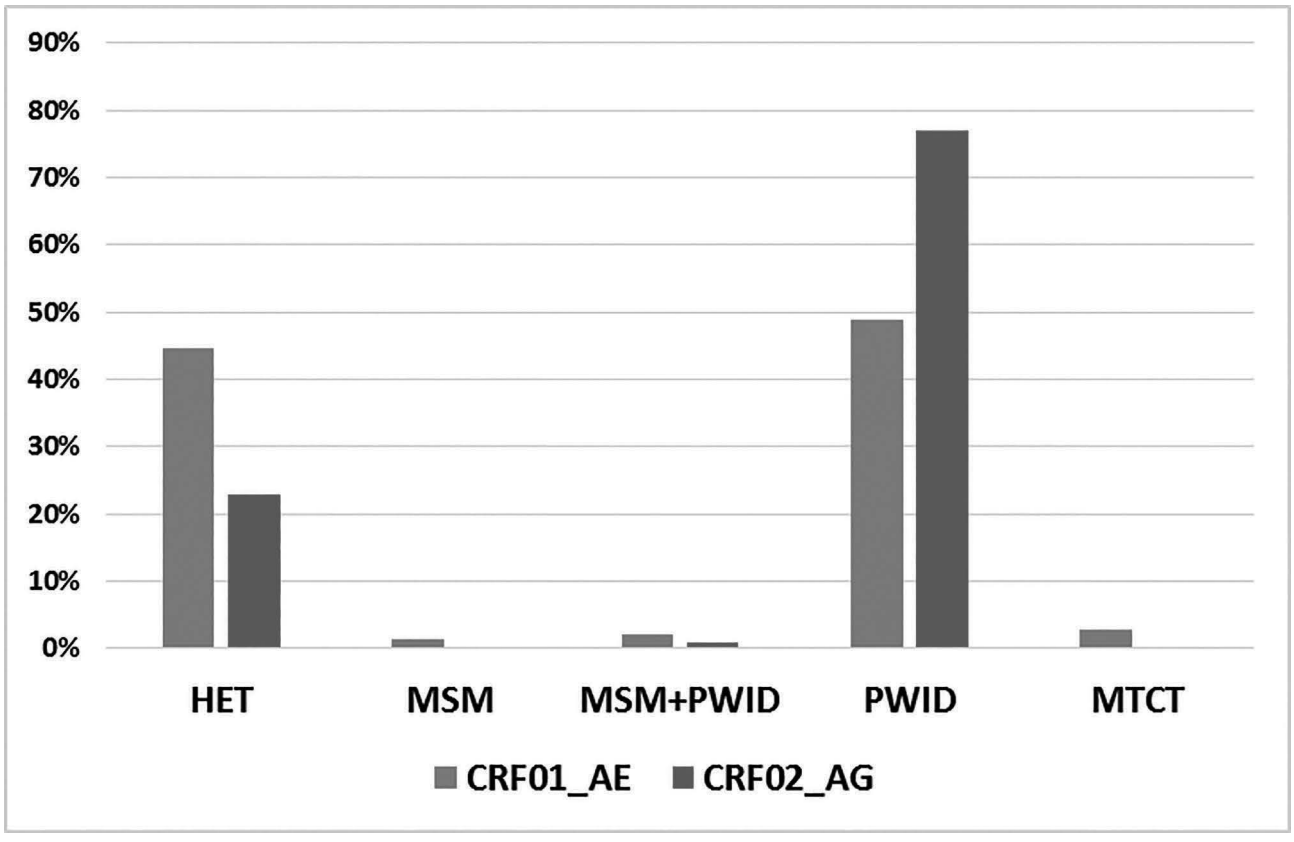

Figure 2. Distribution of CRF01_AE and CRF02_AG in different transmission groups.

Phylogenetic analysis and cluster definition

ClusterPicker software was implemented to analyse the phylogenetic clusters of the two viral strains, CRF01_AE and CRF02_AG. The phylogenetic clusters were defined with two parameters for genetic distance: 0.5% and 1.5% corresponding to 0.005 and 0.015 nucleotide substitutions/site, indicating recent and more remote transmission events. The initial cluster support threshold was defined to have bootstrap values >0.9. Two different options were defined for sequence positions on the topology of the phylogenetic tree: single sequences and phylogenetic clusters. Clusters with threshold size of 10 or more sequences were defined as large clusters.

When analysing the 141 CRF01_AE sequences with genetic distance of 0.5%, two clusters (representing very recent transmission) were identified with two sequences each (Fig. 3 A). At genetic distance of 1.5%, 13 clusters (representing remote in time transmission) with 2 or more sequences were identified, of which 9 clusters were composed of 2 sequences, 2 clusters of 3 sequences, and 2 clusters of 4 sequences (Fig. 3 B).

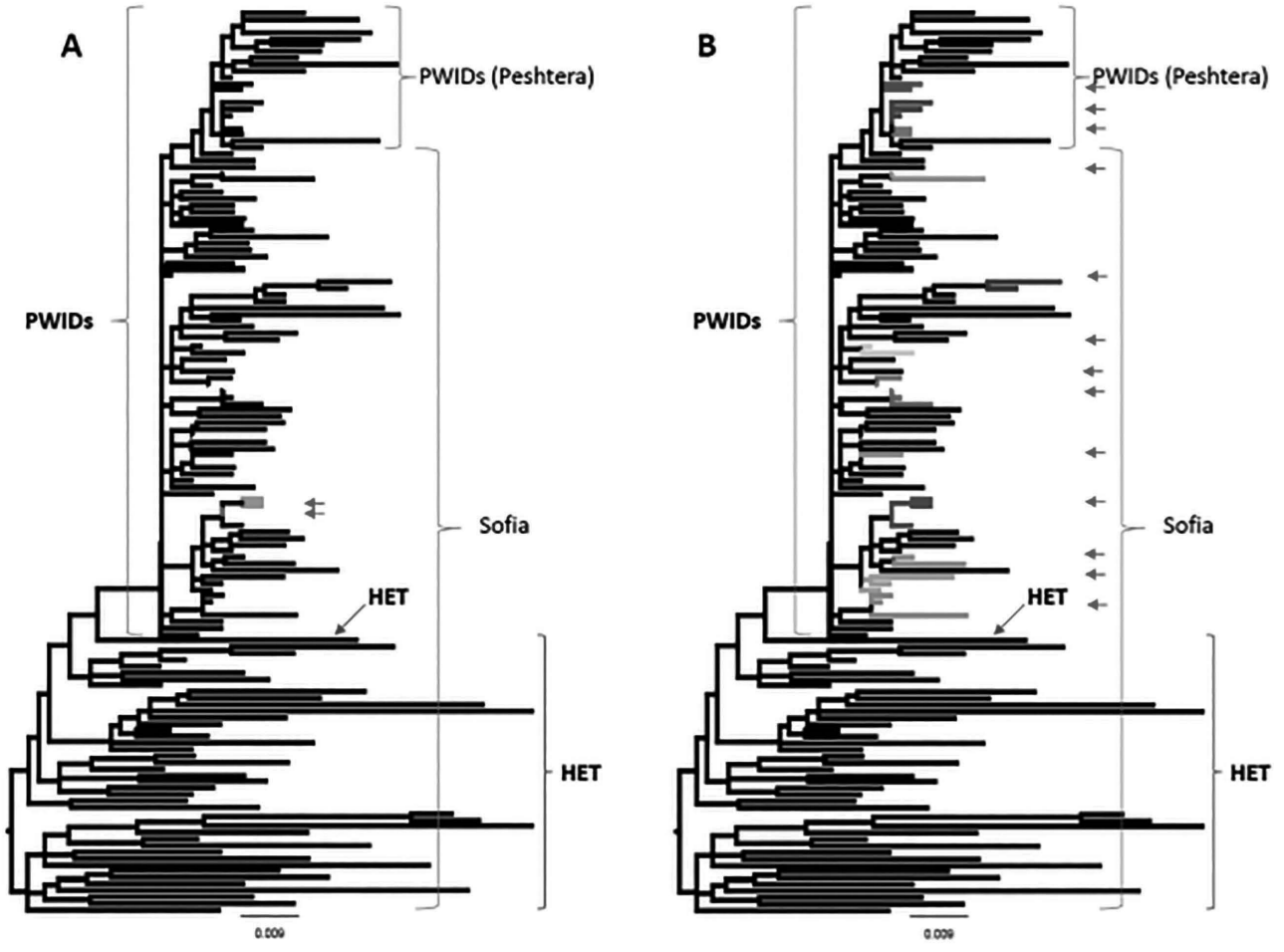

Figure 3 A and B. Phylogenetic tree of CRF01_AE strains reconstructed with ML algorithm using IQ-tree program. **A.** ClusterPicker analysis with genetic distance of 0.005 nucleotide substitutions/site. **B.** ClusterPicker analysis with genetic distance of 0.015 nucleotide substitutions/site. The red arrows indicate the location of the phylogenetic clusters with the corresponding genetic distance. One sequence isolated from HET indicated with blue arrow is at the basis of the sequences isolated from the group of the PWIDs. The size and digital expression of nucleotide substitutions/site are shown at the bottom of the phylogenetic tree.

