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ACKNOWLEDGEMENTS

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CARBAPENEMASE-PRODUCING GRAM-NEGATIVE BACTERIA IN BULGARIA – CURRENT STATUS

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ABBREVIATIONS USED IN THIS ARTICLE:

CR – carbapenem-resistant
UN – United Nations
MDR – multidrug-resistant
CRE – carbapenem-resistant *Enterobacteriaceae*
CRAb – carbapenem-resistant *Acinetobacter baumannii*
ESBL – extended-spectrum β-lactamase
EARS-Net – European Antimicrobial Resistance Surveillance Network
MLVA – multiple-locus variable-number of tandem repeat analysis
MBL – metallo-β-lactamase

ABSTRACT

The emergence and spread of carbapenem resistance among Gram-negative bacteria is a serious clinical and public health concern. Carbapenem-resistant (CR) pathogens can acquire genes encoding multiple antibiotic resistance mechanisms and have the potential to develop multidrug-resistant and pan-resistant phenotypes. Early diagnosis, active surveillance, and prevention of infections are necessary in order to develop strategies to limit their spread. For the past ten years CR *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. have been identified in major regional hospitals in the country.

KEY WORDS: carbapenem-resistant, Bulgaria, carbapenemase-producing *Enterobacteriaceae*

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INTRODUCTION

Dealing with infections caused by multi-resistant Gram-negative pathogens is becoming a growing challenge for clinicians because of the exhaustion of the antibiotic reserve. Concerns that we may soon find ourselves in a situation comparable to the pre-antibiotic era have already acquired real dimensions on a global scale (1). The gravity of the problem also prompted the convening of a high-level meeting during the 71st session of the UN General Assembly in September 2016, on resolving important issues related to antimicrobial resistance. As a result of this meeting, the 193 member states have signed a declaration to combat growing antibiotic resistance. The 71st UN Assembly also noted that more than 700,000 people die annually from infections caused by resistant pathogens worldwide.

With Gram-negative bacteria, the problem becomes more and more important, because they have the potential to develop resistance to one as well as almost all available antibiotic groups and to emerge as pandemic phenotypes (2). The spread of pathogens with such characteristics would have a negative and even devastating effect not only on public health but also on the main fields of modern medicine such as surgery, organ transplantation, chemotherapy, etc. MDR Gram-negative pathogens tend to become more prevalent in the society (2).

In 2013, the Centre for Disease Control and Prevention (CDC) published a report in which infections caused by resistant bacteria can be classified into three groups: “urgent”, “serious”, and “troublesome” (1). In general, the first two groups require increased attention, surveillance, and prevention. The first group, which requires the introduction of urgent measures to limit the spread of these pathogens, is referred to carbapenem-resistant *Enterobacteriaceae* (CRE). Polyresistant *Pseudomonas aeruginosa* and *Acinetobacter* spp, in which carbapenem resistance is more common, are classified in the second group, which requires implementation of serious measures (1). In 2015 the ECDC surveillance report revealed that the distribution of carbapenem-resistant isolates indicates a growing prevalence of CRE and CRAb in Europe (3). The published report highlights the need for coordinated efforts

of the European Union countries for early diagnosis, active surveillance, and prevention of infections caused by carbapenem-resistant pathogens (3).

Carbapenem resistance in *Enterobacteriaceae*

Enterobacteriaceae are part of the intestinal flora and are often the cause of community-acquired and nosocomial infections (4). Resistance to broad-spectrum cephalosporins due to the production of extended-spectrum β-lactamases (ESBL) continues to increase in this family (5).

Since the early 2000s there is an increase in ESBL production in some enterobacterial species such as *Escherichia coli* from 1.2% in 2000 to 10.0% in 2005 (5). Taking into account the fact that antibiotic consumption in Bulgarian hospitals is dominated by the cephalosporin group, it has been demonstrated that there is a correlation between antibiotic use and the incidence of ESBL-producing bacteria (6). There are several studies indicating the dissemination of ESBL-producing bacteria in different hospital care units in the country with CTX-M-like and SHV-like among the most prevailing types (5, 7, 8). The plasmids bearing ESBL genes also carry genes encoding resistance to other antibiotics, such as aminoglycosides (9) and quinolones (7). Studies have defined carbapenems as superior to quinolones for the treatment of serious infections caused by ESBL-producing organisms (10). In many cases carbapenems are the key drugs for infections caused by various enterobacterial species and they must be preserved as they are.

The spread of carbapenem-resistant *Enterobacteriaceae* (CRE) in Europe has continued to increase over the past few years with 13 countries reporting for regional CRE spread or endemic situation in 2015 (3). Sporadic cases and single and/or sporadic hospital outbreaks have been reported by 21 European countries. In Greece, Italy, and Malta CREs have been regularly isolated from patients in most hospitals, which corresponds to an endemic situation (3). Data from an ECDC study covering 30 European countries shows nearly two-fold increase in invasive CRE over 2009-2012 from 3.2 to 6.3%, respectively. In Bulgaria, the percentage of carbapenem-resistant invasive isolates of *Klebsiella pneumoniae* is 3.2% for 2015, according to EARS-Net data.

Enterobacteriaceae become resitant to carbapenems mainly through production of carbapenemases from Ambler classes A, B, and D (11). The most commonly found carbapenemases in *Enterobacteriaceae* are KPC (*Klebsiella pneumoniae* carbapenemase), VIM (Verona integron-encoded metallo-β-lactamase), IMP-type metallo-β-lactamase (IMP), NDM-1 (New Delhi metallo-β-lactamase), and OXA-48 (OXA-48-like carbapenem-hydrolysing oxacillinase). Less common is the carbapenem resistance due to the production of ESBLs and/or AmpC cephalosporinases in combination with reduced permeability of the outer membrane (12). Genes responsible for carbapenemase production are generally associated with mobile genetic elements resulting in their rapid dissemination (11, 12). Among the measures for limiting the spread of carbapenem resistance are precise identification of resistance mechanisms and knowledge of the current situation on local country level.

In our country carbapenemases of all classes have been identified among *Enterobacteriaceae* with reports on sporadic occurrence during the last five years.

In 2013, Markovska and colleagues identified three *Proteus mirabilis* isolates with reduced susceptibility to cephalosporins and carbapenems that produced VIM-1 carbapenemase and the acquired AmpC β-lactamase CMY-99 at the Alexandrovska University Hospital in Sofia (13). In the same year was reported the first identification of a *K. pneumoniae* isolate, producing KPC-2 and VIM-1 (14).

Usually the genes responsible for rRNA methyltransferase production are associated with ESBLs but they also have been found in association with carbapenemases: NDM-1 and VIM-1 carbapenemase genes associated with RtmB and ArmA methyltransferase in *Enterobacteriaceae* isolated from cancer patients. Considering that carbapenems and aminoglycosides may have a synergistic effect in therapy, the prevalence of strains resistant to these antimicrobial agents would have

a dramatic effect on the choice of optimal therapy (15).

Although *E. coli* is not among the usual causes of nosocomial outbreaks, the first one caused by NDM-1-producing *E. coli* was documented in 2013 in Bulgaria at the MMA (Military Medical Academy), Sofia. According to the authors, all isolates belonged to ST101 and co-produced the extended-spectrum β-lactamase CTX-M-15 as well as 16S rRNA methylase RmtB leading to high-level aminoglycoside resistance (16).

OXA-48-type carbapenemases are increasingly reported in enterobacterial species (17). Up to date there is limited data on the detection of this carbapenemase in our country, though it has gradually disseminated in the wider Mediterranean area and Europe, especially in Turkey (17). The low level of carbapenem resistance and the lack of specific OXA-48 inhibitor may result in misidentification of the strains, but phenotypic detection by routine antibiogram was proposed for enterobacterial isolates (18). Data on the first full genetic characterisation of OXA-48-producing *K. pneumoniae* isolate form Bulgaria were published in 2015 (19). The isolate had reduced susceptibility to carbapenems but remained susceptible to broad-spectrum cephalosporins. PCR and sequencing confirmed the presence of $bla_{\text{OXA-48}}$ gene flanked by two intact copies of IS1999 on truncated ΔTn1999.1. This transposon was located on unusual non-typeable 29-kb plasmid that could be transferred only by transformation. Multilocus sequence typing (MLST) indicated the presence of sequence type ST530. This isolate has a number of unique characteristics that distinguish it from the typical OXA-48 producers established in Europe. The non-conjugative nature of the OXA-48 plasmid is likely to prevent its effective spread in the future and no such strain has actually been reported in the country so far. There are few reports on *K. pneumoniae* carrying $bla_{\text{OXA-48}}$ in Bulgarian hospitals: from Markovska et al. (20) and Syracov et al. (21).

Recently Pfiefer et al., published data on clonal transmission of Gram-negative bacteria harbouring different β-lactamase genes in a Bulgarian hospital (22). NDM-1-producing *Morganella morganii, E. coli,* and *Providencia rettgeri* were identified (22). The authors define the identification of NDM-1-producing *P. rettgeri* strain as a rare finding, because the isolate had a set of additional resistance genes, such as $bla_{\text{CMY-6}}$, $bla_{\text{VEB-1}}$, $bla_{\text{OXA-1}}$, aac_{A4}, qnr_{B1}, qnr_{A1}, *armA*. The NDM-1-producing *M. morganii* had different resistance gene combinations including ($bla_{\text{DHA-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, aac_{A4}). The study included also a carbapenem-resistant *Enterobacter cloacae* strain but carbapenemase activity was not detected and the resistance to ertapenem was linked to modification or loss of outer membrane proteins in combination with β-lactamase production (22).

Almost exclusively high-risk international *K. pneumoniae* clones were detected in the National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance at the NCIPD after implementing MLST typing for different carbapenemase producers of this bacterial species (23): ST11 (NDM-1), ST15 (KPC), ST147 (VIM), and ST258 (KPC-2). The molecular epidemiology of *K. pneumoniae* needs further investigation because this microorganism seems to possess the ability to acquire various carbapenemase genes and carbapenemases of all classes (A, B, and D) have already been reported in our country.

Carbapenem resistance among *P. aerugionosa* and *A. baumannii*

P. aeruginosa and *A. baumannii* are opportunistic pathogens that are often isolated in hospital environment, mostly from the intensive wards. The treatment of infections caused by these species is more and more challenging due to the increasing resistance to several classes of antibiotics such as β-lactams, quinolones, and aminoglycosides (24, 25) In Bulgaria, the percentage of carbapenem-resistant invasive *A. baumannii* and *P. aeruginosa* isolates for 2015 are 78.6% and 27.3%, respectively, according to EARS-Net data.

P. aerugionosa often becomes the cause of difficult-to-treat nosocomial infections (24). This bacterium is known for its intrinsic resistance and the ability to acquire additional resistance mechanisms that result in emergence of MDR strains frequently isolated among patients receiving intensive care treatment (26).

Carbapenem resistanse in *P. aerugionosa* for 2003 was estimated as 8.6% according to BulSTAR data (27). One of the first studies of antimicrobial resistance in 203 *P. aerugionosa* isolates collected for a 5 year period (2001-2006) from five university hospitals in Sofia revealed that 49.8% of the strains were multidrug-resistant. The authors found a variety of β-lactam resistance mechanisms such as AmpC and ESBL production. Carbapenem resistance was a result of OprD protein deficiency and active efflux and was not related to production of class B carbapenemases (26). The first report on VIM-producing *P. aerugionosa* isolates from Bulgaria comes in 2008 with the identification of two new VIM-2 variants: VIM-15 which differs from VIM-2 by one amino acid substitution that caused a significant increase in its hydrolytic efficiency, and VIM-16 with hydrolytic activity not influenced by Ser54Leu substitution (28). The genes encoding VIM-15 and VIM-16 were described as a part of class I integron (28).

The first data on the molecular epidemiology of MDR *P. aerugionosa* showed 23 different MLVA profiles among 29 isolates collected from seven Bulgarian hospitals (29). The phenotypic and genetic tests for carbapanemase detection showed absence of enzyme production but revealed the presence of other resistance mechanisms. The majority of isolates produced OXA-type enzymes (OXA-10, OXA-7, 0XA-17, OXA-47, OXA-42, OXA-2) and six isolates carried the gene encoding the extended-spectrum β-lactamase VEB-1. All VEB-1 producers recovered from four hospitals were defined as ST111or ST244 after MLST analysis. The authors observed a lack of OprD production in 97% of the isolates which was considered the main reason for carbapanem resistance. Overexpression of AmpC was documented in 17% of the studied strains. The percentage of isolates overexpressing MexXY-OprM was high - 72% (29).

A. baumannii carries the species-specific OXA-51 that results in carbapenem resistance only in cases of overexpression of the encoding gene, and the most common mechanism of carbapenem resistance in this species is related to the acquired OXA-23-like, OXA-24-like, OXA-58-like, OXA-143, and OXA-235 (25).

The first OXA-23-producing *A. baumannii* were reported for the first time in Bulgaria by Stoeva et al. (30). OXA-23 carbapenemase has also been identified as the main mechanism responsible for carbapenem resistance in *A. baumannii* isolates at the University Hospital in Pleven for a period of seven years (30). Later, OXA-23- and OXA-58-producing *A. baumannii* were described at the MMA Sofia, confirming the prevalence of class D carbapenemases and their concurrent occurrence in this frequently isolated nosocomial pathogen (31). The first *A.baumannii* isolate, producing OXA-24, was identified in the National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance at the NCIPD (32). Molecular genetic assays have demonstrated the presence of both $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-23}}$ genes in the isolated strain (32).

The epidemic and endemic potential of *A. baumannii* as well as persistence of clones over time in hospital settings was demonstrated in a study from two Bulgarian hospitals (the University Hospital “St. Marina” and Municipal Hospital “St. Anna” in Varna). The multidrug resistance in this pathogen was associated with intrahospital dissemination of IC1, 2, and 8 and with unique local clones (33).

The latest data on the persistence of carbapenem-resistant *A. baumannii* in a Bulgarian hospital over a period of 5 months (September 2014 – January 2015) identified the existence of nine different clones belonging to eight sequence types (ST350, ST208, ST436, ST437, ST449, ST231, ST502, and ST579). All isolates possessed genes encoding the production of OXA-23 and/or OXA-72 carbapenemase (22).

CONCLUSION

For the past ten years, carbapenemase producers of all classes have been identified among *Enterobacteriaceae* in major regional hospitals in the country (13, 14, 15, 16, 19). The fact that the majority of carabapenemase-producing isolates in our hospitals were represented by different enterobacterial species (*K. pneumoniae, E. coli, P. mirabilis, M. morganii, P. rettgeri*) harbouring carbapenemases of classes A, B, and D, confirms the potential for spreading this resistance among some of the most common

causes of nosocomial and community-acquired infections.

In summary, while the production of metallo-β-lactamases is uncommon among *P. aeruginosa* isolates from Bulgarian hospitals, MDR profiles frequently result from the production of ESBLs combined with the lack of production of the carbapenem porin OprD and the overexpression of the MexXY-OprM efflux pump (26, 29).

Frequent hospital outbreaks caused by carbapenem-resistant *A. baumannii* have been reported, with no evidence of MBLs. OXA derivatives are primarily identified and their detection may be challenging due to the lack of class D carbapenemase inhibitor and the additional mechanisms that can lead to carbapenem resistance (30, 31, 32, 33). The misidentification of plasmid-encoded carbapenemases in one of the most commonly isolated nosocomial agents, such as *A. baumannii,* could facilitate their spread to other bacterial species with the ability to cause hospital outbreaks.