When analysing 101 CRF02_AG sequences with genetic distance of 0.5%, four clusters (representing very recent transmission) with two sequences each, were identified (Fig. 4 A). At genetic distance of 1.5%, 12 clusters (representing remote in time transmission) with 2 to 5 sequences were identified. Of these, 9 clusters were composed of 2 sequences, 1 cluster of 3 sequences and 2 clusters of 5 sequences (Fig. 4 B).

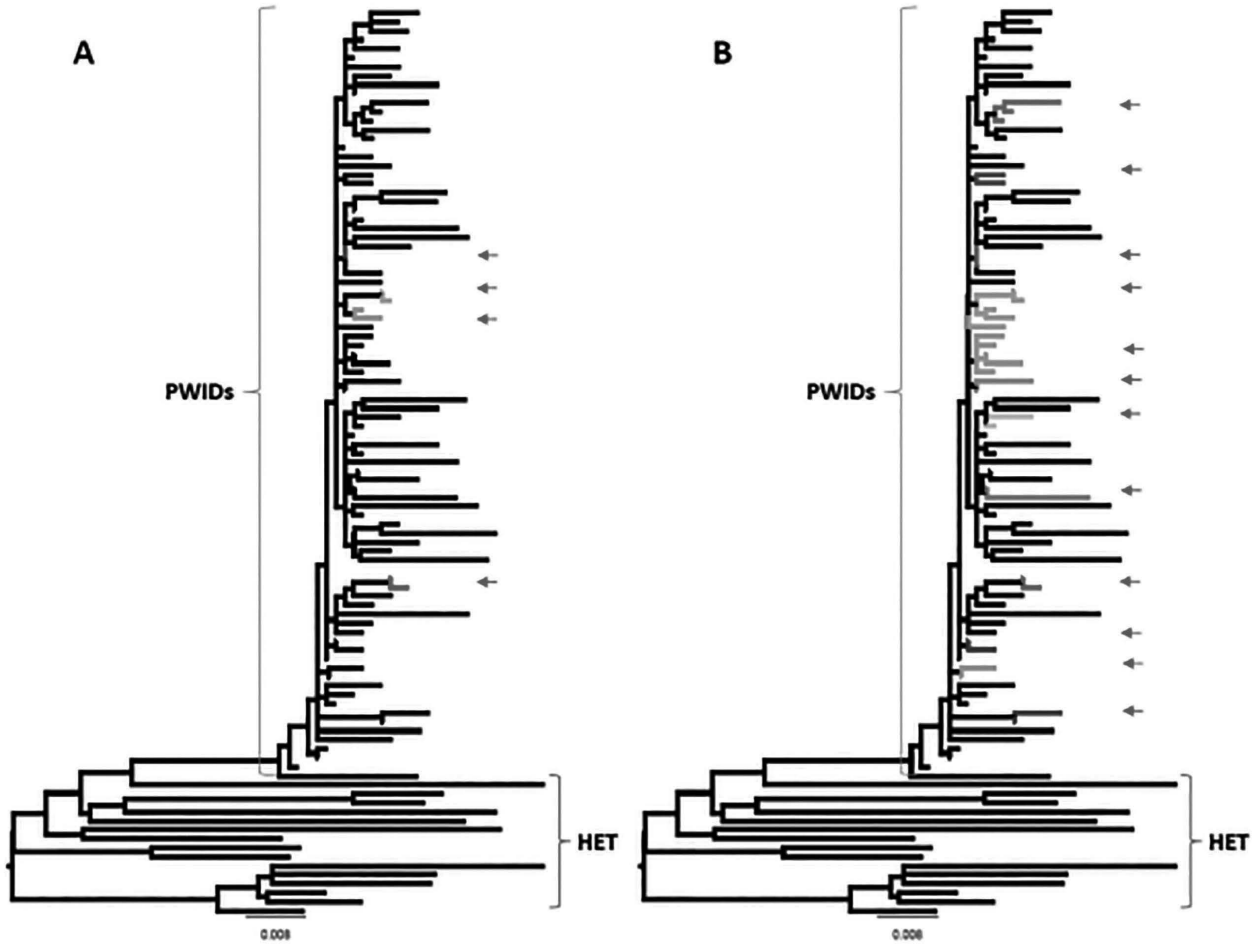

Figure 4 A and B. Phylogenetic tree of CRF02_AG strains reconstructed with ML algorithm using IQ-tree program. **A.** ClusterPicker analysis with genetic distance of 0.005 nucleotide substitutions/site. **B.** ClusterPicker analysis with genetic distance of 0.015 nucleotide substitutions/site. The red arrows indicate the location of the phylogenetic clusters with the corresponding genetic distance. The size and digital expression of nucleotide substitutions/site are shown at the bottom of the phylogenetic tree.

Discussion

In this study, we analysed two of the most prevalent HIV-1 strains in Bulgaria – CRF01_AE and CRF02_AG. The spread of these two recombinant forms is significant both at the country and at the global levels as demonstrated by previous national and global worldwide distribution surveys (4). Of additional interest are the findings that these two strains have been introduced among the most vulnerable individuals to blood-borne infections such as PWID, where viruses have a chance of accelerated spread in limited geographical areas causing an epidemic outbreak among the affected populations. For example, in the late 1990s-early 2000s in Finland, HIV affected a marginalised population of PWID with high rates of imprisonment and homelessness and CRF01_AE, a prevalent variant in Southeast Asia that was circulating in Finland in the early 1990s, was the cause of the outbreak. CRF01_AE was imported from Helsinki, Finland to Stockholm, Sweden leading to an outbreak there among PWID that started probably in around 2003 and was detected in 2006. Similarly, in a PWID-related outbreak in the early 2000s in Northern Italy, HIV-1 diagnoses among PWID formed a monophyletic cluster of subtype G with origin in West Africa (19).

Our phylogenetic analysis uses bioinformatics programs and a range of phylogenetic proximity and genetic distance parameters to analyse isolated viral sequences and to identify phylogenetic clusters that represent transmission events occurring at different time frame.

The smaller genetic distance of 0.005 nucleotide substitutions/site allows identification of transmission events that occurred within one year. Using this time constraint, only two clusters of two

sequences each were detected in the CRF01_AE phylogenetic tree, representing transmission events within the time frame of one year (Fig. 3 A). These transmission events took place within PWIDs in Sofia. In contrast, four clusters with two sequences each were identified for the other HIV-1 strain – CRF02_AG, indicating more turbulent transmission events occurring within a short time frame among the group of PWIDs (Fig. 4 A).

At a greater genetic distance of 0.015 nucleotide substitutions/site, many phylogenetic clusters have been identified in both phylogenetic trees, representing the unfolding of broad-scale transmission events that have taken place over a longer period of time during the expansion of independent local epidemics in remote geographic regions.

Individual groupings of sequences stand out on the topology of phylogenetic trees reconstructed by both recombinant forms of HIV-1. On the phylogenetic tree of CRF01_AE sequences, PWIDs are separated into a distinct group of sequences with relatively short branches generally representing short evolutionary history. The sequences from the HET individuals are positioned closer to the root of the tree. The sequences isolated from HET individuals are at the basis of all sequences and precede all PWIDs sequences showing that these viruses were first introduced and spread within HET transmission group, and later were transferred to PWIDs. This finding is in line with our epidemiological data and previous reports indicating that HIV was introduced into the vulnerable group of PWID later as compared to HET individuals (4). In addition, there are grouped sequences with geographical division and sequences from the town of Peshtera that stand out separately from those of Sofia. Moreover, although the phylogenetic trees have not been dated, the isolated sequences from Sofia appear to be the ancestors of those from Peshtera.

Furthermore, the topology of the phylogenetic tree of CRF02_AG sequences also demonstrates division into two major transmission groups: PWIDs and HET. Similarly to the CRF01_AE phylogenetic tree, in the CRF02_AG tree the sequences isolated from HET individuals are closer to the root of the tree while the distinct group of sequences from PWIDs are with relatively short branches representing short evolutionary history. Most of the patients with CRF02_AG were from Plovdiv and only a small number of sequences have been isolated from persons living outside this region.

Both recombinant forms of HIV-1 – CRF01_AE and CRF02_AG appear to have been introduced in Bulgaria initially among HET individuals and subsequently introduced into the vulnerable group of PWIDs, where the viruses spread rapidly (4). This rapid spread of HIV among vulnerable populations has evoked urgent and widespread actions to curb the epidemic by the Ministry of Health. A campaign was launched for educational initiatives, free tests and counselling, including distribution of needles and disposable syringes among PWIDs. These initiatives led to a significant decrease of the number of newly diagnosed PWIDs with HIV.