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MULTILOCUS SEQUENCE TYPING OF MULTI-DRUG RESISTANT BACTERIA IN THE ERA OF WHOLE GENOME SEQUENCING

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ABBREVIATIONS USED IN THIS ARTICLE:

MDR – multidrug-resistant
XDR – extensively-drug resistant
PDR – pan-drug resistant
MLVA – multiple-locus variable-number of tandem repeat analysis
MLST – multilocus sequence typing
RAPD – random amplification of polymorphic DNA
PFGE- pulsed-field gel electrophoresis
AFLP - amplified fragment length polymorphism
PCR – polymerase chain reaction
VRE – vancomycin resistant *Eneterococcus*
VSE – vancomycin-sensitive *Eneterococcus*
ST – sequence type
CC – clonal complex
CG – clonal group
SLV – single locus variant
DLV – double locus variant
TLV – triple locus variant
NDM – New Delhi metallobeta-lactamase
KPC – Klebsiella pneumoniae carbapenemase
VIM – Veronaintegron-encodedmetallo-β-lactamase
MRSA – methicillin resistant *Staphylococcus aureus*
ESBL – extended-spectrum β-lactamase

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ABSTRACT

Over the past three decades molecular genetics methods for identification and typing of high-risk strains are developing very rapidly. Their role is crucial in elucidating epidemiological dissemination of multi-drug (MDR) and pan-drug resistant (PDR) bacterial clones. While the Whole Genome Sequencing (WGS) technologies are increasingly adopted for typing and surveillance of antimicrobial resistant bacteria, the basic concepts and bioinformatic databases used are derived from the Multilocus Sequence Typing (MLST) that long has been considered the “main stage” for phylogenetic studies. Here we review the MLST approaches for typing of the MDR bacteria with public health significance.

INTRODUCTION

Antimicrobial resistance is one of the distinctive traits of the problematic bacterial strains because it reduces the appropriate treatment options and thereby contributes to increased patient mortality. The term “multidrug-resistance“ has stayed poorly defined in literature for a long time but presently MDR is considered as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. MDR bacteria are hosts for multiple genetic elements like genes, integrons, transposons and plasmids that determine their antibiotic resistant phenotype (1). Multi-resistance needs to be investigated especially when it is associated with an outbreak in healthcare or community settings and this is achieved by molecular-epidemiology analysis.

Over the past three decades molecular methods for identification and typing of high-risk strains are developing very rapidly. The most common methods currently used for epidemiological purposes are Multilocus Sequence Typing (MLST), Pulsed-Field Gel electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP) analysis, Multiple-Locus Variable number of tandem repeat Analysis (MLVA) and other PCR fingerprinting methods. Through the years a set of laboratory methods based on the above were applied for species identification, for determining antibiotic resistance profiles, virulence factors of the strains and the epidemiological relationship between them, but now all of these time and resource consuming techniques can be replaced with one single molecular method MLST.MLST was first

introduced by Maiden et al. in 1998 for typing of the human pathogen *Neisseria meningitidis*, causing bacterial meningitis and sepsis. They studied DNA sequences of approximately 470 bp fragments from 11 housekeeping genesin a reference set of 107 isolates *N. meningitidis*(2).

The principle of MLST is based on PCR amplification of seven or eight housekeeping genes by specific primers, and subsequent bidirectional DNA sequencing of the resulting amplicons using the same or additional internal primers. Housekeeping genes undergo mutations rarely and from an evolutionary perspective are the least variable compared to other genes in the genome. When differences in the nucleotide sequence of the genes under examination occur, they are mostly adaptive and reflect the pathogens process of adaptation to new environmental conditions. The optimal size of fragments is about 450-500 bases (with few exceptions), as this size is sequenced with a minimal number of errors, with a single pair of primers, using an automated DNA Sequencer. Often the studied gene is present in the genome of multiple related species, which will provide sufficient variations for recognizing many alleles within the population. When comparing genes of two strains, it is possible to find difference in their sequence, caused by mutations. Each different sequence of studied genes is recorded as a new allele. By the number and location of mutations, one can judge about the similarities between different strains. The method characterizes the strains by their unique allelic profiles. The sequence (allele) of each studied gene is compared with the others sequence in a global database for the respective microorganism. Combination of all allele profiles determines sequence type (ST) of the strain. The method is suitable to establishing phylogenetic relationships between different strains of a species (3). The sequences are processed in 3 stages: data collection, data analysis and multilocus analysis. At the first stage, localization of the mutations occurs after determination of the nucleotide sequence of the house-keeping gene fragments. In the next stage (data analysis), all unique gene sequences are designated by a number, and the combination of all allele profiles determines the sequence type (ST) of studied strain. If new alleles and sequence types are found, they are stored in web-based database after verification by database administrator. At the final stage of the assay, the relatedness among isolates is determined, by comparing the allelic profiles.This method allows epidemiological and phylogenetic studies to be performed, by comparing the sequence types of various monoclonal complexes, thus monitoring the evolution of the studied pathogens (4)

MLST advantages

Multilocus sequence typing has several major advantages over other typing methods (PFGE; MLVA; AFLP and etc)

- Bidirectional DNA sequencing ensures unmatched reproducibility
- High discriminatory power is achieved by studying multiple house-keeping genes
- MLST involves genes with relatively low mutation rate; variations in these genes occur when microorganism develops antibiotic resistance or adapts to changing environmental conditions which helps in understanding evolution of studied microorganism;
- MLST is easy to use and does not require sophisticated equipment and reagents with the exception of DNA sequencer
- Scalability from typing of a single strain to hundreds of samples (high-throughput).
- Accessibility - processed data can be easily transferred between laboratories and shared globally via web-based databases (2). Protocols and primers for each individual species are easily accessible, as they are published in general database.
- Versatility- the method is applied in many areas, for example in research microbiological laboratories, in veterinary laboratories, public health centers, in the food industry and also for studying plant pathogens (2)(5).

MLST disadvantages

- One of disadvantage of MLST is the use of only seven housekeeping genes, which limits the ability to describe relationship between more diverse strains.
- MLST is one of the most discriminative methods for population genetics currently available is relatively expensive. The cost of determining an allele of one gene is approximately 3-5 euro, therefore, the establishment of a sequence type, is about 35€, provided that perfect determination of the nucleotide sequences occur in all samples

at the first sequencing attempt. If there are sequence errors or incomplete sequence(s), it is necessary to modify sequencing parameters or concentration of agents in the reaction, and then sequence reaction must be repeated, which makes it even more expensive.

- Another disadvantage is that the primers and protocols for some of the epidemic types have not been optimized, which limits their use in epidemiological studies.

To improve the discriminatory power of MLST, a multi-virulence-locus sequence typing (MVLST) method, i.e. sequencing of virulence genes based on studies on *Listeria monocytogenes*, was introduced MVLST is directed against the virulent genes of pathogens and expands the benefits of the typing method; the disadvantage of this method is that virulence genes have a high incidence of variability compared to house-keeping genes and are much more difficult to group into common clonal groups and complexes (6).

Some authors suggest that, MLST is not suitable for routine infection control or outbreak investigation due to high cost, labor intensity, and lack of broad access to high-throughput DNA sequencing (7)

Before considering the applications and interpretation of a method for different microorganisms one must provide **definitions of certain terms used when working with MLST.**

Allele- the unique nucleotide sequence of sequenced housekeeping gene fragment. Every unique sequence receives an integer number.

Sequence type (ST) – the unique combination of allele of different genes. For example combination of seven housekeeping genes of *K. pneumoniae,* gapA-1; infB-1; mdh-1; pgi-1; phoE-1; rpoB-1 and tonB-1. This allele combination corresponds to sequence type 15 (ST15). Other sequences types have a different combination of alleles. Allele sequences, which are determined by MLST and ST profiles are stored in curated databases hosted at different sites worldwide. One of the largest databases is maintained by the University of Oxford (http://pubmlst.org) (8).

SLV (single locus variant) represents the differences between two sequence types in only one of studied housekeeping genes

DLV (double locus variant) - STs differ in two gene sequences

TLV (triple locus variant) Difference between STs in three housekeeping genes

CG or clonal group - When the difference between several sequence types is only in one gene (SLV), then these types is united in a common clonal group.

Founder ofCG - a sequence type, with the largest number of established strains that is central to a number of SLV sequence types deriving from it.

Clonal complex (CC) – related **c**lonal groups form a clonal complex, if founders of each group have certain level of similarity. These genotypes are formed by a series of comparisons between the results obtained from different laboratories that are subsequently processed by the Java program eBurst. Groups, that are included in one clonal complex, should have a similarity in at least five of seven housekeeping genes. Each sequence type must have a difference between his closest type only in two alleles to be in a common clonal complex; they also must possess an identical allele of the gene, which is the most conservative. If one ST has a different sequence of most conservative allele, it is moved to other clonal complex. **http://eburst.mlst.net/2.asp**

eBURST - eBurst is algorithm, which identifies mutually exclusive groups of related genotypes in the population and attempts to find the founding genotype of each group. The algorithm of eBURST predicts the relationship from the other predicted founding genotype to the other genotypes in the group, displaying as a radial diagram, centered on the founder genotype. **http://eburst.mlst.net.** (9)

Currently there are four online MLST databases: PubMLST- https://pubmlst.org/; MLST.net - http://www.mlst.net/databases, database of the Institute Pasteur - http://bigsdb.pasteur.fr/ and Warwick EnteroBase - http://enterobase.warwick.ac.uk/. The PubMLST database is supported by Department of Zoology, University of Oxford UK. MLST.net is hosted at Imperial College, London UK. Warwick EnteroBase is supported by Warwick Medical School, Coventry UK. These databases contain MLST data of over 140 microorganisms. The species that are most frequently associated with nosocomial infections are *E. coli, P. aeruginosa, A. baumannii, K. pneumoniae, S. aureus, S. pneumoniae, E. faecium,* and *S. enterica.* These bacteria have over 2000 sequence types each and databases are regularly with new ones. (10)

MLST for *Klebsiella pneumoniae*

*Klebsiella pneumoniae*is one of the major sources of hospital infections associated with high rates of morbidity and mortality and is among the most common pathogens isolated in intensive care units. The limited treatment options for the infections caused by MDR *K. pneumoniae* are due to its capability to accumulate antibiotic resistance genes under selective antibiotic pressure. This happens by de novo mutations or via acquisition of transferable genetic elements leading to emergence of extremely drug resistant strains. The carbapenem resistance in this pathogen is well documented, as well as resistance to quinolones and aminoglycosides (11). Recent studies highlight the emergence of multidrug-resistant *K. pneumoniae* strains which show resistance to the last-line antibiotic colistin (12). The novel methods for molecular typing provide useful information for the dissemination and the evolution of this pathogen. For characterizing isolates and detecting the epidemiological relationships between different strains several molecular methods are used: RAPD, PFGE, and AFLP which are useful for outbreak investigation at the local level but often lack the reproducibility needed to achieve highly informative and specific data. The difficulties with tracing epidemiological relationship between various isolates are overcome by MLST (13).

The MLST scheme for *Klebsiella pneumoniae* was developed by Laure Diancourt(11).

Initially the sequences of seven housekeeping genes were obtained for 67 *K. pneumoniae* strains, among which 19 ceftazidime- and ciprofloxacin resistant isolates. The isolates were collected during the SENTRY Antimicrobial Surveillance Program and selected from different European hospitals and clinical sources. The group of isolates with various origins was aimed to estimate the discrimination of MLST within strains with no epidemiological links.

For amplification and sequencing of internal sections of the housekeeping genes specific primer pairs were designed by Diancourt et.al (11). The *K.pneumoniae* MLST scheme, developed by Diancourt uses internal fragments of the following housekeeping genes, that encode proteins essential for the cell metabolism, such as: beta-subunit of RNA polymerase (rpoB), glyceraldehyde 3-phosphate dehydrogenase (gapA), malate dehydrogenase (mdh), phosphoglucose isomerase (pgi), phosphorine E (phoE), translation initiation factor 2 (infB), and periplasmic energy transducer (tonB). These genes are selected because they show low level of mutations (11).

The selected genes were located far from each other on the chromosome and could be amplified by PCR. Nucleotide variation was identified in all genes, with 6 to 21 distinct alleles. After combining these seven genes 40 sequence types (STs) were defined. eBURST analysis (http://eburst.mlst.net/) revealed the existence of two clonal complexes, one including ST14 and ST15, the other including ST16 to -22. When considering only the isolates without known epidemiological link, the discriminatory index (Simpson index) was 96%. For further validation of the method all the strains used in the survey were analyzed by rybotyping (a method highly discriminatory in this bacterium). Simpson's index, calculated for ribotyping data on the set of isolates with unidentified epidemiological relationships, was 98%. The number of the distinguished ribotypes was 46, four STs (ST5, ST15, ST23, and ST42) were subdivided into two ribotype profiles. Noteworthy all the different ribotypes within a ST were very similar, probably connected with a common evolutionary ancestor. The authors concluded that MLST data of larger sets of isolates can provide much wider understanding of the evolution and dissemination of *K. pneumoniae* multi resistant strains (11)(14).

Up to 2017 the MLST database for *K. pneumoniae* contains 2878 sequence types (ST) and is one of the largest databases. During the last two decades, numerous MDR and XDR *K. pneumoniae* sequence types have emerged showing superior ability to cause cross-continent outbreaks, and continuous global dissemination. The most frequently reported MDR sequence types are ST258; ST15; ST11; ST147; ST14; ST101 and ST307. Strains belonging to these sequence types cause nosocomial infection in many countries – China; USA; Greece; Australia; Switzerland; Czech Republic; Spain; Italy; Germany; Thailand; India; Taiwan; Argentina; Turkey; Bulgaria e.g. (15)(16)(17)(18).

It is noteworthy that a lineage, known as ST258 carrying genes for the carbapenemase KPC has become an example of MDR *K. pneumoniae*

clonal spread. ST258 has been identified worldwide, emphasizing its role in the spread of carbapenem resistance (19). *K. pneumoniae* ST258 and ST11 are often responsible for hospital outbreaks. Recently in China was reported a large outbreak caused by hypervirulentcarbapenem resistant *K. pneumoniae* belonging to ST11. Genomic analyses showed that the emergence of these carbapenem-resistant *K pneumoniae* with sequence type ST11 strains was due to the acquisition of pLVPK-like virulence plasmid by classic ST11 *K. pneumoniae* carbapenem-resistant strains, which is the prevalent ST in the country (15).

The genes encoding class B carbapenemasebla_{NDM} were found in ST340 in South Korea hospital units (20). In United Kingdom diverse STs contribute to the dissemination of bla_{NDM} –ST14, ST11, ST149, ST231 and ST147 (21). Another class B carbapenemase, often produced by *K. pneumoniae* is bla_{VIM}. The sequence types reported to harbor that resistant mechanism are ST147, ST36, ST273, ST283. In France ST395, a single locus variant of ST134 was involved in hospital outbreak caused by bla_{OXA-48} producing *K.pneumoniae*(20).

The data for evolution of MDR, XDR and PDR (pan drug resistant) *K. pneumoniae* complexes indicates transfer and spread of antibiotic resistance genes, and epidemic plasmids in highly successful disseminated *K. pneumoniae* clones.

The reportedESBL producing *K. pneumoniae* in Bulgaria represent the highest percentage (75%) in Europe (22). Eight different sequence types have been identified in four Bulgarian hospitals. MLST-type of the isolates from Varna and Pleven were ST15 (n=27), ST76 (n=6), ST1350 (n=2), ST101, ST258 (n=1), and ST151 (n=1) were detected. All except for one KPC-2 producing *K. pneumoniae*isolate were mostly in combination with CTX-M-15. ST11 was dominantin one hospital, while in another hospital the very rare ST530 was detected. ST11 was associated with carbapenemase NDM-1, while ST530 possessed thebla_{OXA-48} gene(17)(23)(24). This underlines the need for tracing the dissemination and evolution of the *K. pneumoniae*strains and their ability to acquire and spread diverse antimicrobial resistance determinants.

MLST for*Escherichia coli*

Escherichia coli is opportunistic pathogen and causes over 160 million infections in patients with compromised immune system and 1 million deaths per year (25). *E.coli*is the most common agent causing infections of urinary tract and bacteremia. Since the 2000s there is an increase of the isolates resistant to beta-lactams, mainly due to production of ESBLs and recently by carbapenemases. As a result researchers are interested in the biology of multi-resistant, extra intestinal pathogenic *E. coli* strains.

There are three MLST schemes available for *E.coli,* but the most widely used are the 'Achtman' hosted at the Warwick Medical School (Coventry, UK) http://mlst.ucc.ie/mlst/dbs/Ecoli) and 'Pasteur' developed by by Sylvain Brisse and Erick Denamur and hosted at the Pasteur Institute (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi. html). The *E. coli* MLST database created by Thomas Whittam was developed forenteropathogenic*E. coli.* These schemes are based on different gene combinations and have only one gene on common gene. The coherence observed between phylogenies derived from the sequence data from each of the three MLST schemes and the phylogeny based on whole-genome data showed that the Warwick MLST genes provide the most consistent results (26).

For the development of Warwick MLST scheme fragments of seven housekeeping genes distributed across the *E. coli* chromosomewere sequenced in 462 isolates from diverse sources (from 421 humans and 41 animal species). The strains were isolated in different geographic area in Europe, Africa, North America and the Pacific Rim. The sequence data of the following genes- adk (adenylate kinase), fumC (fumarate hydratase), gyrB (DNA gyrase), icd (isocitrate/isopropylmalate), mdh (malate dehydrogenase), purA (adenylosuccinate dehydrogenase) and recA (ATP/GTP binding motif) are available on the public website http://mlst.warwick.ac.uk/mlst/. The usage of this MLST scheme led to the knowledge that the majority of CTX-M-15 ESBL-producing isolates belong to ST131 (defined also as clone, B2,O25:H4). ST131is classified as virulent phylogroup B2, a reported causefor urinary tract infections that can be recognized by specific PCR of the *pabB* gene (27). ST131 is disseminated worldwide and can be subdivided in to multiple variants by PFGE. For instance

the five most frequently isolated CTX-M-producing *E. coli* strains in United Kingdom were recognized as A-E by PFGE and the fact they all belong to ST131 was unnoticed for several years (27). This underlines the advantages of MLST as a population-focused approach and the parallel application of PFGE as a strain typing method for studying of emergence events. If the clones are successfully disseminated they acquire the locally prevalent resistance plasmids. A good example is ST131 that may carry various resistant determinants and display multiple resistance profiles. According to a Japanese study there are two ESBL-producing *E. coli* clones prevailing in health care settings -O25:H4-ST131 and O86:H18-ST38. The first one is a producer of various ESBLs such as CTX-M-14, CTX-M-2 or -35, while the other clone (O86:H18-ST38) is associated with CTX-M-9 or CTX-M-14 enzymes (28). ESBL negative ST131 isolates have been found in 7% of stool samples from healthy residents in Paris. In Madrid ST131 strains were reported as TEM-30, -33 and -37 producers, and all encoded genes were found to be a part of IncFII plasmids. ST131 is considered to be the founder of a clonal cluster containing 13 single locus variants and 3 double locus variants. There are several lineages of virulent *E. coli*phylogroup D that are associated with multi-resitance: ST69 (clonal group A; CgA), ST405 and O15:K52:H1. ST405 is increasingly reported worldwide, associated or not with production of CTX-M-15 ESBL. The CMY-2 enzyme was found in eight phylogroup D STs in Spain, mainly STs 57, 115, 354, 393 and 420 (29).

Several *E. coli* clones belonging to phylogroup A (STs 10 and 23) are increasingly associated with resistance to beta-lactams due to ESBLs production and hyperexpression of AmpC enzymes. Two members of phylogroup B -STs 155 and 359 have been reported in Spanish, Portuguese and Brazilian hospitals (30).

Though *E. coli* is recognized as the main producer of CTX-M-type ESBLs, even in community setting, the carbapenemase production in this species is not such a rare event anymore. There are several reports of NDM-1 producing *E. coli* isolates, belonging to the successfully spreading ST131. As carbapenemases are becoming widely distributed among *Enterobacteriaceae*, their detection in successful clones such as ST131, would create a significant public health concern, given the disease burden caused by *E. coli.*Nevertheless at currently the most frequently detected carbapenemase producing enterobacteria is *K. pneumoniae* (31).

MLST for*Pseudomonas aeruginosa*

Pseudomonas aeruginosa is opportunistic pathogen causing a huge number of nosocomial infections as main cause of bacteremia, pneumonia and abscesses (32)(33). Furthermore, it is characterized by rapid adaptability under changing conditions and acquired resistance to multiple antibiotics. Representatives of the genus *Pseudomonas* and in particular *P.aeruginosa* are among the most common causes of nosocomial infections, both in Bulgaria and worldwide.

P. aeruginosa is the most common pathogen isolated from lungs of patients with cystic fibrosis (CF), approximately 80%. Most patients are infected with the microorganism from the environment, but there is evidence that some strains can cause cross-infection from other patients with CF (34)(35). At present there is a great need for a universal technique for *P. aeruginosa* typing that is reproducible and applicable to epidemiological studies. MLST is the method of choice as it effectively types all strains from a diverse collections of *P. aeruginosa* isolates (36).

MLST scheme for typing of *P. aeruginosa* was developed by Barry Curran in 2004 (36). This scheme comprises seven housekeeping genes, initially identified by using the *P. aeruginosa* PAO1 genome database (http://www.pseudomonas.com/)(37)**.** The main criterion for selecting genes for typing is their biological role, supporting the function of the bacterial cell (housekeeping genes responsible for replication reparation and translation of DNA). The selected size of amplified gene fragments is approximate 600bp, located minimum of 6 kbp upstream or downstream from known virulence factors, or insertion sequence (IS) elements. The *P. aeruginosa* MLST scheme uses internal fragments of the following seven house-keeping genes coded these enzymes: *acsA* (Acetyl coenzyme A synthetase); *aroE* (Shikimate dehydrogenase); *guaA* (GMP synthase); *mutL* (DNA mismatch repair protein); *nuoD* (NADH dehydrogenase I chain C, D); *ppsA* (Phosphoenolpyruvate synthase); *trpE* (Anthralitesynthetase component I); http://pubmlst.org/paeruginosa. PCR primers were

designed for the loci listed above by using the published *P. aeruginosa* sequences(36). Each unique sequence (i.e., a new allele) is denoted by a number (the next available in the database). Each sequence type is defined by a unique combination of the seven alleles and receives a number of the next available sequence or is assigned to a previously defined one. Allele profiles and STs can be accessed at http://pubmlst.org/paeruginosa(36). The MLST database is updated regularly and currently contains 2680 MLST profiles from all over the world. The important extensively resistant (XDR) and multidrug-resistant (MDR) strains belongs to sequence type (ST) - ST175; ST111; ST235 and ST1725. The four sequence types occur in almost all parts of the world, especially ST235. These types are located in distinct clonal complexes and clonal groups, due to the high frequency of mutations in housekeeping genes. ST175 is grouped to CC175 and CG175; ST111 (CC 833; CG111); ST235 (CC235; CG235) and ST1725 (CC309; CG 309). Highly resistant strains are isolated in Spain, Colombia, Serbia, Hungary, Brazil, Russia, Belarus, Kazakhstan, Scotland, Poland, England, Tanzania; China; Mexico; France; South Korea; Belgium, e.g. These four sequence types dominate over the others owing to their association with acquired carbapenemases (e.g)blaVIM and ESBL (mostly oxacillinases) that adapts them to changing antibiotic therapies and facilitate acquiring further resistance genes more quickly and effectively. Other Sequence Types associated with MDR - ST17; ST27; ST146; ST233; ST244; ST253; ST309; ST357; ST520; ST539; ST540; ST622; ST733; ST1216; ST1492 are epidemiologically related to the above ST, but they are limited in their distribution, i.e. not developed mechanisms for resistance to changing conditions within the hospital environment. In the last five years many new sequence typeshave been reportedfrom South Africa, Poland, Spain, Mexico and Belgium (38)(39)(40).

Two sequence types, ST111 and ST244, have been reported among isolates from four Bulgarian hospitals associated with carbapenem resistance as well as Vietnamese Extended β-lactamase 1 (VEB-1) and blaOXA-10 (41).

MLST for *Acinetobacter baumannii*complex

*A.baumannii*causes infections mostly in patients with impaired immunity andis among the most problematic pathogens, due to its intrinsic resistance to numerous antibiotics. Infections caused by *A.baumannii*are often associated with epidemic spread, and strains, isolated in hospitals, are frequently (MDR) or (XDR). Numerous studies have supported the observation that *A. baumannii* is the species most commonly involved in nosocomial infections such as pneumonia, wound infection, and bloodstream infection (42)(43)(44).

Determination of relationships between different stains is needed for understanding the epidemiology and studying the relationships between different genotypes and phenotypes of *A. baumannii* isolates. MLST is the most robust method for investigating the clonal structure of this bacterial species and has a high potential to discriminate strains within *A. baumannii*(45).

Two MLST schemes have been developed for typing of *A. baumannii*. The first one is maintained by Bartual in The Oxford University, and is based on the sequencing of *gltA, gyrB, gdhB, recA, cpn60, gpi,* and*rpoD* (Bartual et al., 2005). The second scheme is called the Pasteur scheme and relies on the sequencing of *gltA, recA, cpn60, fusA, pyrG, rpoB,* and*rplB*(46). Both schemes were shown to generate comparable results. However, the Oxford scheme has been shown to have a higher resolution than the Pasteur scheme (47).

The clonal structure of clinical isolates of *A. baumannii* is dominated by three clonal lineages - European clones I, II and III, corresponding to CC1, CC2 and CC3 in the MLST scheme of Diancourt et al. (2010). Representatives of these clonal complexes appear as a major cause of hospitaloutbreaks. In Bartual's scheme, reference strains of European clones I corresponded to ST12 and ST6whereas ST22,and its SLVs clustered into CC22 belonging to European clone II reported widely in outbreaks (48)(49)(50)(51). Antimicrobial resistance as well as biofilm formation may represent the main reason for the evolutionary success of international clones.

Some other CCs from European clones I to III, have been described. They include the novel international clonal complex (CC15 associated with multiple antibiotic resistances (Diancourt et al., 2010). Carbapenem resistance among strains from CC15 is common and mainly characterized by acquisition of OXA carbapenemase genes (blaOXA-23-like,

blaOXA-58-like, blaOXA-40-like, and blaOXA-143-like), but it may involve insertion sequence-mediated up-regulation of the intrinsic blaOXA-51-like gene as well (52)(50).

There is a geographical variation in the distribution of acquired OXA genes, with blaOXA-58-like associated with Greece and Italy ((53)(54)(55), blaOXA-40-like with Spain and Portugal (56) (57) and blaOXA-23-like with North European countries, Asia and South America (58)(59)(60) (61)(48)). The ability of CC15 to adapt changes in antimicrobial therapy is likely to be a key factor in their success.

A recent report have shown multiple STs in a Bulgarian hospital :ST350 (CC118), ST208(CC118), ST231 (CC109), ST436, ST437 (CC118), ST449, ST502(CC118), and ST579, which were established by MLST. All strains possessed eitherblaOXA-23 or blaOXA-72 genes, confirmed by PCR and sequencing (62).

MLST of *Enterococcus faecium*

E. faecalis and *E. faecium* often found as a part of the normal human gut flora, can cause infections especially in hospitalized patients (63). These opportunistic pathogens have been considered as relatively innocuous bacteria, but since the 80s gained a different perspective, being found to sustain high-level resistance to ampicillin. These data were followed by reports from US hospitals in the 90s of rapid emergence of vancomycin resistant strains VRE (64). Over the last 20 years the aminoglycoside, penicillin, ampicillin, and vancomycin resistance in *E. faecalis* and *E. faecium* increased rapidly due to acquisition of antibiotic resistance genes (65)(66).

Nine genes are able to promotevancomycin-resistance in enterococci but the major vancomycin-resistance phenotypes are VanA and VanB(67)(68)

Although PFGE is considered as “the gold standard” for genoyping VRE in nosocomial outbreaks, MLST is the standard method for epidemiological investigations for large scale international comparisons (2). Detailed analysis of the evolutionary relatedness of *E. faecium* genotypes has been achieved by MLST (69).

The MLST scheme for *E. faecium* is based on seven housekeeping genes selected either because of their function, previous use in other MLST schemes or availability of sequence data from *E. faecalis.* The scheme was validated by testing 123 epidemiologically unrelated isolates from humans, livestock including outbreak isolates from the USA, Australia, United Kingdom, and the Netherlands. As a result a total of 62 STs were identified among vancomycin-sensitive *E. faecium* (VSE) and vancomycin-resistant *E.faecium* (VRE)isolates (70).

The development of MLST scheme for *E.faecium*allowed the application of alternative method in the molecular epidemiological investigation of VRE worldwide. It may not be as discriminative as PFGE but as the sequence types are defined they can be easily exchanged between laboratories (70).

The first *E. faecium* population-wide study using MLST revealed that that the majority of the globally representative hospital isolates were genotypically and evolutionary closely related and belonged to a single clonal-complex CC17 (71). Further studies confirmed the global dissemination of CC17. In Sweden a MLST study encompassing 60 *E. faecium* VRE isolates indicated that the predominant ST was ST-192, less frequent types were ST18, ST78, ST787 ST721, and ST203 (72).The MLST analysis of 46 isolates from Poland revealed the presence of 14 STs. Four of them STs (78, 192, 341, 412) were classified in lineage 78 after the eBURST analysis and the rest (with one exception) STs (17, 18, 64, 80, 117, 202, 262, 877, 878) in lineage 17/18. ST879 represented a singleton (73).In Turkey are described ST 117, ST 11, ST 17 and ST 78 the last two belong to the clonal complex-17 (CC-17) lineage. That clonal complex is hospital- adapted and the cause of most of the nosocomial VRE outbreaks worldwide including France, whereST78 and ST18 are the most frequently isolated (74).

The highest rates of VRE associated with hospital-acquired infections in Europe has been reported in South European countries with levels up to 45% detected in Greece and Portugal. Therefore for the prevention and control of the spread of hospital outbreaks caused by VRE, it is crucial to identify resistance patterns and relationships between different strains of these organisms(75)(76).

MLST for*Staphylococcus aureus*

Staphylococcus aureus is one of the most significant bacterial pathogens, causing

both mild skin infections in community and severe nosocomial infections, such as sepsis, pneumonia, post-operative wound infections, and catheter-related infections. Most strains of *S. aureus* are opportunistic pathogens, which cause infections in immunocompromisedhosts. The main feature of this pathogen is its adaptability to unfavorable environmental conditions. An example of this is the development of resistance to practically to all antibiotics used for the therapy of staphylococcal infections (77).