Our study has some potential limitations. Firstly, it comprises the period between 1986 and 2011, and may not reflect the overall picture of HIV-1 CRF01_AE and CRF02_AG epidemic in Bulgaria, which is characterised by high dynamics due to the introduction of these HIV-1 strains in different transmission groups of the population. Secondly, our data sample includes only the individuals from whom the HIV-1 *pol* gene was successfully generated, and not those from whom no viral sequence has been obtained due to successful therapy and low viral load.

Conclusions

A large variety of HIV-1 subtypes and recombinant forms has been introduced in Bulgaria. The two recombinant forms CRF01_AE and CRF02_AG have been introduced into geographically distant groups of PWID and triggered local epidemic outbreaks. The phylogenetic analysis indicated the presence of accelerated transmission of HIV, which is characteristic of the spread through injection practices. The presence of a reservoir of a large number of HIV-infected individuals from vulnerable groups is of concern because there is a possibility of rapid dissemination and transmission into the general population. The monitoring of transmission clusters is important for better understanding of the epidemic and for identification of risk indicator population groups.

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PREVALENCE OF ANTI-HBC IN HBSAG-NEGATIVE POPULATION: SCREENING OF PATIENTS WITH UNSPECIFIED ACUTE HEPATITIS AND REVIEW OF THE LITERATURE

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ABSTRACT

Background: Among the various serological markers employed in the hepatitis B virus (HBV) differential laboratory diagnosis, serum HBsAg is considered the most reliable. In order to characterise the HBV infection, another important diagnostic marker is employed – the HBc antibody (anti-HBc). There are three categories of anti-HBc-positive individuals: patients with HBV immunity, patients with chronic HBV and individuals with the so-called isolated anti-HBc pattern. The current study aimed to evaluate the presence of anti-HBc in patients negative for HBsAg whose clinical diagnosis was acute viral hepatitis.

Material and methods: A total of 88 specimens were examined, of which 75 sera were from prospective patients diagnosed with acute viral hepatitis, and 13 sera from breast milk donors. Antibodies against the hepatitis B core antigen were detected by enzyme-linked immunosorbent assay (ELISA).

Results: Twenty-eight (32%) of all tested samples were positive for anti-HBc. Nineteen samples belonged to male and 9 to female patients. One positive sample was from a breast milk donor. Two age groups, namely 46-55 years and 56-65 years, demonstrated the highest rate of anti-HBc positivity. Among the other age groups positivity rates varied from 15% to 36%. The results demonstrated a linear trend of increasing anti-HBc prevalence with increasing age.

Conclusions: Considering the highest rate of anti-HBc positivity being demonstrated in the age range 46-65 years, it could be assumed that a sufficient number of risk factors accumulate over time resulting in greater population susceptibility to HBV infection.

KEYWORDS:

HBV, anti-HBc, acute hepatitis

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INTRODUCTION

Hepatitis B virus (HBV) is a causative agent of acute or chronic viral infection that represents a major public health problem with significant morbidity and mortality. More than 500 000 newly infected cases are reported each year. According to the World Health Organisation (WHO), about 248 million people are chronically infected, and approximately 686 million deaths per year are due to secondary complications of HBV infection such as hepatocellular carcinoma and cirrhosis (1).

The worldwide distribution of the virus has traditionally been determined by the seroprevalence of the surface antigen (HBsAg) in a given population. Highly endemic countries demonstrate HBsAg prevalence greater than 8%. In countries with intermediate endemicity, the HBsAg prevalence is subdivided into lower-intermediate (2-4.99%) and higher-intermediate (5-7.99%) and, finally, in low endemic areas, less than 2% of the population is affected (2). In hyper-endemic regions, the vast majority of cases is comprised of individuals that were infected perinatally or later in early childhood through horizontal transmission. Perinatal transmission is also possible in areas with intermediate endemicity, but the primary mode of transmission remains horizontal. In hypo-endemic areas, infections tend to be incidental and are most commonly the result of unprotected intercourse, injecting drug use or other unsafe exposure to blood products (3, 4). In a large multicentre study on the presence of major HBV markers among the general population in Bulgaria during the period 1999-2000, HBsAg prevalence was found in 3.87% of

the total population, i.e. Bulgaria falls into the group of intermediate endemicity with respect to HBV (5)**.** In 2017, a total of 3132 cases of viral hepatitis were reported in Bulgaria, and the rate of viral hepatitis B was 7.95%, meaning that 249 cases were reported in 26 districts of the country – Stara Zagora with 12.14%$_{000}$ morbidity, followed by the districts of Pernik (9.70%$_{000}$), Sliven (6.85%$_{000}$), Montana (6.68%$_{000}$) and Gabrovo (6.23%$_{000}$). In 22 (8.83%) of the reported cases, the affected were aged 19 years or less and 12 (54.55%) of the patients had been previously immunised against hepatitis B (6). In accordance with Ordinance No. 21 on the Procedure for Registration, Reporting and Control of Infectious Diseases, the acute form of viral hepatitis B is subject to mandatory registration and reporting (7)**.**

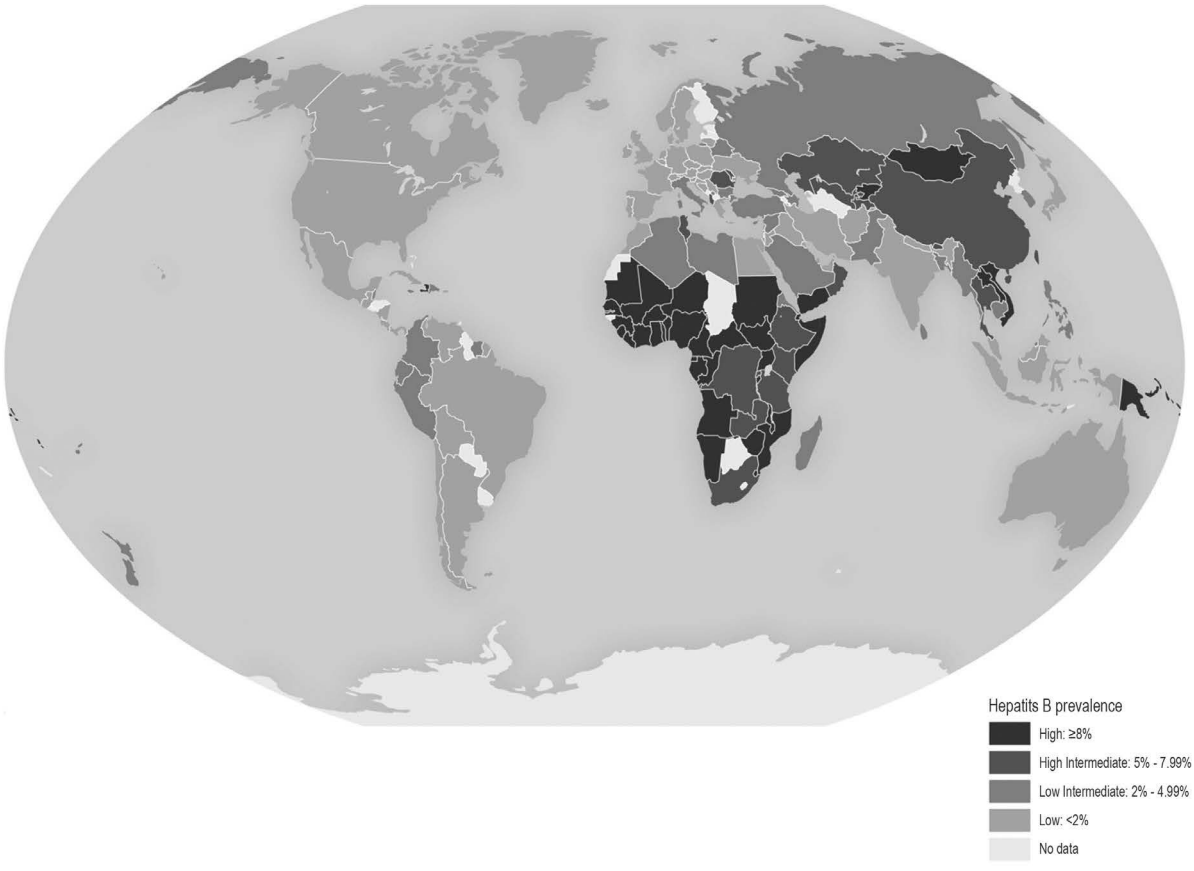

Figure 1. Prevalence of hepatitis B virus infection estimated from data on HBsAg antigenemia. (Source: Centres for Disease Control and Prevention. Infectious Diseases Related to Travel: Hepatitis B, https://wwwnc.cdc.gov/travel/yellowbook/2018/infectious-diseases-related-to-travel/hepatitis-b)

Hepatitis B virus can be found in almost all bodily fluids. However blood, semen and vaginal secretions have the greatest infectious potential, and thus, transmission occurs through percutaneous or mucosal exposure to infective fluids (8). The virus is not found in urine, sweat and stool. Serological tests for the detection of HBV antigens and antibodies are the mainstay of diagnostic screening and utilise blood with subsequent serum or plasma isolation. Both HBV antigens and antibodies are stable for days at room temperature, for months at -4°C and years if stored at -20°C to -40°C.

Following infection, the first marker to appear in the blood circulation is the HBV surface antigen (HBsAg**)**, which becomes detectable 2-4 weeks prior to biochemical evidence of liver damage or the onset of jaundice (9**)**. This is the main viral protein that induces protective immunity. The marker becomes negative upon infection elimination after a period of 1-2 months and its persistence beyond 6 months is an evidence for chronic infection (9, 10). The HBsAg loss is marked by the development of protective antibodies – anti-HBs. The presence of anti-HBs only is evidence for vaccination. An anti-HBs serologic test result

demonstrating more than 10 mIU/mL is indicative of protective immunity (11). Some patients may be positive for both HBsAg and anti-HBs implying ineffective virus neutralisation and a chronic carrier state. Another specific HBV viral protein that could be found in serum is the HBV e-antigen (HBeAg). Since HBeAg is associated with the acute phase of HBV infection, i.e. with high levels of viral DNA, it is an indicator of a high degree of replication and infectivity. Seroconversion to anti-HBe may occur after years in patients with chronic HBV infection. The disappearance of HBeAg, even without seroconversion, is a sign of a significant decrease in the viral titre. Some HBV variants carrying certain mutations do not produce HBeAg or produce very low levels (pre-core and core mutants), but continue to have high HBsAg levels and are associated with poor clinical prognosis, i.e. prolonged and severe disease with a substantial risk of cirrhosis (10). Another important diagnostic marker is the HBV core antigen **(**HBcAg**)**, which is essentially a capsid protein. It is not readily detectable in serum as the antigen is incorporated into the virion, but it is highly immunogenic and its antibody (anti-HBc) is detectable in blood with the onset of clinical symptoms. The IgM class antibodies against HBcAg (anti-HBc IgM)**,** are the first to appear approximately 6 weeks after HBV exposure. The high titre of anti-HBc IgM may be the only marker detectable in the "window" period of acute HBV infection prior to seroconversion of HBsAg to anti-HBs (12).

In recent years, the emphasis has been placed on the extent to which HBsAg tests alone can be relied upon for acute or chronic HBV infection diagnosis. Serum HBsAg detection is considered a reliable marker of HBV infection, but it is insufficient to differentiate between an inactive carrier state, acute or chronic HBV infection (13). False-negative results during the window period, false-positive results, occult infections, and the presence of HBsAg mutants that cannot be demonstrated by standard assays have to be taken into consideration (14). A particular case is fulminant hepatitis, where HBsAg disappears too early due to the fulminant course of the disease, which also delays seroconversion to anti-HBs (15). Typical example is a study of 27 cases of fulminant hepatitis, conducted by Shimizu et al., of which 11 patients demonstrated both HBsAg and anti-HBc IgM but were initially diagnosed by testing for anti-HBc IgM. For the remaining 9 patients from the same study, anti-HBc IgM titres were demonstrated without detecting HBsAg (16).

Detection of HBsAg, anti-HCV, anti-HAV IgM and before the year 2020 occasionally detection of anti-HEV IgM, was requested for differential diagnosis of patients with acute viral hepatitis. In the National Reference Laboratory (NRL) "Hepatitis viruses" in case of HBsAg-positive results, samples were evaluated for the presence of anti-HBc IgM and/or HBeAg for confirmation of acute HBV infection. The present study further analysed the prevalence of anti-HBc total in 75 patients and 13 control subjects (breast milk donors) with the main aim to evaluate the presence of anti-HBc in patients negative for HBsAg whose clinical diagnosis was acute viral hepatitis.

MATERIAL AND METHODS

Seventy-five sera of prospective patients diagnosed with acute viral hepatitis as well as 13 sera of breast milk donors (as negative controls) were investigated in the present study. Samples collected from January to December 2016 were selected from sera bank of the NRL "Hepatitis viruses". All samples were tested previously in the NRL "Hepatitis viruses" and met the criteria to be negative for HBsAg, anti-HBc IgM, anti-HAV and anti-HCV. For *in vitro* detection of anti-HBc total in human sera a qualitative enzyme-linked immunosorbent assay (ELISA) ANTICORASE B-96 (TMB) (General Biologicals Corporation) was employed according to the manufacturer's instructions. The diagnostic specificity and sensitivity of the test are 99.8% and 100%, respectively. Specimens with absorbance values greater than 1.1 multiplied by the cutoff value are considered negative for anti-HBc. Specimens with absorbance values less than 0.9 multiplied by the cutoff value are considered positive for anti-HBc. All positive specimens along with specimens with absorbance value falling within the retest range, which is the cutoff value ± 10%, were retested and interpreted as above. The tested samples were divided into 6 groups depending on the age of the patient: patients between 15 and 25 years; 26 to 35 years; 36 to 45 years; 45 to 55 years; 56 to 65 years and patients between 66 and 75 years old.

RESULTS

For the period January-December 2016 in the NRL "Hepatitis viruses" 1802 samples were received for differentiation of viral hepatitis or for screening of hepatitis markers. From these samples, 1356 (75%) were tested for the presence of HBsAg and 190 (14%) were positive; 1019 (57%) were tested for anti-HCV and 127 (12%) were positive; 306 (17%) were tested for anti-HAV IgM with 84 (27%) positive results; and 501 (28%) were tested for the presence of anti-HEV IgM and 162 (32%) were positive.

During a training practice conducted in the NRL "Hepatitis Viruses" at the National Centre of Infectious and Parasitic Diseases, within the framework of the "Student Practices – Phase 1" project implemented by the Ministry of Education and Science in partnership with higher education institutions and scientific organisations in Bulgaria, blood serum samples were screened for the presence of anti-HBc. The studied patients were from Sofia, Pernik, Gabrovo, Haskovo and Shumen. The breast milk donors were from Sofia only. All tested samples met the selected criteria to be HBsAg-, anti-HBc IgM-, anti-HAV- and anti-HCV-negative and to be with sufficient quality and quantity for testing. The mean age was 40 years ± 15, the youngest individual being 15 and the eldest – 69 years of age; male to female ratio was 46:42. For 8 patients the age was not recorded in the medical profile. With regard to anti-HBc presence in sera, 28 (32%) of all tested samples were positive. From the positive samples 19 were male and 9 female patients. One positive sample belonged to a breast milk donor. Eighteen of all positive samples were from patients from Sofia, 7 from Pernik, and 1 from Gabrovo, Haskovo and Shumen, respectively. We evaluated the prevalence of anti-HBc total among the different age groups. Three age groups demonstrated the highest rate of anti-HBc positivity – 46% for the age group 46-55 years, 50% for 66-75 years and 55% for 56-65 years (Fig. 2).