In a short time after the introduction of methicillin in 1959 the first Methicillin-resistant S. aureus (MRSA) strains emerged. MRSA became a general clinical problem within hospitals during the 1960s in Europe and the 1970s in the United States and elsewhere. Many MRSA strains are resistant to other classes of antimicrobial agents and are susceptible only to glycopeptides, linezolid and new investigational drugs. The recent studies of MRSA strains, showed decreased susceptibility to glycopeptides (glycopeptide-intermediate *S. aureus*) [GISA]) that threatens to further compromise our ability to treat hospital-acquired *S. aureus* infections (7).

S. aureus acquires resistance to meticillin and other beta-lactam agents through expression of the exogenous mecA gene, which encodes a variant of the penicillin-binding protein (PBP2a) having much lower affinity for beta-lactams, thus preventing the inhibition of cell wall synthesis. In some methicillin/oxacillin resistant mecA -negative MRSA, a novel mec gene, mecC was described in 2010 (78).

According to data from ECDC the percentages of invasive MRSA isolates in Europe ranged from zero (Iceland) to 57.2% (Romania)(22).

Transfers of mobile genetic elements, leading to the emergence of new clones and acquisition of new pathogenicity factors are important elements in the evolution of MRSA(77). One of the first typing methods used for MRSA typing is plasmid profiling(79), which however had shown low discriminatory power and reproducibility due to the instability of extra-chromosomal mobile elements. Other genetic methods are PFGE, restriction fragment length polymorphism-RFLP, RAPD, Repetitive-element PCR (rep-PCR), multi locus VNTR analysis (MLVA), MLST, and recently Whole genome sequencing (WGS) (80).

The MLST scheme for *S.aureus* is performed by sequence analysis of approximately 450 bp internal fragments of seven housekeeping genes: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) (7). Isolates that have identical sequences at all seven genetic loci are considered a clone, and assigned to a unique sequence type (ST). Sequence types that differ by single nucleotide polymorphisms (SNP) at fewer than three loci are considered closely related, and are grouped into clonal complexes (CC). This process involvesthe application of the eBURST algorithm (http://eburst.mlst.net), which uses MLST data to group closely related strains into a clonal complex. It also predicts the probable founding clone (i.e., sequence type) of each group and recent evolutionary descent of all other strains within the CC from the founder. The analysis can be further refined to identify specific subclones by the addition of other methods, such as *spa* typing, PFGE, or by studying other genetic markers (e.g., toxin genes or specific plasmids) (81).

The first MLST study of *S.aureus* encompassed 155 isolates and more prevalent allelic profile were MRSA, they had the same profile as reference strain of the epidemic MRSA clone 16 (EMRSA-16). Among methicillin-susceptible *S. aureus* (MSSA) the most prevalent clone was very closely related to EMRSA-16. MLST methodology provides data about MRSA and MSSA strains to known clones or assigning them as novel clones via the web based database. In study of Enright, the genotypes of MRSA and MSSA isolates with the same allelic profiles were very closely related. The most likely explanation is that the MSSA isolates represent the genotype from which the MRSA isolates recently arose by the acquisition of the methicillin resistance determinant (*mec*) by horizontal gene transfer (7).

In September 2017 the *S. aureus* MLST database https://pubmlst.org/saureus/ numbered 32,920 isolates and 4300 STs from global sources. These isolates represent mixture of MRSA and MSSA originated from infections and asymptomatic carriage. Most of the clonal complexes of *S. aureus* have simple structure, where the majority of STs differ only by a single gene sequence from the primary founder (SLVs).

The ST30 and ST239 have more complicated structure. The ST239(initially grouped into CC8) includes the earliest known MRSA clone (ST250) and three other STs that represent MRSA clones commonly encountered within hospitals (ST8, ST239, and ST247). ST30 is the predicted primary founder (99% bootstrap support) of this clonal complex. All of the MRSA isolates within this complex belong to ST36, with the exception of one SLVof ST36 that has probably descended from it. ST36 is a well-characterized epidemic MRSA clone (EMRSA16) that appears to have emerged following the acquisition of methicillin resistance by an SLV of ST30 (82). At present ST36 (EMRSA-16) is among the most disseminated hospital acquired MRSA (HA-MRSA) along with ST22 (EMRSA15), ST225 and ST228. The most common community-aquired MRSA (CA-MRSA) in Europe is ST80. Another frequently isolated CA-MRSA is ST152.
There is no full and comprehensive data about the circulating STs MSSA and MRSA in Bulgaria however, there is information about the prevailing HA-MRSA defined as ST239 and ST10, less common are ST72 and ST30 (83).
Relatedness between the major MRSA clones described by different laboratories in different countries is stillambiguous.

MLST of *Streptococcus pneumoniae*

Another Gram-positive bacterium associated with infections with high morbidity and mortality rates is *Streptococcus pneumoniae*.This pathogen is the leading cause of community-acquired pneumonia but the *S.pneumoniae*infection spectrum includessinusitis, otitis media, bloodstream infections and meningitis among others(84).
Several molecular typing methods have been developed for use in population structure studies of *S. pneumoniae*(85). Multilocus sequence typing (MLST) is an excellent tool for tracking the national and global spread of *S. pneumoniae* clones, and for identifying lineages that have an increased propensity to cause pneumococcal disease. Groups of *S. pneumoniae* with highly related sequence types (STs) can persist for many years and spread as clonal populations. The MLST scheme for *S. pneumoniae* was developed by Enright and Spratt in 1998 (86) and involves sequencing of housekeeping gene fragments of: *aroE* (shikimate dehydrogenase), *ddf* (D-alanine-D-alanine ligase), *gdh* (glucose-6-phosphate dehydrogenase) , *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I) and *xpt* (xanthine phosphoribosyltransferase). Among the studied of 295 pneumococcal isolates the sequence diversity was low but 18-34 alleles were distinguished. Twelve clusters of pneumococcal isolates with identical STs were indentified(86).
Recently, 169 invasive (IPD) and noninvasive pneumococcal disease (NIPD) strains were tested by MLST byZhoua et.al and 85 sequence types (STs) were observed. The seven prevalent STs were ST271, ST320, ST876, ST3173, ST236, ST81 and ST342, mainly associated with serotypes 19F, 19A, 14, 6A, 19F, 1, and 1/23F, respectively (87).
ST81 is often associated with antimicrobial resistance and the isolates most closely related to this cluster are more likely to be MDR (9). The three pneumococcal clones that were most frequently associated with invasive disease prior to vaccination were also penicillin non-susceptible: Spain^{23F}-1, Spain^{9V}-3 and Taiwan^{19F}-14. Using MLST these and related strains may be assigned to 'clonal complexes' (CCs): CC 81, CC 156 and CC 236. All three clones have persisted into the post-vaccine population with a new serotype not targeted by the vaccine, and in each the predominant serotype is 19A. The most successful and clinically significant is ST320, which is a close relative of ST 236, Taiwan^{19F}-14(88). Another ST associated with MDR is Poland- B-20 clone, which is registered in Argentina and correspondsto ST315.
MLST can also be used to characterize unusual isolates (e.g. optochin-resistant, non-serotypable strains), that may be either atypical pneumococci or viridians group streptococci. MLST is effectively applied for typing of penicillin-resistant invasive isolates. Comparing their allelic profiles via the online database, provides an unambiguous approach that has substantial advantages over the current methods for the characterization of these strains and contributes to the surveillance of antimicrobial resistance on all levels.

MLST and Whole Genome Sequencing

During the last decade the Whole Genome Sequencing technologies have revolutionized the infectious disease surveillance on a global scale and especiallyour understanding of the antimicrobial resistance dynamics and

evolution.The WGS determines the DNA sequence of prokaryoticchromosomesmaking it the only method that offers complete information about the genetic material(89). It is becoming more and more applied in clinical practice as arapid diagnostic toolfor bacterial infections, and determining of the most appropriate antibiotic for treatment, as determined sensitive of bacterial agent for only 4-6 hours. In near future WGS will be a major method in personalized medicine being able to provide a lot of useful information for directing doctors to a proper selection in the choice of therapy for individual patient treatment (90) .

Many laboratoriesspecialized in bacterial typing are now moving to WGS approaches. Nevertheless MLST still have its impact as the basic principle remains the same.The evolution step of Maiden's MLST method is called core genome MLST (cgMLST) and is based on the WGS approach. The cgMLST schemes consists of around 1500 target genes whose sequence should be determined by NGS and results obtained by one such study give more rich and detailed information for establishment of phylogenetic relationship between various pathogens. cgMLST method is more cost-effective provides better discriminatory power, and faster results than the previous methods applied in laboratory practice for detection of nosocomial outbreaks (91).

As next-generation sequencing is increasingly replacing Sanger sequencing, conventional MLST can be extended to whole genome MLST (wgMLST). Since many more loci (typically 1500 – 4000) are considered in wgMLST, a much higher typing resolution can be obtained.However the bioinformatics tools capable of handling such large datasets are still in development and the few commercial software packages could be very expensive.

Recently MLST and WGS have been applied simultaneously in the investigation *Campylobacter jejuni* infections andisolates in three districts in Finland (92). Authors applied WGS of all isolates belonging to the four most prevalent MLST STs. Up to three clusters were differentiated by WGS in each ST with highly homogenous genomesin each cluster. Authors concluded that wgMLST analysis resulted in recognition of genetically closely related isolates within the STs, suggesting that sporadic infections may sometimes have a common infection source.

Another recent study reports on the development of wgMLST schemes for ESBL producing *Enterobacteriaceae*(93). Representative genus-, genetic-complex-, and species-specific wgMLST schemes were developed for the five most prevalent *Enterobacteriaceae* species in clinical practice and used to type a large national collection of ESBL-E isolates obtained from patients in Dutch hospitals.As expected, wgMLST and cgMLST enabled further differentiation of isolates that belong to the same ST, however the ranges of genetic distances overlapped for *E. cloacae, E. coli, and K. pneumoniae*, indicating that some isolates with different STs can be genetically more closely related than isolates with the same ST.

CONCLUSION

MLST have been established as one of the most informative tools for analysis of phylogenetic relationships between strains causing nosocomial outbreak in different parts of the world. In the recent two decades, through MLST, epidemiologists have been able to studyassociation between antimicrobial resistance and sequence types in pathogens, causing both hospital and community-acquired infections. Although Whole genome sequencing is being increasingly applied for bacterial typing its application as a routine method is still beyond the capabilities of many laboratories in terms of equipment, reagents, bioinformatics and software. Besides the WGS databases are still in development compared with MLST and until thousands of isolates are sequenced by WGS, MLSTwillkeep its place.

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MYCOPLASMA GENITALIUM: PREVALENCE AND CLINICAL SIGNIFICANCE IN SOFIA MUNICIPALITY

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ABSTRACT

Mycoplasma genitalium as an etiological cause of bacterial sexually transmitted infections (STIs) has been of increasing interest in recent years. This study is one of the very few prevalence studies concerning *M.genitalium*among Bulgarian population. The recorded prevalence of *M. genitalium*was2.45%. Moreover, the association-between*M. genitalium*and genital tract infections such as urethritis and cervicitis was likewise affirmed in the present study.Further research is still needed for obtaining antimicrobial resistance data on*M. genitalium*to secure effective control and optimal treatment of bacterial STIs.

KEYWORDS: *M. genitalium*, prevalence, urethritis, cervicitis

INTRODUCTION

Chlamydia trachomatis and *Neisseria gonorrhoeae* are generally accepted pathogens in urethritis and cervicitis but due to the many cases where no pathogen can be identified, *Mycoplasma genitalium* has been of increasing interest in recent years. *M. genitalium* was first isolated in 1981 following a prolonged incubation of urethral swabsfrom men with urethritis. Two of the samples showed changes in the medium acidity, and an electron microscopy study proved the presence of bacteria, which were later named *Mycoplasma genitalium*(1). Due to difficulties in detection by conventional laboratory methods such as cultivation and serological tests, the etiological role of *M. genitalium* as a cause ofsexually transmitted infections(STIs) (in particular, the non-gonococcal non-chlamydial urethritis) was confirmed only after the introduction of PCR diagnostic methods in 1991(2). Nowadays it is well known that *M. genitalium* frequently causes urethritis in men, and urethritis and cervicitis in women (3). *M. genitalium* is also associated with an increased risk of pelvic inflammatory disease, preterm birth, spontaneous abortion, and tubal infertility(4).A meta-analysis performed by J.S. Jensen shows that the prevalence of *M. genitalium* infections is lower than that of *C. trachomatis*(5). In a recent study at the Medical University in Sofia, the observed prevalence of *M. genitalium* was as low as 0.29%(6). The aim of this study is to obtain further information on *M. genitalium* concerning prevalence and association with symptoms and microscopic signs of urethritis and cervicitisin Sofia Municipality.

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MATERIAL AND METHODS

From January 2017 through June 2017 clinical samples to be tested for *M. genitalium* were collected from 204 consecutive patients attending the National Reference Laboratory for Mycology and STIs in Sofia, Bulgaria for routine STIs check-up. A total of 80 women and 124 men were included. The median age was 35 years (range 17-61) and37 years (range 20-60)for the women and for the men, respectively.

Patients with dysuria, urgency, and/or dischargewere regarded as symptomatic. Microscopic urethritis was diagnosed if more than 10polymorphonuclear leucocytes (PMNLs) were observed in high-power field (hpf) magnification (x 1000) (averaged over five fields) in a Gram-stained smear preparedfrom spun deposit of a first-void urine (FVU) specimen. Microscopic cervicitis was diagnosed if more than 30 PMNLs were observed over 3 fields per hpf in a Gram-stained smear of endocervix collected with a cotton-tipped swab.

During the study FVU specimens were placed in sterile tube and transported to the laboratory within 1 hour. Urethral and endocervical swabs were taken with a cotton tipped swab and processed immediately.

Genomic DNA from first-void urine, urethral and endocervical swabs was isolated with the Quick-DNA™ Universal Kit (Zymo Research, United States) according to the manufacturer's instructions.

Detection of *M. genitalium* in the clinical samples was performed using AmpliSens® Mycoplasma genitalium-FRT PCR assay (InterLabService, Moscow, Russia) according to the manufacturer's instructions.

RESULTS

M. genitalium was detected in 5 (2.45 %) patients: 4 (3.23 %) of the 124 men and 1 (1.25 %) of the 80 women. The median age of the *M. genitalium* infected men was 31 years (range 28-38) and the infected woman was 30 years old.

Among the 204 examined patients 51 had symptoms as well as microscopic signs of urethritis and/or cervicitis and 55 had microscopic signs of urethritis and/or cervicitiswithout any symptoms. *M. genitalium* was detected in three (7.84 %) of those with both symptoms and microscopic urethritis/ cervicitis and in two (1.81 %) with only microscopic signs of urethritis/cervicitis. No *M. genitalium* was detected among those only with symptoms (n=32) and those without any symptoms and microscopic signs of urethritis/ cervicitis (n=66) (Table 1).