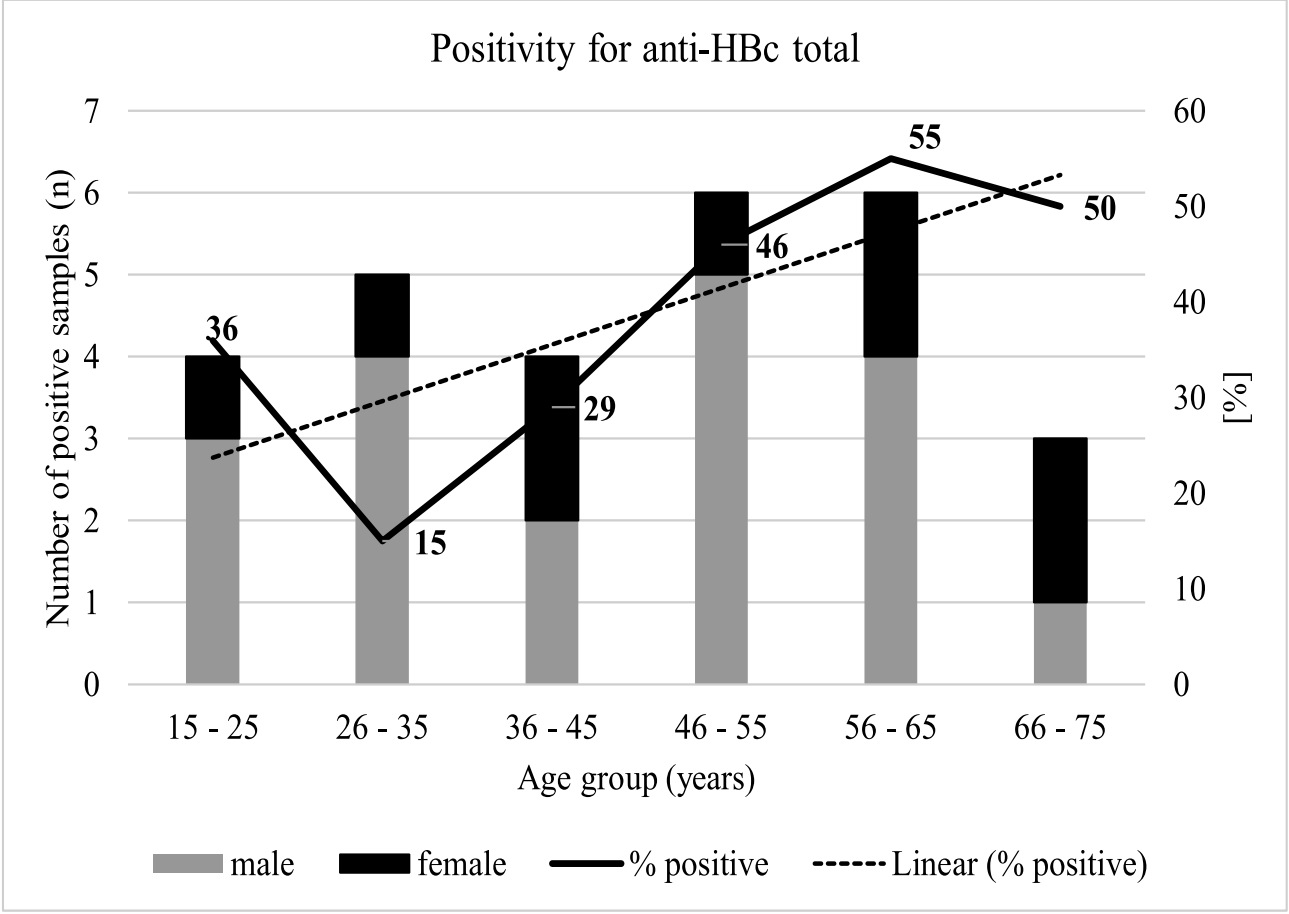

Figure 2. Age distribution of anti-HBc-positive individuals.
Legend: Percentage refers to a proportion of positive individuals for each age group.

Among the other age groups, positivity varied from 15% to 36%. In the present study, the peak of anti-HBc positivity is in the active age over 45 years, with males being predominantly anti-HBc-positive except for two age groups, namely 36-45 years and 66-75 years.

DISCUSSION

The current study revealed that 32% of the studied population were positive for the presence of anti-HBc total with prevalence in male individuals. The results demonstrated a linear trend of increasing anti-HBc prevalence with increasing age. Similar results were reported in the multicentre study conducted in Bulgaria in the period 1999-2000 (5). The apparent steady increase in seropositivity with age confirms the fact that a sufficient number of risk factors and occasions for exposure accumulate over time rendering the population susceptible to HBV infection. Given that anti-HBc antibodies remain in the serum for a significantly long period of time and are indicative of HBV exposure, they are suitable as a screening test for blood donation or for distinguishing whether a patient has acquired immunity through exposure or vaccination. These antibodies may be the only serological marker for HBV infection and potentially infectious blood.

Individuals who show no sign of liver disease but are anti-HBc-positive can be divided into three categories: 1) those with HBV immunity, i.e. individuals who are anti-HBc- and anti-HBs-positive and HBsAg-negative; 2) individuals with chronic HBV that are HBsAg-positive, and 3) individuals with the so-called isolated anti-HBc pattern (IAHBc) – they are positive for anti-HBc and negative for both anti-HBs and HBsAg (17). The IAHBc pattern is not unusual given that it is reported in 10-20% of patients with positive serology for HBV without the presence of other serological markers for HBV infection (18). This serological profile is often observed in intravenous drug users, hepatitis co-infections and pregnant women (18, 19). In HIV-positive patients, IAHBc occurs in 7% to 40% of the cases (20). In the evaluation of the IAHBc serological profile, several interpretations may be considered, of which the most significant are the window period in the acute infection, a false-positive result, past or occult HBV infection (21, 22). During the acute phase of HBV infection, it is impossible to detect HBsAg and HBeAg and their corresponding antibodies anti-HBs and anti-HBe in serum by conventional tests due to the formation of immune complexes which underlies the serological profile of isolated anti-HBc (19, 21). The IAHBc pattern could be observed years after recovery of the patient owing to a decline in the anti-HBs titre (21). This profile is also observable in chronic infections with waning anti-HBs titre or in the presence of HBsAg escape mutants (19, 23). Patients who are carriers of anti-HBc alone should be retested to rule out false-positive results (21). These cases are more commonly encountered in low prevalence areas and result from non-specific cross-reactivity reactions, circumstances related to the applied detection method and technical preparation. The primary mechanism that explains the IAHBc serostatus in HCV co-infected individuals is inhibition of HBV replication by the direct effect that the HCV core protein exerts on HBV (19). Another study suggests an alternative mechanism in which a stronger immune response to HBV leads to the formation of anti-HBs with partial clearance of HBV and partial HCV suppression. The subsequent loss of anti-HBs due to possible cross-reactivity and other mechanisms is a prerequisite for reactivation of HCV causing active HCV infection with IAHBc serostatus (24). Carriers of isolated anti-HBc ought to be considered potentially infectious, as instances of HBV transmission from blood or grafts from anti-HBc only-positive donors have been reported (22, 23, 25, 26).

In conclusion, the prevalence of anti-HBc in the studied population increases with age. It could be assumed that a sufficient number of risk factors accumulate over time resulting in greater population susceptibility to HBV infection. We should note the main limitation of the study – the small number of samples tested; however, the study was carried out in the framework of a project with the main purpose to familiarise medical students, under the supervision of a mentor, with the laboratory differential diagnosis of hepatitis viruses and the activity of NRL "Hepatitis viruses". The primary focus was the acquisition of practical and analytical skills while working in a laboratory setting and with scientific literature to design and conduct a study leading to publication of a scientific article.

ACKNOWLEDGMENTS: This study was carried out in the context of "Student Practices – Phase 1" project, implemented by the Ministry of Education and Science.

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RETROSPECTIVE ANALYSIS OF TAENIASIS IN BULGARIA CAUSED BY THE BEEF TAPEWORM FOR THE PERIOD 2008-2017

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ABSTRACT

The aim of the present study is to analyse the dynamics of the distribution of taeniasis caused by the beef tapeworm (*Taeniarhynchus saginatus*) among the population of Bulgaria for the period 2008-2017.

Material and methods. The analysis is based on laboratory and clinical data on patients diagnosed with taeniarhynchosis in DPTM at NCIPD, as well as data from epidemiological studies carried out by RHIs in accordance with the current legislation in the country.

Results. For the studied period, cases of human taeniarhynchosis were reported from 21 districts of the country, with 246 infected persons. The areas with the highest number of cases during the whole period were Shumen – 14.6%, Plovdiv – 13.8%, Targovishte – 10.9 and Sofia – 10.6%. Annual morbidity ranges from 0.63 per 100 000 (2008) to 0.23 per 100 000 (2017), with average morbidity of $0.37\%_{000.}$ The prevalence was higher among female subjects (61%), and the distribution by age showed a significantly higher incidence in adults (88%) compared to the group of children and adolescents (22%). The most affected were the age groups 35-39 (11.79%) and 55-59 (11.38%) years. Patients of different ethnic origin represent 54% of cases.

Conclusion. Data from our study shows that cases of taeniarhynchosis, even though sporadic, are reported annually in the country. The distribution is highest in regions with well-developed private livestock farming and high rate of home meat production without veterinary control. Poor health literacy of the population resulting in environmental contamination with human excreta and the possibility of infection of intermediate hosts, as well as consumption of uncooked homemade products and insufficient veterinary health control mainly contribute to the endemic spread of taeniarhynchosis in the country.

KEYWORDS:

taeniasis, beef tapeworm, incidence

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INTRODUCTION

Taeniasis caused by the beef tapeworm (taeniarhynchosis) is a parasitic food-borne disease the source of which is the infected person and cattle being the intermediate hosts (1). The causative agent of the disease is the beef tapeworm *Taeniarhynchus saginatus* (*Taenia saginata*) belonging to class Cestoda. *T. saginatus* is a flat, hermaphroditic parasite composed of 1000 to 2000 proglottids and reaches 4 to 10 metres in length. One proglottid may contain between 50 000 and 80 000 eggs (2, 3). There are four suckers, a spherical scolex and a rostellum without hooks. The large number of bilateral branches of the uterus is the main element used in species identification of the helminth (Fig. 1) (4). The eggs of the beef tapeworm are spherical with a thick dark brown cross-furrowed shell and an oncosphere with a 6-hooked embryo (1).

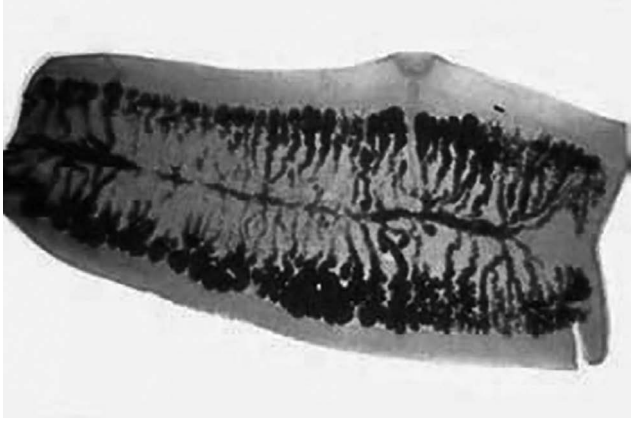

Fig. 1. Proglottid of *T. saginata* (Source: Kurdova, R (Ed.). Laboratory diagnosis of parasitoses in humans. Sofia, ARSO, 2009, 254 p.).

T. saginatus has a typical life cycle. Human is the ultimate host where the tapeworm develops into a sexually mature parasite in the small intestine. Mature proglottids actively exit the anus of the infected person, the uterus ruptures and the eggs fall in the external environment where they remain invasive for 2-3 months. Oncospheres are released from the swallowed eggs in the bovine intestines, penetrate the intestinal wall and spread throughout the body. The cysticercus that develops from them reaches invasive stages after 4 months. The larval form of *T. saginatus* is called *Cysticercus bovis* and develops in the chewing muscles and heart of the cattle. People become infected after consumption of uncooked beef or veal. Animals are infected when feeding in areas contaminated with human faeces containing *T. saginatus* eggs (1-4).

The incubation period of the disease is about 3 months. The complaints can start before releasing of proglottids and continue for a long time afterwards with loss of appetite, nausea and navel pain. During the later stages of the disease fatigue, headache, dizziness, itching in the anal area is observed as well as urticaria. In most cases, the clinical symptoms are either mild or absent. The proglottids are released during the day, actively crawling through the anal opening (1, 2).

In Bulgaria the disease is widespread throughout the country. During the 1950s, 750-1000 patients were registered annually (1). The disease is widespread in Central and South America, South Asia, the Philippines, China and Africa, and has been reported in Europe (1).

In Bulgaria taeniasis caused by the beef tapeworm and bovine cysticercosis is a subject to mandatory registration and notification.

The aim of the present study is to analyse the dynamics of the distribution of taeniasis caused by the beef tapeworm among Bulgarian population for the period 2008-2017.

MATERIAL AND METHODS

The analysis is based on laboratory and clinical data on patients diagnosed with taeniarhynchosis in the Department of Parasitology and Tropical Medicine (DPTM) at the National Centre of Infectious and Parasitic Diseases (NCIPD), as well as data from epidemiological studies carried out by the Regional Health Inspectorates (RHIs) in accordance with the current legislation in the country, and the annual analyses of parasitic diseases in the country performed by DPTM.

Mean values, standard deviation and confidence interval were determined with statistical software. We used Student's t-test to determine if the means of two data sets differ significantly at $p < 0.05$.

RESULTS

For the studied period, cases of human taeniarhynchosis were reported from 21 districts of the country with 246 infected persons. The areas with the highest number of cases during the whole period were Shumen – 14.6%, Plovdiv – 13.8%, Targovishte – 10.9 and Sofia – 10.6% (Fig. 2).

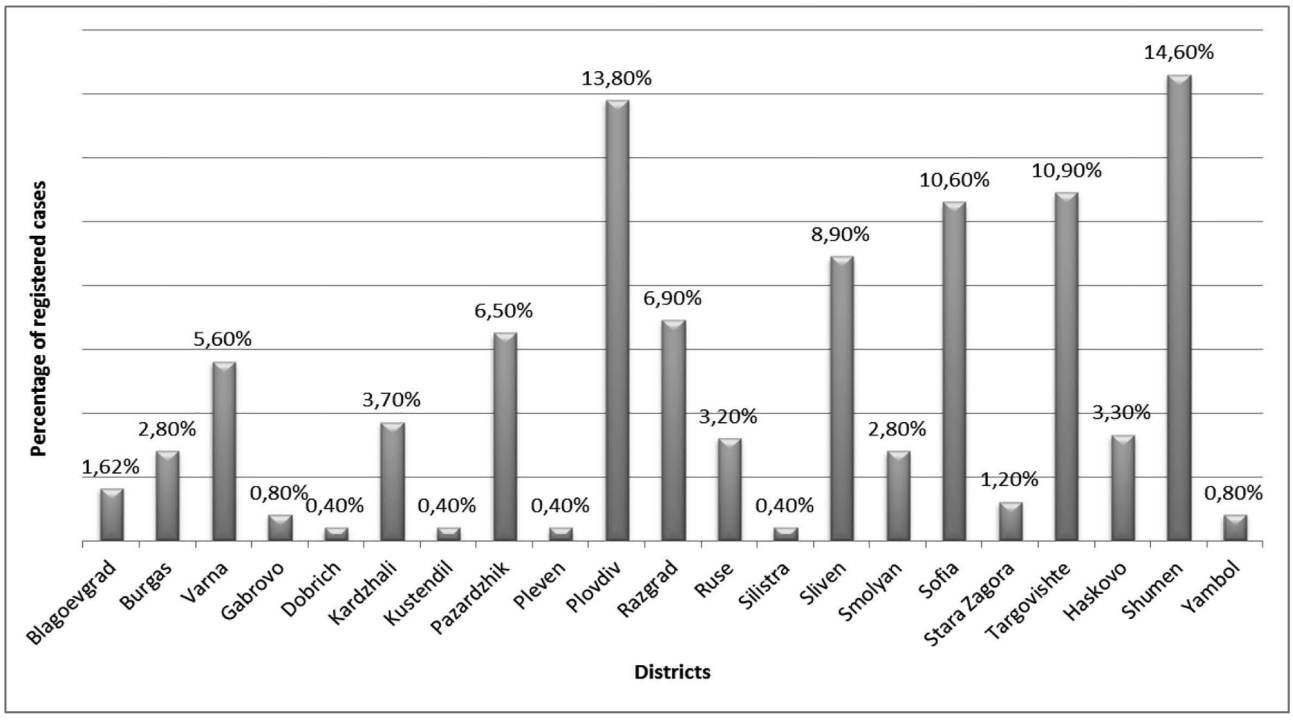

Fig. 2. Percentage of registered taeniosis cases caused by *T. saginata* by district.

Annual morbidity ranges from 0.63 per 100 000 (2008) to 0.23 per 100 000 (2017) with average morbidity of 0.37‰ (Fig. 3). The prevalence was higher among female subjects (61%) (Fig. 4). The distribution by age showed a significantly higher incidence in adults (88%) compared to the group of children and adolescents (22%) (Fig. 5). The most affected were the age groups 35-39 (11.79%) and 55-59 (11.38%) years (Table 1). Patients of different ethnic origin represent 54% of cases.

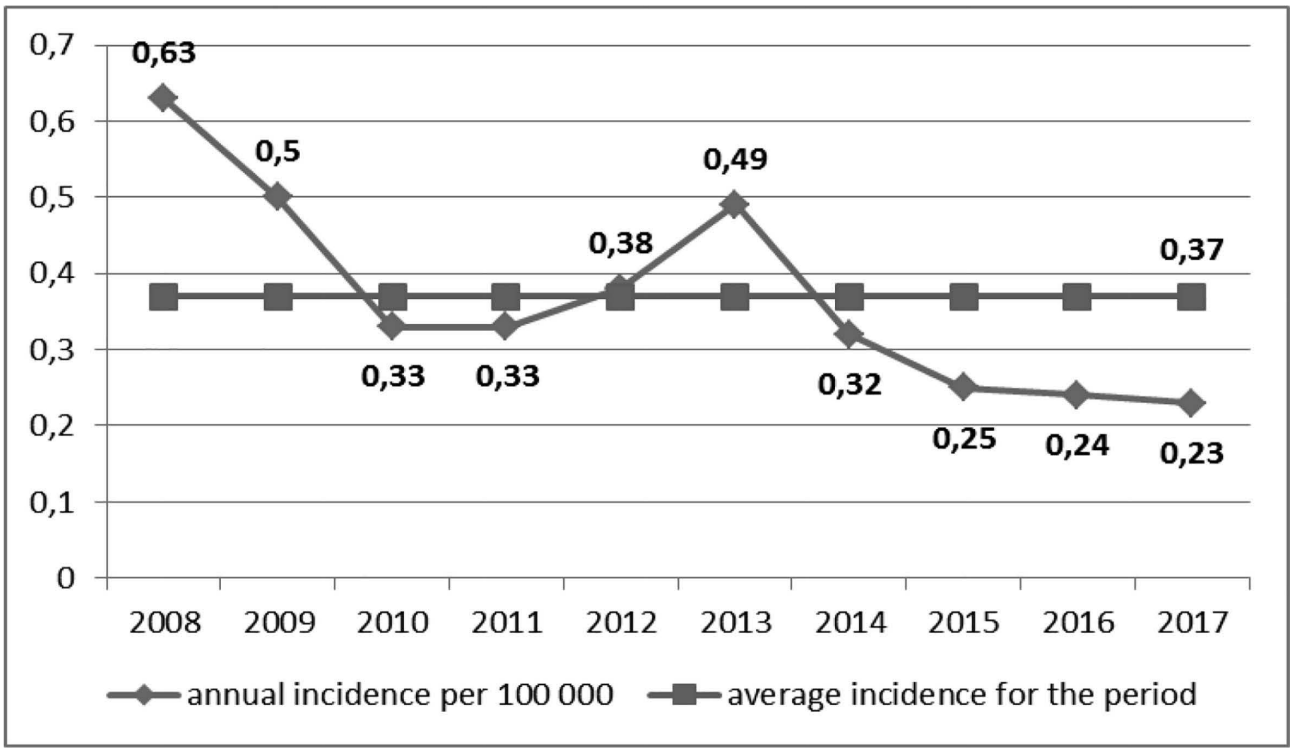

Fig. 3. Incidence of taeniosis caused by *T. saginata* by year (2008-2017).