Table 1. Symptoms and microscopic signs in patients infected with *M. genitalium.*

	Symptoms and microscopic signs (n=51)	Microscopic signs (n=55)	Symptoms only (n=32)	No symptoms and no microscopic signs (n=66)
M. genitalium-positive samples (n=5)	3 (7.84 %)	2(1.81 %)	0 (0 %)	0 (0 %)

DISSCUSSION

This study provides information on *M. genitalium* concerning prevalence among Bulgarian population from Sofia Municipality and association between *M. genitalium* and symptoms and microscopic signs of urethritis and cervicitis in men and women, respectively. In this study, *M. genitalium* was found less often as an infectious agent causing urethritis/ cervicitis than *C. trachomatis* in Bulgarian population(7-10)which is in accordance with the meta-analysis performed by J.S. Jensen (5).Our findings confirm the results from other studies regarding the association between *M. genitalium* and non-gonococcal urethritis (NGU) and suggest promptly that these bacteria should be considered in the differential diagnosis of NGU(11). Among the *M. genitalium*-positive cases virtually all patients had urethritis but only two thirds had symptoms. Although more patients had symptomatic urethritis/cervicitis in accordance with results from other studies (12, 13), the high proportion of asymptomatic carriers would facilitate the spread of the infection.

In contrast with the previous investigation on the prevalence of *M. genitalium* in Bulgarian population we found a significantincrease in prevalence (from 0.29% in 2011(6) to 2.45% in the current study). Back in 2011 it was speculated that the low prevalence of *M. genitalium* was due to low circulation of the pathogen in our population because of the high consumption of macrolides in the clinical practice. Unfortunately the emerging macrolide resistance of *M. genitalium* in the last decade had seriously compromised the effective treatment and control of this bacterial STI(14-17). As antimicrobial resistance data for *M. genitalium* strains in Bulgaria are entirely lacking, further research would be valuable to secure effective control and optimal treatment of urogenital mycoplasma infections.

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MYCOBACTERIUM TUBERCULOSIS INFECTION RATES IN EXPOSED BULGARIAN HEALTHCARE WORKERS BASED ON INTERFERON-GAMMA RELEASE ASSAYS

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ABSTRACT

Background: The incidence of tuberculosis (TB) in Bulgaria remains higher than the average for EU/EEC countries. Healthcare workers (HCWs) providing TB care are at increased risk of MTB infection. Interferon-gamma release assays (IGRAs) are an accurate means for diagnosis of MTB infection. **Objectives:** The study aims to assess the prevalence of MTB infection among exposed HCWs in Bulgaria based on IGRAs as screening tools. **Methods:** Data about age, sex, professional category and employment history were collected. Screening was performed in four rounds (2012, 2014, 2015 and 2016), using QuantiFERON-TB® Gold in-tube assay (QFT-GIT) and T-SPOT.TB assay. **Results:** A total of 633 HCWs from 37 specialized healthcare facilities were tested, including medical doctors (22.7%), nurses (34.8%), laboratory workers (16.6%), cleaning staff (20.1%) and administrative personnel (5.8%). A positive IGRA result was obtained in 260 (41%) HCWs. Positive results were associated with higher age but not with gender, professional category or employment duration. Unclassifiable results (4.3%) were obtained mostly with T-Spot, and in the elderly HCWs. Consecutive testing in 125 (19.7%) HCWs revealed a significant share of reversions and conversions (30.4%) requiring retesting/follow-up. The quantity of MTB-specific IFNg measured by QFT-GIT was not directly associated with infection duration, activity, or MTB-specific treatment. **Conclusion:** HCWs providing TB care in Bulgaria are at increased risk of MTB infection. Occupational screening programs with focus on the elderly HCWs should be routinely applied in high-risk settings. Retesting "close to zero" results and combining sequential IGRAs with detailed history, clinical examination, and radiography would improve the efficiency of screening.

KEY WORDS: *MTB infection, Healthcare workers, IGRA*

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INTRODUCTION:

Tuberculosis (TB) remains a significant health issue, being the most prevalent infectious disease, and is the ninth cause of death worldwide. The active infection may remain undiagnosed for months leading to a high spreading risk. According to the latest World Health Organization (WHO) TB report, every single day 4,400 people die from TB (1). In 2016, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 6.7 million (65%) were among men, 3.7 million (35%) among women and 1.0 million (10%) among children. There were 600,000 new cases with resistance to rifampicin (RR-TB), the most effective first-line drug, of which 490,000 had multidrug-resistant TB (MDR-TB). The average proportion of MDR-TB cases with extensively drug-resistant TB (XDR-TB) was 6.2% (2). Bulgaria is one of the six European countries with TB incidence above 20 %0 000. Although the newly registered cases tend to decrease (27%0 000 in 2016 vs. 54%0 000 in

2005, the rate of relapses and resistant forms remains high; 22 cases with MDR-TB were registered in Bulgaria in 2016 (3.3%) of which 1 (5%) were with XDR-TB (3, 4).

One third of the world population is estimated to have latent TB infection (LTBI): evidence of persistent immune response against *Mycobacterium tuberculosis* (MTB) in the absence of clinical signs (5). The risk for activation of LTBI within a lifetime is about 10%. The risk of infection increases with the duration and closeness of contact. However, brief occasional contacts may also lead to infection (6) Therefore, MTB infection is a major occupational hazard for healthcare workers (HCWs) worldwide (7 – 9). The proportion of occupation-related tuberculosis in healthcare workers varies between 32 and 80% according to recently published molecular epidemiology studies (4, 9 – 11).

In many countries, TB screening is performed following risk assessment. HCWs in regular contact with TB patients are screened at different frequencies (one to three-year intervals) (11). In addition, accidentally exposed HCW may be screened urgently (12). A specific aim of the National program for prevention and control of tuberculosis in Bulgaria during the period 2017 – 2020 is the preventive testing and treatment of HCWs at high risk (working specialized tuberculosis departments/hospitals for TB treatment or research institutes and laboratories who have regular contact with infectious patients or materials) (4). Understanding the infection risks and implementation of adequate occupational safety programs is crucial both for protecting HCWs and their patients (1, 13).

Interferon – gamma release assays (IGRAs) are a new generation *in vitro* tests that measure IFNg response of peripheral blood cells stimulated with peptides from the RD1 complex, specific of virulent MTB strains. As compared to the classical tuberculin skin test (TST), IGRAs are characterized with much higher specificity, and provide an accurate, reliable and efficient tool for diagnosis of TB infection, convenient for BCG-vaccinated populations (14). We present here the first results from a large scale screening of Bulgarian health-care workers at high risk for TB infection, performed with IGRAs during the period 2012 - 2016 in the context of the National Program for Prevention and Control of tuberculosis in Bulgaria.

MATERIAL & METHODS:

Study design. The screening was performed at four rounds (2012, 2014, 2015 and 2016) in the context of the National Program for Prevention and Control of tuberculosis in Bulgaria (2012 – 2016). *Participants*. HCWs from specialized health-care facilities for pulmonary diseases and tuberculosis, including medical doctors, nurses, laboratory technicians, medical attendants, and administration personnel were tested based on voluntary and random choice. *Methods.* The following commercially available assay versions of IGRAs were used: QuantiFERON-TB® Gold in-tube (QFT; Qiagen, Hilden, Germany) in 2012, 2015 and 2016, and T-SPOT. TB (Oxford Immunotech, Abingdon, UK) in 2014. Both types of tests were performed and interpreted according to manufacturers' instructions. Questionnaires with standard demographic data as well as data about the type and duration of employment were collected from all participants at screening. Additional data (availability of symptoms, radiography, and treatment) were collected from a limited number of IGRA-positive participants. Statistical analysis was performed with parametric T-test or Spearman's rank correlation test for quantitative data, and Chi-Square test for categorical data (GraphPad v.6).

RESULTS:

1. Demographic characteristics of the studied group.

In the period 2012-2016, a total of 791 samples from 633 HCWs were tested: 490 samples with QFT, and 301 – with T-Spot. A total of 126 (19.9%) HCWs were tested more than once and only 4 of them - at every single round (**Table 1**). Samples were collected from 37 specialized health facilities for pulmonary diseases and TB, in all 28 administrative districts of Bulgaria. The mean (min-max) age of the participants was 51.4 (19-76), including 54 men (9%) and 579 women (91%). Most of the participants fell in the age groups " > 55", and "41 – 50" **(Table 2).** The mean (min-max) length of service at the present working place was 15.2 (1 - 46) years. According to the specific occupation, the participants were distributed as follows: medical doctors (22.7%), nurses (34.8%), laboratory workers (16.6%) (including microbiologists, immunologists, biologists, lab technicians, and lab assistants), medical attendants (20.1%) and administrative personnel (5.8%).

Table.1 Distribution of participants according to the number and type of IGRA test

Test	1xQFT	2xQFT-GIT	3 x Q F T - GIT	Q F T - GIT+ T-Spot	2 x Q F T - GIT+ T-Spot	3 x Q F T - G I T + T-Spot	1xT-spot
Number of tested HCW (n=633)	305	30	4	72	16	4	202

Table 2. Age and sex distribution of IGRA test results

		QFT				T-Spot			
		Total	**Neg (%)**	**Pos (%)**	**U (%)**	**Total**	**Neg (%)**	**Pos (%)**	**U (%)**
	All	431	255 **(59,2)**	170 **(39,4)**	6 **(1,4)**	294	154 **(52,3)**	118 **(40,1)**	22 **(7,5)**
Age intervals	20-30	23 (5.3)	21 (91.3)	2 (8.7)	-	12 (4.1)	8 (66.7)	3 (25.0)	1 (8.3)
	31-40	52 (12.1)	36 (69.2)	15 (28.8)	1 (1.9)	26 (8.8)	21 (80.8)	5 (19.2)	-
	41- 50	131 (30.4)	82 (62.6)	46 (35.1)	3 (2.3)	79 (26.9)	47 (59.5)	25 (31.6)	7 (8.9)
	51- 55	77 (17.9)	45 (58.4)	32 **(41.6)**	-	54 (18.4)	26 (49.1)	27 **(50.0)**	1 (1.9)
	> 55	148 (34.3)	71 (48.0)	75 **(50.7)**	2 (1.3)	1 2 3 (41.8)	52 (42.3)	58 **(47.1)**	13 **(10.6)**
Gender	Male	44 (10.2)	27 (61.4)	16 (36.3)	1 (2.3)	17 (5.8)	10 (58.8)	6 (35.3)	1 (5.9)
	Female	387(89.8)	228 (58.9)	154 (39.8)	5 (1.3)	277 (94.2)	144(52.0)	112 (40.4)	21 (7.6)

2. Association of IGRA results with the demographic and occupational characteristics of the participants

The results were classified as positive, negative and unclassifiable (indeterminate/borderline), according to manufacturer's instructions. A positive result (regardless of the type of IGRA used) was established in 260 (41%) of the HCWs tested. The share of positive results did not differ significantly among men and women (35, 2% vs. 41,6%, X^2=0.04, p>0.05), (**Table 2**). The IGRA-positive HCWs were significantly older as compared to the whole studied group: mean (min-max) 53.7 (26-76) years vs. 51.4 (19-76), (T-test p<0.01). In addition, the share of positive results with both IGRAs increased significantly with age **(Table 2).**

The distribution of positive results according to the employment position was as follows: 20.0% medical doctors, 37.7% nurses,16.5% laboratory staff, 22.3% hospital attendants, and 3.5% administration, and did not differ significantly as compared to the whole group (X^2= 4.92, p>0.05). Interestingly, the length of service at the present working place did not differ significantly either: mean (min-max) 17.8 (1 - 45) years for IGRA(+) HCWs vs. 15.2 (1 - 46) for the whole group, p > 0.05. No significant dynamics in the share of positive results was established during the 4 rounds: 34.4% in 2012 (11/32); 40.1% in 2014 (118/294); 44.4% in 2015 (71/160), and 35.1% in 2016 (103/293), (X^2=4.03, p>0.05).

There were a total of 28 unclassifiable results of 27 (4.3%) HCWs, mostly obtained with T-Spot (QFT, n=6 vs. T-Spot, n=22). A significantly higher share of borderline T-Spot results were obtained in the elderly HCWs (10.6% for T-Spot vs. 1.3 for QFT in the age group > 55). Indeterminate QFT results were either due to a high value of the negative control (n=3), or low response to mitogen (n=3). Ten from the 28 non-classified results were retested one year later. In 60% of

the retested cases, the indeterminate result was followed by a negative one, in 33% the indeterminate result was followed by a positive one, and in just one case the indeterminate result was repeated.

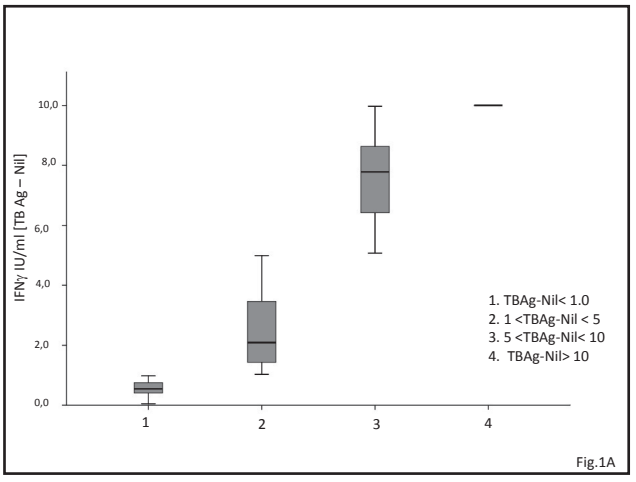
3. Dynamics and quantitative characteristics of individual IGRA results

Due to the random study design, only 125 HCWs (19.7% from the studied group) were tested repeatedly in different years: 34 HCWs underwent 2 or 3 QFT-GIT tests, and 92 – T-Spot, preceded or followed by QFT-GIT (Table 1). Of these, 53 (42.4%) had consistently negative, and 33 (26,4 %) – consistently positive results. Conversion of MTB status (a negative or indeterminate result, followed by a positive one) was documented in 26 cases (20.8%). A „reversion", ie positive/ indeterminate result followed by a negative one, was observed in 12 cases (9.6 %). Finally, in one HCW a negative result was followed by an indeterminate one. The significant share of reversions and conversions (30.4%) prompted us to analyze in detail the quantitative data from the QFT-GIT tests.

According to manufacturer, all cases whereby MTB-specific IFNg secretion corrected for background (TBAg –Nil) is > 0.35 are interpreted as positive, except of those with too high background (TBAg < 25% of Nil value). However, positive results clustered in four subgroups according to the quantity of IFNg measured: very low (TBAg-Nil ≤ 1 IU/ml), 24.7%; low (TBAg-Nil >1 ≤ 5 IU/ml), 43.5%; medium (TBAg-Nil >5 ≤ 10 IU/ml), 10.0% and high (TBAg-Nil > 10 IU/ml), 21.8% **(Fig.1A).** The measured MTB-specific IFNg secretion was not associated with age, specific occupation, employment duration or the time after the first positive testing.

An important share of QFT results ranged close to 0. As shown in Fig.1A, 42 (24.7%) from the positive results fell in the group TB Ag-Nil ≤ 1 IU/ ml (0.35 – 0.98). Noteworthy, among retested positives, 50% of those with "very low" response reverted to negatives vs. only 7% from those with medium to high response **(Fig.2A)**.