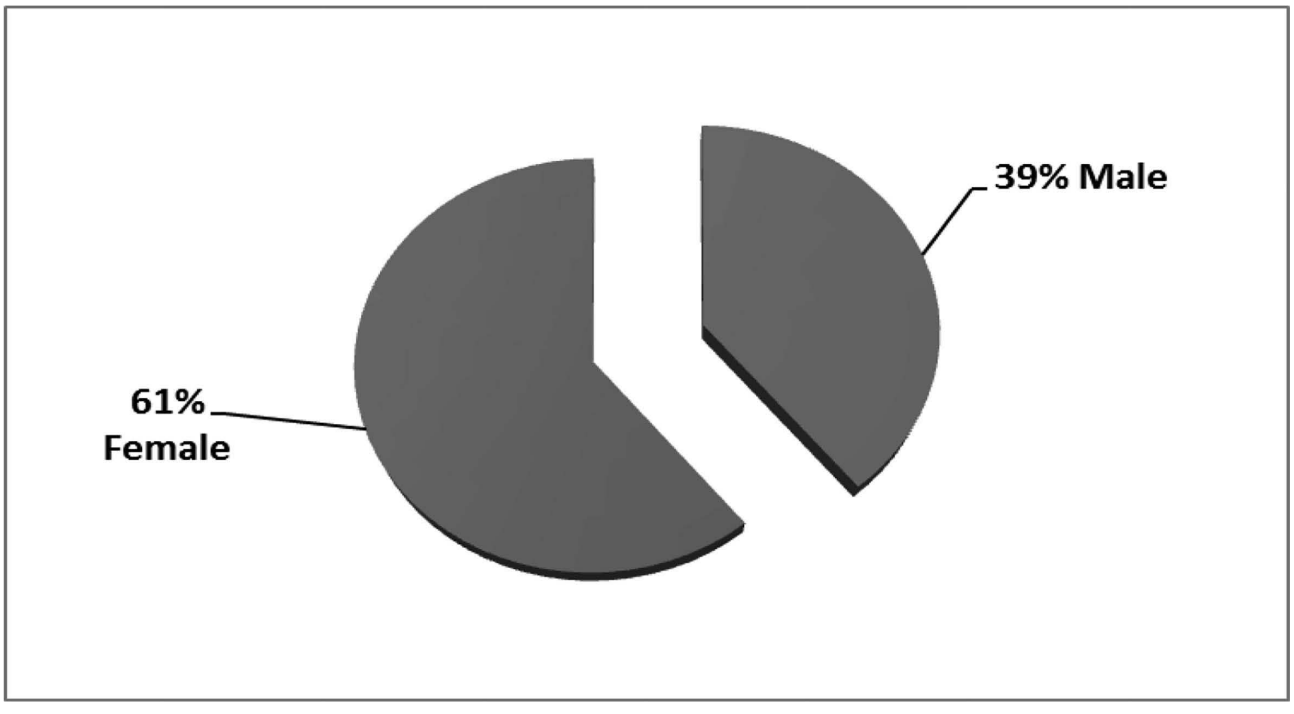

Fig. 4. Gender distribution of taeniosis cases.

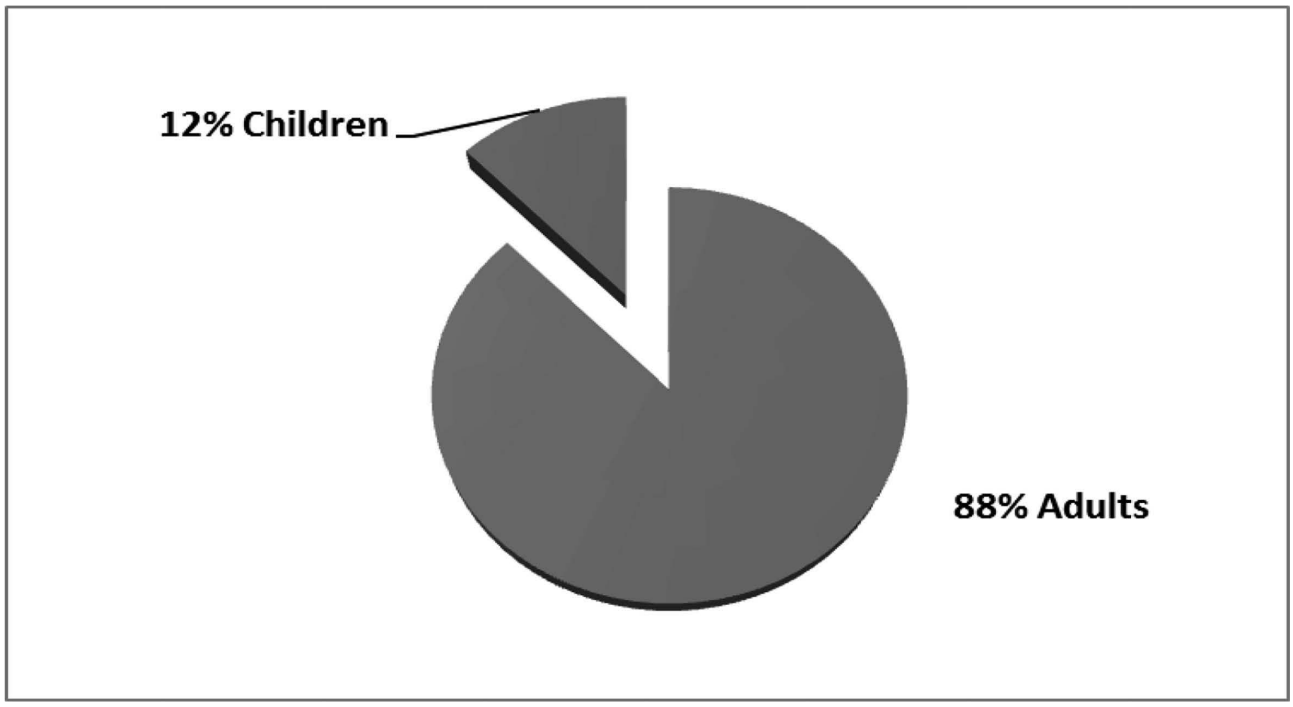

Fig. 5. Distribution of taeniosis cases among children and adults.

Table 1. Distribution of taeniosis cases by age group.

Age group/ Year	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
0 - 4	2	1	1	–	–	–	–	–	–	1
5 - 9	2	1	–	–	2	1	–	1	–	1
10 - 14	3	–	2	–	1	1	1	2	2	1
15 - 19	3	4	–	1	–	–	3	–	2	1
20 - 24	5	1	–	–	1	–	3	–	2	1
25 - 29	3	2	1	1	2	3	1	1	4	1
30 - 34	4	2	3	1	1	5	3	–	1	1
35 - 39	3	6	3	4	2	3	2	2	1	3
40 - 44	4	2	1	4	2	6	1	1	1	1
45 - 49	4	2	1	2	–	2	3	4	1	1
50 - 54	3	4	3	3	1	3	–	–	–	2
55 - 59	8	4	3	1	4	1	2	2	1	2
60 - 64	3	1	2	1	2	–	1	3	–	1
> 65	1	3	3	–	2	5	2	1	1	2
Mean	3.43	2.36	1.64	1.29	1.43	2.14	1.57	1.21	1.14	1,36
Standard Deviation	1.65	1.65	1.22	1.44	1.09	2.07	1.16	1.25	1.10	0.63
Confidence Level (95.0%)	0.953	0.950	0.702	0.830	0.629	1.195	0.669	0.723	0.635	0.366
Total	48	33	23	18	20	30	22	17	16	19
Percentage	19.51	13.41	9.35	7.32	8.13	12.20	8.94	6.91	6.50	7.72

DISCUSSION

Taeniarhynchosis is a disease with a cosmopolitan spread. *Taenia saginata* is thought to be more widespread in developing countries where hygiene and sanitary standards are below average and routine meat inspections are not always performed. However, in countries where standards of hygiene and sanitation are considered high and routine meat inspection is in place, for example in Europe, bovine cysticercosis is still widespread and the prevalence of the disease in humans ranges from 0.01% to 10% (3, 5). Data on the prevalence of taeniarhynchosis in the New World countries shows similar results with prevalence of 0.04% to 8.8% (5).