In most HCWs with a negative QFT-IT result (144 or 56%) TBAg-Nil was a positive value. In 32 cases (12.3%) TBAg-Nil value was within the interval (0.2 – 0.5), practically overlapping with the positive ones **(Fig.1B).** HCWs with elevated negative results were significantly older as



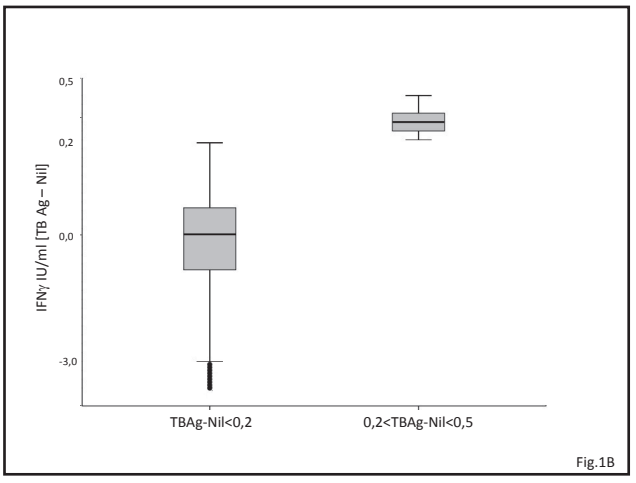


Fig. 1. Quantitative QFT-GIT results based on TB Ag-Nil IFNγ concentration (U/ml)
A Distribution of positive test results B Distribution of negative test results

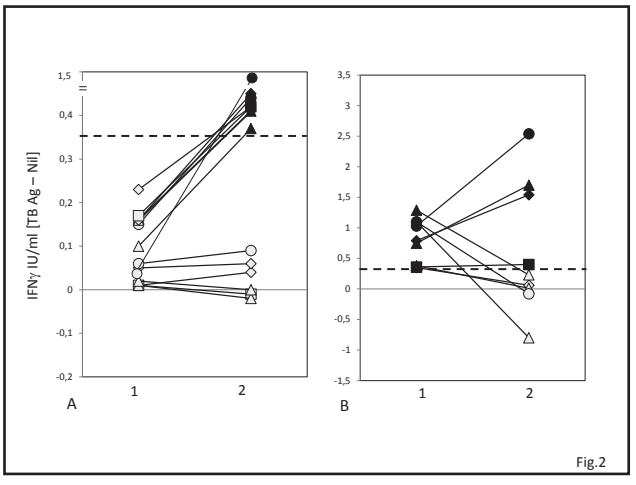


Fig.2. Dynamics of borderline negative (A) and positive (B) QFT-IT result. MTB-specific IFNγ values [TB Ag-Nil] obtained during consecutive tests are presented. Positive results are presented with black, and negative results – with grey symbols.

compared to the rest of the MTB-negatives (mean age 53.2 vs. 47.4; p<0.001). Among retested negatives, 41% from the negative results with initially positive TBAg-Nil values converted to positive vs. only 6% from the rest of negatives (p<0.01), **(Fig.2B)**.

4. Correlation of IGRA results with other clinical and laboratory data.

New MTB infection, activation of existing LTBI, and/or application of MTB-specific treatment are the principal factors that determine dynamics of consecutive IGRA results.

Only 230 (36%) from the tested HCWs have completed the additional questionnaire. According to these data, 12 (5.2%) HCWs reported MTB-specific therapy in the past. Most of them (10/12) were treated before the screening (in the period 1974 – 2010). The patterns of IGRA response observed in this group were either a single or repeated positive result, or - negative result, followed by a positive one. Positive results ranged between 0.36 and >10 IU/ml IFNg. Only 2 cases of active TB were registered in the period of screening (2015), and both received MTB-specific treatment. Interestingly, one of them had a borderline IGRA result in 2014, and the other has converted after a negative result in 2012 to weakly positive in 2015 (TB Ag-Nil = 0,36). Only 52 (20%) of the HCWs with positive IGRA result reported receiving prevention Isoniasid therapy. Those who were retested after prevention (11/52, 21%) were repeatedly positive, with high TB Ag-Nil values (4,13 – 10). Overall these data indicate that the quantity of MTB-specific IFNg measured by QFT-IT is neither associated with the probable infection duration/activity, nor is directly affected by MTB-specific treatment.

DISCUSSION

The first nation-wide IGRA-based study on the prevalence of MTB infection among Bulgarian HCWs at risk established a mean of 41% infected. This rate seems alarmingly high in the context of recently published European studies. LTBI among German HCWs was reported to be around 10% (15), a study among French HCWS reported 18.9% positivity (16), and a screening of 4735 hospital workers over 3 years period in Portugal yielded a rate of 32.6% (17). Similar to our results (40% QFT+) were reported by a screening among HCWs in the rural regions of India, ie – in settings of high TB prevalence (18). Logically, MTB infection rates among HCWs reflect the incidence of active TB among the general population. According to the latest ECDC report for 2015, Bulgaria remains a country with intermediate incidence (24 $\%_{0\ 000}$), similar to Portugal (23 $\%_{0,000}$), and unlike Germany (8.1%$_{0\ 000}$) and France (8.2 $\%_{0\ 000}$) (3). A metaanalysis of IGRA-based serial studies published between 2006 and 2012 reported 8 – 31% initially positive IGRA results in countries with intermediate TB incidence (20 – 100 $\%_{0\ 000}$) (11). Several factors might explain the elevated rates established by us. Most published data concern HCWs in general hospitals while our study was targeted at health-care facilities specialized in the treatment of pulmonary diseases and TB, and exposed to a regular and higher occupational risk of MTB infection. Thus, the prevalence of LTBI among Georgian HCWs in TB healthcare facilities was reported to be 55% vs. 31% for those from non-TB facilities. (19) Besides, our study was not exhaustive but based on voluntary participation. Therefore, one may expect that HCWs with a history of positive TST and/or IGRA test, or radiography, or clinical symptoms would have preferably opted for testing at each round. The stable rates during the years indicate that the personnel of the included healthcare facilities was relatively constant, and so were the volunteers for the study.

Similar to other reports (15) our results show that susceptibility to MTB infection depends primarily on the individual immune competence which decreases with age. The probability of MTB infection was not associated with a specific professional category or the length of service in high risk environment. Based on these results, regular screening is warranted for all HCWs in high risk settings with appropriate occupational policy for those aged above 55.

Both QFT and T-Spot have been recommended for screening in HCWs, especially in the BCG-vaccination settings, as higher sensitivity tests. However, a significant impact of age on the sensitivity was recently demonstrated exclusively for QFT, in a cohort of patients with active TB (20). In our hands, a significantly higher share of borderline results were obtained using T-Spot in the elderly HCWs. At least a part of these could have been positives, masked by a secondary immune deficiency, and therefore require

follow-up, and additional laboratory and clinical evaluation. Thus, using T-Spot for screening of HCWs aged above 55 might decrease the rate of false-negative results.

We observed a significant dynamics of individual results, including 21% conversions and 10% reversions. A conversion is usually due to a new infection with MTB and is directly related to the prevalence of active *Mycobacterium tuberculosis* infection (ATB) among the general and the specific patient population. In may be also observed following a successfully treated infection in the past (i.e., after reversion). In our hands, 10/10 (100%) of HCWs treated in the past were positive or converted to a positive result in the present study, indicating an important risk of reinfection in the studied health care facilities.

Reversion indicates that MTB has been successfully eliminated or suppressed, so that T-cells are no longer sensitized (10). Different studies report between 32.1% and 44.4% reversion rates in MTB patients after 3 to 6 months of specific treatment (5, 21, 22). Whether preventive treatment of LTBI may lead to reversion, or whether MTB-specific responses may gradually decline in the absence of reinfection, is still a matter of debate. Reversions might be also due to the biological variation in IFNg levels, or variability in laboratory and test procedures. A high rate of conversions and reversions has been observed in serial screening studies, and were associated with the "zone of uncertainty", around the manufacturer's cut-off (6). Weekly testing among HCWs has demonstrated 24 to 28.6% conversions and reversions, the greater majority with weak TB antigen-specific response (18, 23). We also observed significant dynamics of "close to zero" QFT results, as well as in HCWs in advanced age, underlying the impact of age, and of non-specific inflammation for the individual variations in IFNg levels. A number of recent studies have shown that repeated testing decreases the share of false negatives and positives, and have proposed as a new upper limit for "retesting" zone 0.7, 1.1, or 2.0 IU/mL (24). In order to avoid overtreatment, a result within the zone should be retested within about four weeks, and monitored for changes in risk profile before preventive chemotherapy is recommended (11). Validating IGRA thresholds suitable for serial testing and corresponding to the overall MTB prevalence (18) as well as clinical guidance is needed for interpreting QFT results around the "retesting" zone (25). If we reconsider our data in the context of "retesting zone", the rate of "true" positives would drop to about 34% which would correspond better to the overall incidence of TB in the country. Further on, positive or negative result should always be interpreted in the context of additional information including patient's medical history, physical evaluation, and previous IGRA and radiography results, when available. In our study only 36% of the tested HCWs provided such information which greatly impeded the analysis.

The main purpose of screening populations at risk, alongside with LTBI detection, is the prognosis and prevention of ATB by specific treatment. According to the manufacturer, IGRAs results are interpreted in a binary mode (infected vs. not infected), and the quantity of measured IFNg is not considered indicative of infection activity or therapy efficiency. Never-the-less, an important heterogeneity of individual quantitative results exists, and requires further elucidation. Several early studies in high incidence settings have demonstrated low, moderate and high IFNg responses to ESAT-6 and CFP-10 among close household contacts (26), and have associated vigorous early IFNg response with an increased risk of ATB in the next 1-2 years. Indeed, the quantity of IFNg measured in response to MTB-specific stimulation depends on the number and functional activity of circulating RD1-specific CD4 and CD8 T cells that are proportional to the infectious dose and depend on the quality of host response. The large number of IFNg(+) antigen-specific T cells detected during the acute response to MTB, gradually declines, but a heterogeneous pool of long-living memory cells is still present in the settings of chronic infection. A number of observations proposed that response to RD1 peptides is lower in MTB-infected subjects without active disease than in ATB patients, but also decreases in patients with advanced disease, and under effective chemotherapy (27, 28, 29). In this context, the subgroups with medium to high MTB-specific IFNg secretion (TB Ag-Nil >5 IU/ml and > 10 IU/ml) from our study require close follow-up. Only 4 of these patients were retested one year later, and they were consistently positive with no history of clinical symptoms. Therefore, we suggest that a high IFNg response may indicate

not only imminent ATB but restimulation in the settings of repeated contact with MTB. Further on, preventive treatment did not seem to affect significantly quantitative QFT-IT results. To date, it is clear that classical QFT-GIT and T-Spot provide incomplete information about the protective response to MTB. The optimized peptides of the well characterized RD1 and RD11 included in the first generation IGRAs were shown to drive primarily CD4 T cell responses (30, 31). Yet, a number of human and animal studies have confirmed the critical role of CD8 T-cells in the early and definitive control of MTB infection (29 – 34). In this aspect the new generation IGRAs and flow cytometry ICS analysis differentiating between CD4 and CD8 T cell responses might prove much more instrumental for prevention and screening programs.

In conclusion, the significant rate of MTB infection among Bulgarian HCWs in high-risk settings warrants an adapted screening algorithm and its consistent application. Retesting of indeterminate, border-line and close-to-zero results, as well as applying T-Spot in the elderly HCWs and corrected borderline zone for QFT will improve the efficiency of screening and reduce overtreatment. Targeted follow-up of newly infected and convertors, combined when appropriate with medical history, physical examination and radiography data will reduce the rate of occupational ATB. The immune substrate of low, medium and high IFNg responses remains to be elucidated using new – generation IGRAs and flow cytometry.

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CLINICAL AND EPIDEMIOLOGICAL FEATURES OF PERTUSSIS CASES IN PLEVEN REGION

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ABSTRACT

Pertussis is a vaccine-preventable disease. During recent years, there is a trend of increasing incidence especially in non-immunised. Our aim was to study the clinical and epidemiological features of pertussis cases. **Material and Methods:** This study was carried out as a retrospective analysis of 28 pertussis cases treated in the Clinic of Infectious Diseases at University Hospital – Pleven between 2009 and 2016. **Results:** 64% of the cases were up to one year of age, 21% – one to three years old. Male to female ratio was 1 to 1.8. 36% of the cases were registered during February to March and 25% in June. The children up to one year of age were not immunised due to premature birth, hypotrophy, and frequent illnesses. All cases were treated with antibiotics before admission. The initial symptom was racking cough, followed by whooping sound, flushed face or cyanosis. Fever was registered in 29%. Pneumonia and bronchitis were found by X-ray in 14 and 21%, respectively. Laboratory investigations revealed leucocytosis in 86%, lymphocytosis in 93%, and increased platelet count in 86%. Real-time PCR was performed in 57% of the cases and gave positive result in 62.5% of the tested children. The patients were treated with antibiotics (cephalosporins in 71%, ampicillin in 25%), corticoids (in 82%), and expectorant. The median duration of hospital treatment was eight days. **In conclusion:** The trend of increased incidence of pertussis requires further studies on the clinical, epidemiological, and immunological aspects.

KEYWORDS: Pertussis; Vaccine coverage; Real-time PCR

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INTRODUCTION

Pertussis is a respiratory disease, caused by the bacterium *Bordetella pertussis* (1). According to Gordon JE et al. (1951), pertussis was a prominent cause of mortality in early childhood (2). After considering the routine paediatric immunisation with a whole-cell pertussis (wP) vaccine, the number of reported cases were significantly reduced in most developed countries, such as the USA (3) and Canada (4). Despite the success, pertussis remains a public health challenge. Although there is 82% global vaccine coverage, according to 2008 estimates, pertussis caused 16 million cases and 195 000 deaths in children younger than 5 years worldwide (5, 6). Typically, pertussis presents a significant burden for public health in developing countries, but it has also re-emerged in some developed countries with high vaccine coverage, including the USA (3), the UK (7), and Australia (8). Many explanations have been advanced, but the causes of these resurgences remain disputable.

Pertussis is highly contagious, with an 80% contagious index among susceptible individuals. The agent is transmitted from person to person by respiratory droplets or by direct contact with respiratory secretions (9). The most serious infections and most pertussis-related deaths occur in non-vaccinated infants but adolescents and adults also experience a health burden from the disease. There is a trend of increase in pertussis cases among adolescent and adult populations. Parents and other relatives are common source of *B. pertussis* infections for infants (10).

The causative agent *B. pertussis* enters the human host through inhalation of respiratory droplets and adheres to the ciliated epithelium of the respiratory tract. It was considered that *B. pertussis* is an extracellular pathogen, but it has recently been shown that *B. pertussis* can invade alveolar macrophages. The agent rapidly multiplies on the mucosal membrane of the

upper respiratory tract, followed by adhesion to the ciliated epithelium (11). *B. pertussis* survives within the human host by producing a variety of virulence factors in attempt to evade or counter the host immune system of the host (12, 13, 14). These virulence factors include adhesins (filamentous haemagglutinin, agglutinogens, pertactin, and fimbriae) and toxins such as pertussis toxin, adenylate cyclase toxin (ACT), tracheal cytotoxin, dermonecrotic toxin, and heat-labile toxin (9, 11, 12, 13, 14). *B. pertussis* is a Gram-negative pathogen containing lipopolysaccharides with the properties of endotoxin that facilitates the colonisation by agglutinating human cells (15).

As stated by Steele RW (2001), pertussis was first described in 1578 as an epidemic of paediatric respiratory disease that began in Paris and spread throughout Europe. It is unclear if this is the first emergence of the disease or the first more precise recording of clinical observations (15). In the pre-vaccination era (between 1940 and 1945), nearly every child contracted the disease and pertussis was a major cause of infant death throughout the world (16). During the same period, more than one million cases of pertussis were reported in the USA (175,000 cases per year) (9). Since the whole-cell pertussis vaccine was introduced in the United States in the mid-1940s, pertussis sharply declined and cases were reduced more than 90% compared to levels in the pre-vaccination era. Although effective vaccination campaigns have been well established in developed countries for more than 50 years, pertussis still remains endemic. Increased incidence with epidemic peaks occur every three to five years in the USA (17). Furthermore, vaccines are not adequately used in many developing countries. Approximately 50 million cases of pertussis occur over the globe each year with 300,000 deaths annually. This fact makes pertussis the fifth leading cause of vaccine-preventable deaths (15).

Pertussis is a vaccine-preventable disease. During recent years, there is a trend of increased incidence (Table 1), especially in the non-immunised. Our aim was to study the clinical and epidemiological features of pertussis cases.

Table 1. Incidence of pertussis and coverage with DPT1 and DPT3 globally and in Europe (1980 – 2014). Available from: **http://www.who.int/immunization/monitoring_surveillance/data/EUR/en/**

	1980	1990	2000	2010	2011	2012	2013	2014
Global population (in thousands)	4’418’554	5’285’577	6’087’645	6’873’237	6’954’776	7’036’567	7’118’327	7’199’708
Population in Europe (in thousands)	797’842	848’501	869’888	898’752	901’747	904’484	906’996	909’354
Globally registered cases of pertussis (in thousands)	1’982’355	476’374	190’475	160’710	171’740	249’746	161’889	172’940
Registered cases of pertussis in Europe (in thousands)	90’546	129’735	53’675	28’212	34’436	57’539	28’170	43’774
Global coverage with DTP1 (%)	30	88	83	90	91	90	91	91
Coverage in Europe with DTP1 (%)	70	89	96	96	96	97	98	97
Global coverage with DTP3 (%)	20	76	73	85	85	85	86	86
Coverage in Europe with DTP3 (%)	63	80	93	94	94	96	96	95

MATERIAL AND METHODS

This study was carried out as a retrospective analysis of 28 pertussis cases treated in the Clinic of Infectious Diseases at University Hospital – Pleven (2009-2016). Specimens for molecular analysis were nasopharyngeal secretions taken with a dry sterile swab of the upper respiratory epithelium. Laboratory confirmation of the clinical diagnosis of pertussis was performed by real-time PCR. The advantage of the PCR method is that it is not affected by immunisations which is of particular importance in the diagnosis of infants and young children. The method diagnoses only active infection or carrier of *B. pertussis*. Real-time PCR is much more sensitive, specific, and rapid than culture methods, immunofluorescence, and serological diagnostic methods. It is possible to detect very small amounts of pathogen DNA in the examined sample. With these advantages PCR becomes the first choice for diagnostics.

RESULTS

Two-thirds of the cases **–** 64.29% (18/28) were up to one year of age, 21.43% (6/28) – one to three years old (Figure 1). Male to female ratio was 1 to 1.8. Rural residents were 60.71% (17/28) and the remaining living in Pleven and other small towns in Pleven region.

During February and March were registered 35.71% (10/28) of the cases, and in June – 25% (7/28). Monthly distribution of the cases is shown in Figure 2. The children up to one year of age were not immunised because of premature birth, hypotrophy and frequent illnesses. All cases were treated with antibiotics before admission.

The initial symptom was racking cough, followed by whooping sound, flushed face or cyanosis. Fever was registered in 28.57% (8/28). Pneumonia and bronchitis were found by X-ray in 14.29% and 21.43%, respectively. The main clinical symptoms observed are shown in Figure 3.

Laboratory investigations (Figure 4) revealed leucocytosis in 85.71% (ranging 11.4-60.2 x 10^9/L; mean 20.16±11.93), lymphocytosis in 92.86% (ranging 49-81%; mean 63.82±11.7); increased platelet count >360.10^{12}/L in 85.71% (ranging 374-1015; mean 543.11±194.72).

Real-time PCR was performed at the National Reference Laboratory of Molecular Biology (National Centre of Infectious and Parasitic Diseases – Sofia) for 57.14% of the cases, and it was positive in 62.5% of the tested children.

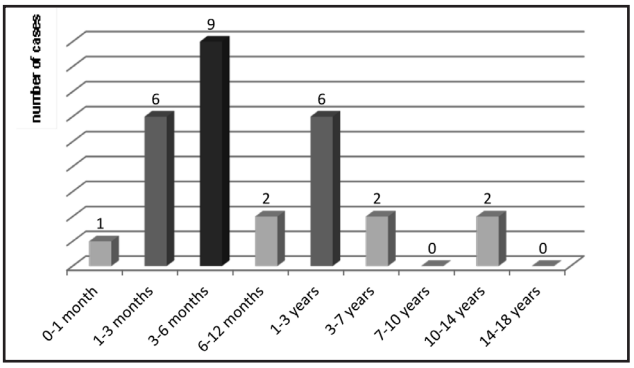

Figure 1. Age distribution of pertussis cases in Pleven Region (2009 – 2016).

All cases were treated with antibiotics

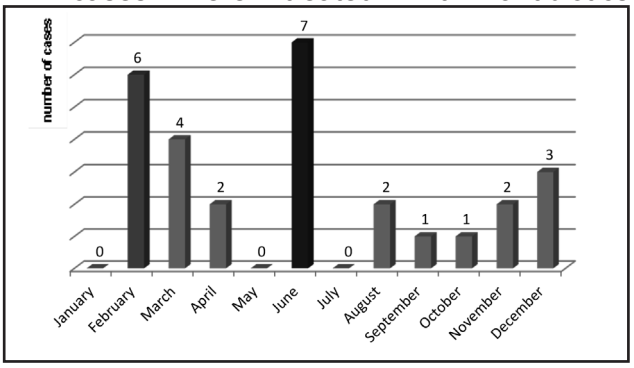

Figure 2. Monthly distribution of pertussis cases in Pleven Region (2009 – 2016).

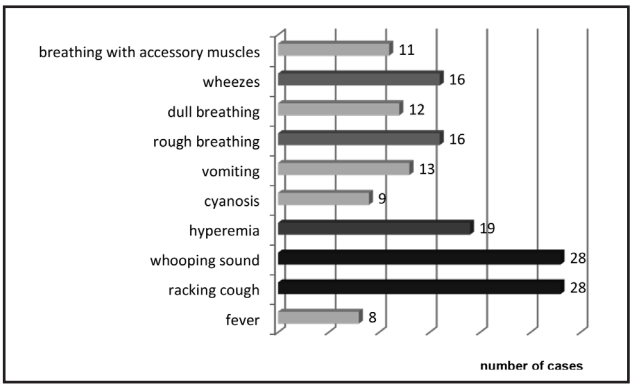

Figure 3. Clinical symptoms of pertussis cases in Pleven Region (2009 – 2016).

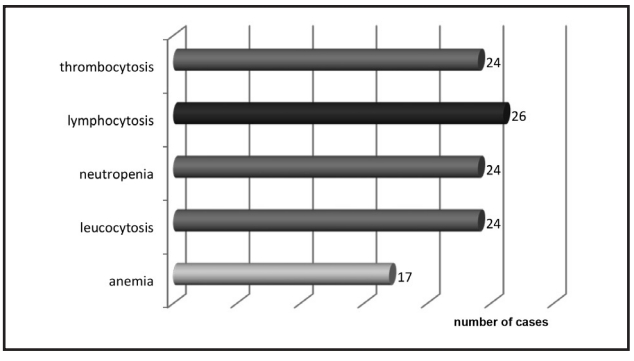

Figure 4. Laboratory investigations of pertussis cases in Pleven Region (2009 – 2016).

(cephalosporins in 71.43% and ampicillin in 25%), corticoids (82.14%), and expectorant. Supportive therapy with inhalations and oxygenation was also performed. The median duration of hospital treatment was eight days with a maximum up to 19 days.

DISCUSSION

Pertussis is an acute infectious disease of the respiratory tract. It is highly contagious, affecting 80 to 90% of exposed and susceptible individuals. During the last 30 years, despite the high level of vaccination coverage, the morbidity and mortality rates of the disease are increasing among all age groups, making pertussis a hot topic to be discussed and hopefully solved in the future. Still there is a misunderstanding among people that pertussis is only a disease of childhood (10, 18). Actually, it affects all ages but it is mostly recognised in children because of the typical clinical presentation and complications (10, 18). In our study, two-thirds of the cases – 64.29% (18/28) were up to one year of age, 21.43% (6/28) – one to three years old. Female cases were nearly two times more than male. Rural residents were 60.71% (17/28) and the remaining living in Pleven and other small towns in Pleven region.

In the pre-vaccination era, most adults suffered from pertussis in their childhood and afterwards they were in contact with many infected people. Their residual pertussis immunity remained high and mothers passed enough antibodies to their babies so they remain passively protected through the first year after birth. For this reason, the disease affects most frequently children between 1-5 years. The introduction of whole-cell pertussis vaccine in the mid-1940s successfully reduced the cases by more than 90% for several decades but also changed the age structure of the disease (17). Protection provided by the vaccine starts to wane 3-5 years after the vaccination and completely disappears after 12 years, and therefore the most protected age group is 2-6 years. In adults and adolescents the immunity is weak or missing and they become vulnerable to the disease. Despite that the most serious pertussis infections and deaths occur in infants and young children, adolescents and adults also carry a health burden although they rarely suffer the typical clinical presentation of the disease. The importance of the increased number of pertussis cases in adolescents and adults is that they become potential source of pertussis infection for non-immune individuals (17, 19, 20, 21). In our study, only two of all cases were in the age group of ten to fourteen years.

Since the early 1980s, pertussis incidence has increased, with peaks occurring every 3-5 years. The incidence rate rose more than 3 times between 1980 and 1998 as the number of reported cases in adolescents and adults greatly contributed to this increase. The number of reported cases above the age of ten years increased from 13 to 47% in Bulgaria during this period (5). There are several explanations of these trends such as increased awareness, improved diagnostic tests and better reporting, increased circulation of *B. pertussis*, genetic changes and strain variations, waning population immunity related to the switch from whole cellular to acellular vaccine. Despite vaccination, pertussis incidence in the USA in children below 1 year of age has increased from 34.2/100 000 in 1980 to 51.1/100 000 in 1990 (rising with 49%). In 2003 the highest incidence was reported in infants (below 6 months of age) – 103.1/100 000 and in teenagers (between 10-19 years of age) – 11.1/100 000 (3, 19). According to CDC, in 2010 pertussis incidence reached 27.550 cases (highest number since 1959) with 27 related deaths. In 2011, adolescents and adults accounted for 47% of the pertussis cases and children aged 7-10 years were 18% of the cases (3, 19). In Table 2 we present the number of all reported pertussis cases in the European Region, Bulgaria, and Pleven region. The global incidence of pertussis is around 48.5 million cases per year with a mortality rate of nearly 295 000 deaths per year.

Although the number of pertussis cases dramatically reduced since the pre-vaccination era, there is still much to be done until the disease is fully controlled. The high incidence in adolescents and adults in highly vaccinated population shows that the current vaccination program is probably not sufficient and more research is needed for new and more successful vaccines. It should be developed a more successful vaccine containing different components of the most commonly circulating *B. pertussis* strains. The vaccine/s should have lower risk of adverse effects and provide longer protective immunity. The development of a booster in adolescents and adults is also

Table 2. Reported cases of pertussis in the European Region, Bulgaria, and Pleven region (2009 – 2016).

	2009	2010	2011	2012	2013	2014	2015	2016→
European region* (in thousands)	?	28 122	34 436	57 539	28 170	43 858	36 626	?
Bulgaria**	285	60	47	101	90	57	37	21
Pleven	5	1	7	6	3	0	3	3

**WHO Database for the European Union*
***NCIPD (National Centre of Infectious and Parasitic Diseases – Sofia, Bulgaria)*

very important as well as encouraging pregnant woman to receive Tdap (tetanus, diphtheria, and acellular pertussis vaccine) during the third trimester of pregnancy to provide effective placental transfer of specific antibodies to their babies (19). In our study all children up to one year of age were not immunised because of premature birth, hypotrophy, and frequent illnesses. Moreover, all studied cases were treated with antibiotics before admission.

Cough is one of most common complaints among children. A racking cough with whooping sound was observed in all studied cases. Flush face or cyanosis were observed in 67.86% (19/28) and 32.14% (9/28), respectively. In 46.43% (13/28) of the cases the cough was followed by vomiting. Only eight children (28.57%) were febrile. Rough or dull breathing was found with auscultation in 57.14% (16/28) and 42.86% (12/28), respectively. Pulmonary wheezes were registered in 57.14% (16/28) of the children. Pneumonia and bronchitis were found by X-ray in 14.29% and 21.43%, respectively. All clinical symptoms were in concordance with other studies (13, 22, 23).

Laboratory investigations revealed typical for pertussis findings (1, 6, 14, 25, 26, 27). Leucocytosis was found in 86%, lymphocytosis and neutropenia in 93% and 86%, respectively. Increased platelet count was found in 86% of the children and probably is due to altered platelet aggregation through increase of intracellular cAMP, provoked by the adenylate cyclase toxin (ACT) (24). ACT triggers an extreme leucocytosis with prevalence of lymphocytes and inhibits chemotaxis of neutrophils facilitating intracellular surviving of *B. pertussis* (24).

Erythromycin treatment for fourteen days was recommended by the American Academy of Paediatrics with considerations about gastrointestinal side effects such as nausea, vomiting, diarrhoea, and increased risk of pyloric stenosis in infants up to two months of age (28). CDC recommended erythromycin, azithromycin (with the highest safety in infants) or clarithromycin (29). Trimethoprim/sulfamethoxazole is an alternative for patients having allergy to macrolides (30). Ampicillin does not reduce intensity of the symptoms and transmission of the infection (30). In our study, 71.43% of the children were successfully treated with cephalosporins and 25% with ampicillin.

Conclusion: Typically, pertussis affects non-immunised infants. The trend of increased incidence of pertussis, including adolescents and adults, requires further studies on the clinical, epidemiological, and immunological aspects aimed to improve the strategy of prevention.

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INFECTIOUS AGENTS DURING PREGNANCY - PRELIMINARY STUDY

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ABSTRACT

The **objective** of this study was to determine the frequency of evidence of any infectious agents during pregnancy.

Material and Methods: The present study included 38 serum samples from women with pathological pregnancy collected in the first half of 2017. The following methods were used: serological - indirect enzyme immunoassay (EIA) test for detection of specific IgM antibodies for measles, rubella, parvovirus B19, *Coxiella burnetii, Chlamydia trachomatis;* and molecular - extraction and detection of infectious nucleic acids with PCR assay and electrophoresis in 2% agarose gel to visualise the PCR products.

Results: During the first half of 2017 a total of 38 serum samples from women with pathological pregnancy were tested with enzyme-linked immunosorbent assay and molecular methods. Specific primary reactive B19-IgM were found in 3/38 (7.89%) of the patients. Positive IgM results for *C. trachomatis* and *C. burnetii* Ph. II were found in 3/38 (7.89%) and 2/38 (5.26%), respectively. B19V seroprevalence (presence of specific B19V-IgG antibodies) of the tested group was calculated as 55.26 %. Positive *C. trachomatis* and *C. burnetii* Ph. II IgG results were shown in 9/38 (23.68 %) and 2/38 (5.26%) of the patients. All tested samples were serologically negative for acute measles and rubella infections and had protective immunity.