Data from our study shows that cases of taeniarhynchosis, even though sporadic, are reported annually in Bulgaria. During the study period 246 cases of taeniarhynchosis were registered in the country which exceeds more than 3 times the number of cases registered in the ten-year period 1991-2000 (6). Similar data is available for the spread of the disease in Croatia where 124 cases of taeniarhynchosis were recorded in 2004-2013. In Serbia and Romania 212 (1997-2004) and 3129 (2007-2014) cases of taeniasis caused by *Taenia* spp. were recorded, respectively, which are not identified to the species level (7). It is worth pointing out that the *Taenia* eggs found with helminthovoscopy are not species-specific and the species can be determined by microscopic examination of the tapeworm proglottids and /or by PCR (1).

The proportion of female patients exceeds that of male persons but there is no statistically significant difference (Student's t-test: t = -2.0275 <2.101). The distribution by age shows a statistically significant difference between the infected adults compared to the group of infected children and adolescents (Student's t-test: t = 6.2923> 2.101). The higher percentage of infected persons of different ethnic origin is explained by the fact that for religious reasons they consume more veal and beef, most of them are engaged in farming and meat production for personal consumption and home trade. In most cases the meat or meat products do not pass veterinary control.

Taeniarhynchosis distribution is highest in regions with well-developed private livestock farming (Shumen, Plovdiv, Targovishte) and high rate of home meat production without veterinary control. The high number of cases registered in Sofia is attributed to the close connection of the residents of the capital with the province. They often travel to the countryside to visit parents and close relatives and for supplying home-produced food, including meat and sausages.

In our opinion, the endemic spread of taeniarhynchosis in the country is mainly due to the poor health literacy of the population regarding the mechanisms of infection with bovine tapeworm, poor sanitation, especially in smaller settlements, which contributes to the contamination of the environment with human excreta and the possibility of infecting intermediate hosts, consumption of poorly-cooked meat products produced at home and insufficient veterinary control.

There are no significant problems regarding the diagnosis of the disease in humans. The eggs of *Taenia* spp. have a characteristic morphology and their detection in faecal samples is not difficult. Species differentiation of the parasite is also not a problem if proglottids are present in the samples. The characteristic uterine branches enable the differentiation of *T. saginata* from *T. solium* (8). There is a problem with the treatment of infected persons due to the lack of first-line drugs of choice in pharmacies (Praziquantel, Niclozamide). In some cases, this may delay the etiological treatment. During this period the infected person is a source of invasive helminth eggs and can contaminate the environment thus leading to infection of the intermediate hosts and the continuation of the epidemic process.

CONCLUSION

Overall, taeniarhynchosis in Bulgaria is not a serious problem for the public health system. However, the annual registration of cases, even though sporadic, requires greater efforts in control measures. On the part of human medicine efforts should be directed towards health promotion measures among the population, and on the part of veterinary medicine – towards improvement of control measures in the field of meat production, especially in small private farms.

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A CASE OF *VIBRIO VULNIFICUS* INFECTION IN A DIABETES PATIENT WITH FATAL OUTCOME

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ABSTRACT

Aim

The aim of this report is to present the case of a 44-year-old male tourist from Belgium with type 1 diabetes who developed necrotizing fasciitis and sepsis caused by *Vibrio vulnificus* during his stay at the Bulgarian Black Sea Coast.

Material and methods

Data was collected from the patient's examination records. *V. vulnificus* was isolated by culture on blood agar and identified with biochemical tests.

Results

During the initial hospitalisation the patient left the clinic without leave and returned 24 hours later. He was surgically treated and supported with intensive care. However, the patient developed severe sepsis which resulted in fatal outcome.

Conclusion

It is important to highlight the need for sufficient awareness among patients with diabetes and other serious chronic diseases of the potential threat posed by *V. vulnificus* infections.

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INTRODUCTION

Vibrio vulnificus belongs to non-cholera *Vibrio* species inhabiting marine waters. The bacteria can cause necrotizing soft tissue infection and primary septicaemia or acute gastroenteritis following the consumption of undercooked shellfish. The skin lesions most often appear in the first 24 hours after the onset of the disease. The infection may be fatal in untreated or immunosuppressed patients (1, 2, 3).

CASE REPORT

This study presents the case of a 44-year-old male tourist from Belgium with type 1 diabetes spending 7 days in Burgas at the Bulgarian Black Sea Coast. The patient reported swimming in the sea every day. Approximately 10 days before admission to hospital the patient complained of fever, pain and a newly appeared wound on the right leg. On examination he was febrile (up to 38.5°C) and the right lower leg region was with oedema and erythematous skin. It was concluded that there is a severe wound infection in the limb which was surgically treated. Specimen was collected for microbiological analysis. After treatment of the wound the patient left the clinic without leave and demanded to receive medical care in his own country. However, the airline refused to allow him to board the plane and after approximately 24 hours the patient returned to the hospital with haemorrhagic necrosis changes in the leg (Fig. 1). Meanwhile, *V. vulnificus* was isolated in the microbiological laboratory of the hospital and subsequently confirmed in the National Reference Laboratory (Fig. 2). Third-generation cephalosporin, vancomycin and metronidazole were administered and the wound was debrided early. Nevertheless, the patient developed clinical symptoms of sepsis with blood pressure 65/50 mmHg, heart rate of 125 bpm and body temperature of 39°C. Consultation with cardiologist concluded that there is a high coronary risk. In the course of the septic condition, the patient became unresponsive. Due to haemodynamic collapse and respiratory failure the patient was placed on mechanical ventilation, but despite the intensive care support, he died 2 days after hospitalisation.

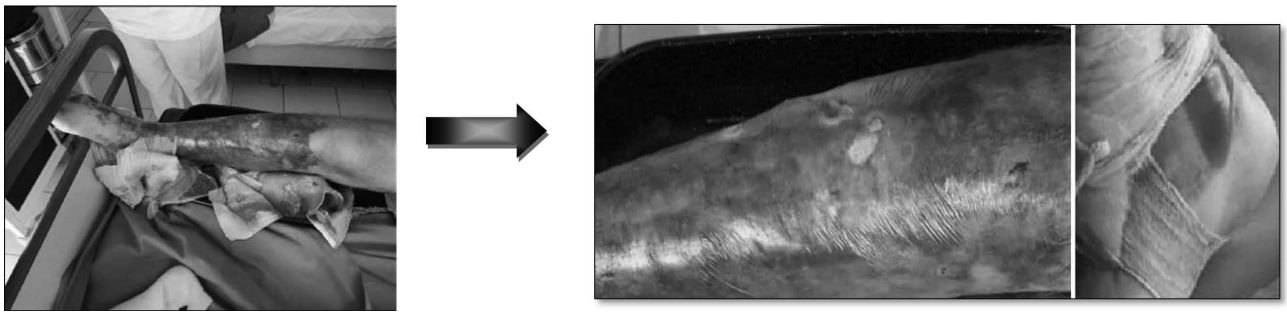

Figure 1. Severe wound infection in the right lower leg surgically treated in the Department of Vascular Surgery at UMBAL Burgas. Haemorrhagic necrosis and bullous changes in the right lower leg during the second hospitalisation.

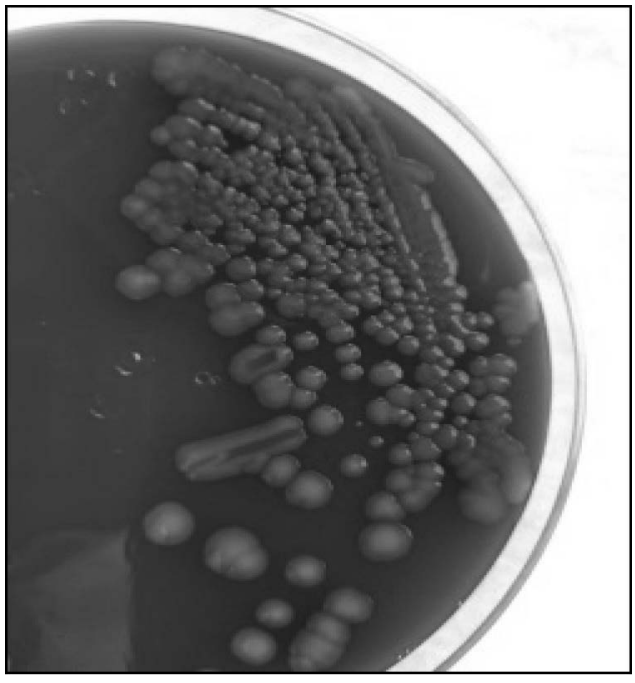

Figure 2. *Vibrio vulnificus* colonies on blood agar isolated from wound sample.

DISCUSSION

In this case, empirical antibiotic treatment was started before microbiological results were available. According to the World Health Organisation's recommendations the patient was treated properly, but there was a delay due to the interrupted hospital stay. Diabetes is also a serious aggravating factor. According to the Centres for Disease Control and Prevention Gulf Coast Surveillance System, diabetes is a risk factor in over 35% of cases (4, 5, 6).

This case highlights the need of including *V. vulnificus* in the differential diagnosis of sepsis and severe wound infections in patients with concomitant chronic diseases and epidemiological evidence of contact with seawater, and to avoid consuming raw seafood.

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