Conclusion: The combined laboratory approach for determining acute infection (detection of specific IgM/IgG antibodies and nucleic acid) is of particular importance for correct diagnosis and in the monitoring of cases of pathological pregnancy. Among the investigated clinical cases of pregnant women, the presence of acute B19V, *C. burnetii*, and *C. trachomatis* infections did not dominate, and measles and rubella were not detected.

KEYWORDS: pregnancy, infectious agents, ELISA, PCR

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INTRODUCTION

Infectious diseases during pregnancy, along with some forms of pregnancy-related illnesses such as diabetes, cardiovascular, gastrointestinal, kidney disorder, etc. are one of the main causes of complications and mortality of the mother and fetus. Infant infections could be transplacental, perinatal (from vaginal discharge or blood) or after birth (from breast milk or other sources). Clinical manifestations of neonatal infections vary depending on the infectious agent and the gestational age of exposure (1). The risk of infection is generally inversely proportional to the gestational age of acquisition and some result in congenital malformation syndrome. As in the first and second trimesters, they can lead to premature leaking of the amniotic fluid and premature infection with disturbances in the fetus. Sexually transmissible infectious agents are the other type of pathogens that are given special attention as one of the causes of premature labour and subsequent complications.

The known infections that lead to birth defects, and to which traditionally special attention is paid during pregnancy, are indicated by the acronym TORCH (Toxoplasma, others, rubella virus, cytomegalovirus, herpes simplex virus). The "others" category is expanding rapidly with the accumulation of research in this area and includes more viruses that have been shown to cause neonatal disease. These include parvovirus B19 (B19V), varicella zoster virus (VZV), West Nile virus, measles virus, enteroviruses,

adenoviruses, and human immunodeficiency virus (HIV). Hepatitis E virus is also important due to the high mortality associated with infection in pregnant women (2).

Aim

The objective of this study was to determine the frequency of evidence of any infectious agents (rubella, parvovirus B19, measles, *Coxiella burnetii,* and *Chlamydia trachomatis*) during pregnancy.

MATERIALS AND METHODS

Materials

The study included 38 serum samples from women with pathological pregnancy, received in the first half of 2017 at the National Reference Laboratory of Measles, Mumps, Rubella and the National Reference Laboratory Rickettsiae and Cell Cultures, National Centre of Infectious and Parasitic Diseases. The average age of the studied patients was 31.33 ± 4.97 years.

Methods

- Blood samples were taken on admission by venipuncture from each patient and tested for anti-Parvovirus B19 IgM/IgG, anti-Rubella IgM/IgG, anti-Measles IgM/IgG, anti-*C. trachomatis* IgM/IgG antibodies, and anti-*C. burnetii* Ph. II IgM/IgG. Blood was centrifuged at 4000 g for 10 minutes and serum was aliquoted and frozen at -20^{0}C until analysed.
- All serum samples were tested for the presence of IgM/IgG antibodies with a commercial indirect enzyme-linked immunosorbent assay (Euroimmun, ELISA IgM/IgG kits and SerionVirion ELISA IgM/IgG kits). The assays were performed as recommended by the manufacturer and the results were interpreted qualitatively as positive, negative or equivocal.
- Molecular methods
  - Extraction of infectious nucleic acids with commercial kit PureLink®Viral RNA/DNA.
  - PCR assay with commercial kit AmpliTaq Gold PCR Master Mix: 18 µl nuclease-free water, 1 µl primer, 25 µl PCR Master Mix, 5 µl DNA template.
  - Electrophoresis in 2% agarose gel to visualise the PCR products: B19V-NS1 region (103 bp), *C. burnetii sodB* region (257 bp) (3).

RESULTS AND DISCUSSION

During the first half of 2017 a total of 38 serum samples from women with pathological pregnancy were tested with enzyme-linked immunosorbent assays and molecular methods. All samples were investigated for the presence of specific IgM/IgG antibodies, as serological diagnostic markers for five infectious agents included in the current study - rubella, measles, parvovirus B19, *C. burnetii* and *C. trachomatis*.

Specific primary reactive B19-IgM were found in 3/38 (7.89%) of the patients. Positive IgM results for *C. trachomatis* and *C. burnetii* Ph. II were found in 3/38 (7.89%) and 2/38 (5.26%), respectively. All patients were tested for two other viral agents – measles and rubella in view of their role in pathological pregnancy and in connection with the measles outbreak in Bulgaria, March-August 2017. Two of the patients were from Plovdiv region, with a total number of 141 reported measles cases during this period and with a potential risk of measles infection (4). All tested samples were serologically negative for acute measles and rubella infections but PCR assay was not performed because nasopharyngeal swabs or urine suitable for viral isolation were not provided.

B19V-IgG antibodies indicating evidence for past infection were found in 21/38 of the women. B19V seroprevalence (presence of specific B19V-IgG antibodies) of the tested group of pregnant women was calculated as a percentage ratio of 55.26 %, which corresponds to data reported from other authors (5, 6). The group of seronegative pregnant women with a potential risk of infection was 44.73% of the tested. Laboratory evidence of this respiratory virus is important in view of its widespread distribution in Europe and Bulgaria (7, 8). Protective immunity for measles and rubella was detected in 38/38 (100%) samples. Positive *C. trachomatis* and *C. burnetii* Ph. II IgG results were shown in 9/38 (23.68 %) and 2/38 (5.26) of patients.

Table1 shows the distribution of tested patients with regard to the two diagnostic markers – ELISA IgM and ELISA IgG.

Table1. Distribution of patients tested by serological ELISA IgM/IgG diagnostic markers.

Total Number of tested pregnant women	Rubella virus ELISA IgM positive (%)	Rubella virus ELISA IgG positive (%)	Measles ELISA IgM positive (%)	Measles ELISA IgG positive (%)	B19V ELISA IgM positive (%)	B19V ELISA IgG positive (%)	*C. trachomatis* ELISA IgM positive (%)	*C. trachomatis* ELISA IgG positive (%)	*C. burnetii* ELISA IgM positive (%)	*C. burnetii* ELISA IgG positive (%)
n=38	0 0%	38 100%	0 0%	38 100%	3 7.89%	21 55.26%	3 7.89%	9 23.68%	2 5.26%	2 5.26%

Molecular assays were performed for all serologically positive samples. Serologically positive *C. trachomatis* patients were not screened by PCR because cervical samples suitable for DNA extraction were not provided. In all patients detected with B19V-IgM antibodies, B19V viral DNA was also isolated and a positive PCR signal for the B19V-NS1 region was detected.

For the two *C. burnetii* IgM/IgG positive samples the PCR was also positive with successfully amplified *sodB* region. These samples were from women from Burgas and Pleven with premature birth as the outcome of pregnancy. The problem with Q-fever is currently worldwide because of its significance as a disease related to human infectious pathology. *C. burnetii* infection in pregnant women can cause serious complications (over 50%) during pregnancy. Observations show that in endemic regions, the disease causes more pregnancy disturbances than TORCH pathogens. In pregnant women infected with *C. burnetii*, it is imperative to compile a history that includes exposure to tick bite, contact with farm animals, visit to endemic areas and consumption of suspect products of animal origin.

C. trachomatis, considered the most common sexually transmitted organism, is responsible for a variety of infections in women, including urethral syndrome, cervicitis, endometritis, and salpingitis. It may colonise the lower genital tract and result in asymptomatic infection, ultimately resulting in sterility. An estimated 20-40% of sexually active women have been exposed to *Chlamydia*, and 4-5% of sexually active US women are estimated to carry *Chlamydia* in the cervix. Infection may involve the lower genital tract (bartholinitis, cervicitis, acute urethral syndrome), the upper genital tract (endometritis, salpingitis, Fitz-Hugh-Curtis syndrome), or both. However, the principal focus of infection appears to be the cervix. The maternal carriage rate of *C. trachomatis* ranges between 2-30%. Infection of the female genital tract during pregnancy may result in spontaneous abortion, premature labour, postpartum endometritis, neonatal conjunctivitis, or pneumonia (9).

Patients with presence of a marker of acute parvovirus B19 infection (IgM and pathogenic DNA) are recommended monitoring (FV monitoring of the fetus and Doppler screening) and laboratory screened during pregnancy.

A number of infectious agents have been linked to adverse outcomes of pregnancy. The purpose of the present study was to determine the implication and prevalence of viral agents (parvovirus B19, rubella, measles), *C. burnetii,* and *C. trachomatis* in the etiology of pathological pregnancy. The infections were transmitted to the fetus perinatally (from vaginal discharge or blood) or after birth (with breast milk). The clinical manifestations of neonatal infections vary depending on the viral agent and the gestational age of obtaining (1).

Among the investigated clinical cases of pregnant women, the presence of acute B19V, *C. burnetii* and *C. trachomatis* infectionsdid not dominate, and measles and rubella were not detected. Considering that this is still a pilot study, the small number of tested patients could be pointed as a limitation of the survey.

The combined laboratory approach for determining acute infection (detection of specific IgM/IgG antibodies and nucleic acid) is of particular importance for correct diagnosis and in the monitoring of cases of pathological pregnancy (10).

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PREVALENCE OF POLYOMAVIRUS KIPyV IN BULGARIAN PATIENTS WITH RESPIRATORY DISEASES

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ABSTRACT

Background: The role of polyomavirus KIPyV as a respiratory pathogen still remains controversial. Little is known about the oncogenicity of KIPyV. The aim of this study was to determine the prevalence of KIPyV in patients with acute respiratory diseases and chronic lung diseases, including lung cancer, in order to evaluate the association between KIPyV infection and respiratory diseases. **Material and methods**: This study included 221 specimens (nasopharyngeal swabs and lung biopsy specimens) obtained from patients with acute respiratory diseases and chronic lung diseases, including lung cancer. Detection of KIPyV was performed by nested PCR.**Results:** KIPyV positivity was 5.9 % on average. All nasopharyngeal specimens were obtained from patients with acute respiratory diseases and10.3% of them were KIPyV-positive. Among children aged 12 years or less KIPyV prevalence was higher than that in patients older than 12 years. We found 2.8%KIPyV positivity rate among patients with noncancer chronic lung diseases. All specimens from lung cancer cases were KIPyV-negative. **Conclusions:**KIPyV was more prevalent among patients with acute respiratory diseases, especially children, indicating that there may be a relationship between the virus and these diseases. The prevalence is very low in chronic lung diseases, suggesting no association with KIPyV infection. We were not able to detect KIPyV in samples from patients with lung cancer, indicating no oncogenic potential of this polyomavirus.

KEYWORDS: KIPyV polyomavirus, nested PCR, respiratory diseases, lung cancer

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INTRODUCTION

The first two human polyomaviruses, BKV and JCV, were isolated in 1971 and since then these viruses have been detected in various samples,collected from patients of different geographical regions, including Bulgaria(1-2). More than 35 years later, in 2007, the third human polyomavirus was identified in nasopharyngeal aspirates ofchildren suffering from respiratory symptoms using high-throughput sequencing technologies(3). The virus was named after the institution where the discovery was made - Karolinska Institute polyomavirus (KIPyV). Subsequent studies revealed a worldwide KIPyV distribution and presence of viral DNA in many different samples: respiratory, blood, faeces, cerebrospinal fluid, lymphoid tissues. It was suggested that primary infection occurs during childhood, establishing a persistent infection.

Most frequently KIPyV has been detected in respiratory samples from patients with acute respiratory symptoms.A higher prevalence of KIPyV was observed in respiratory tract specimens from immunocompromised patients (4). To date, however, the role of KIPyV as a respiratory pathogen has not been clarified andremains controversial.

Little is known about the oncogenicity of KIPyV. So far, KIPyV has not been associated with a specific malignancy, but viral DNA sequences have been found in lung cancer cases(5).

The aim of this study was to determine the prevalence of KIPyV in patients with acute respiratory diseases and chronic lung diseases, including lung cancer, in order to evaluate the association between KIPyV infection and respiratory diseases.

MATERIAL AND METHODS

Clinical samples

This study included 221 specimens: 133 nasopharyngeal swabs (NPS) and88 lung biopsy specimens (LBS). They were obtained from patients diagnosed with respiratory diseasesand aged between 1 and 83 years. NPS specimens were provided by the National Reference Laboratory "Influenza and Acute Respiratory Diseases".LBS were obtained from the Specialised Hospital for Active Treatment in Oncology, Sofia.

Detection of KIPyV by nested PCR

DNA extraction was performed using GeneJET Genomic DNA Purification Kit (Thermo Scientific™) or PureLink® Genomic DNA Mini Kit (Invitrogen™) according to the manufacturer's instructions. Quality of extracted DNA of each specimen was evaluated by β-globin gene amplification with GH20/PC04 primer set as previously described (6).The primer sequences were as described by Allander et al. (3) with slight modification:first round of amplification -5′-AAGGCCAAGAAGTCAAGTTC-3′ and 5′-ACACTCACTAACTTGATTTGG-3′; second PCR-5′-CGCAGTACCACTGTCAGAAGAAAC-3′ and 5′-TTCTGCCAGGCTGTAACATAC-3′. The reaction volume was 15 µl and includedAmpliTaq Gold® 360 Master Mix (Applied Biosystems™), 10 pmol of each primer and nuclease-free water. The first PCR was performed with 4 µl eluted DNA, while 1 µl of the first PCR product was transferred in the second PCR. Aplasmid containing the genome of KIPyV was used as positive control and distilled water as negative control. Amplifications were performed on a DNA Engine Opticon 2 system (MJ Research). The PCR conditions includeddenaturation for 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C,and 30 sec at 72°C, and final extension step of 7 min at 72°C.

Ten microlitres of amplification products were analysed by electrophoresis in 2% agarose gels, stained with ethidium bromide and observed under UV light.

All KIPyV-positive specimens were retested.

Statistical analysis was performed with SPSS for Windows v.10.0 using Fisher exact test to calculate P values. P values of less than 0.05 were considered statistically significant.

RESULTS

Of all 221 specimens, 185were β-globin-positive and were subjected to testing for KIPyV by nested PCR. Of them11(5.9%) samples were KIPyV-positive and amplification of a 211-bp fragment in the second PCR was observed (Fig. 1).

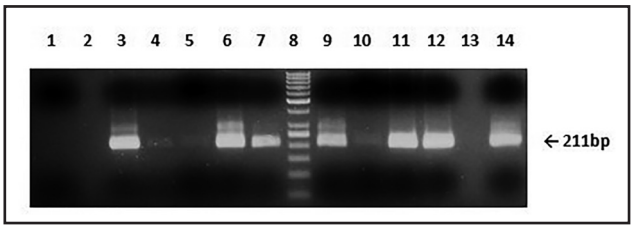

Figure1. Detection of KIPyV in NPS by nested PCR (second PCR). Amplification of 211-bp fragment in KIPyV-positive samples (lines 3, 6, 7, 9, 11, 12). Negative (line 1) and positive (line 14) controls. Line 8: MW marker,50-bp DNA ladder.

We tested two types of specimens: NPS and LBS. Of97NPSsamples 10 (10.3%) were KIPyV-positive. All NPS specimens were collected from patients with acute respiratory diseases of higher and lower respiratory tract (pharyngitis, tonsillitis, common cold, bronchitis, pneumonia, etc.).Four of 27(14.8%) NPS obtained from children aged 12 years or younger were KIPyV-positive, while8.6% (6 of 70) of NPS from patients older than 12 yearswere positive (P = 0.4577).KIPyV prevalence in NPScollected from women and men were12% (7 of 58) and 7.7% (3 of 39), respectively (P = 0.7353).

Lung biopsy specimens were collected from88 patients with chronic lung diseases: 53 lung cancer cases and 35 cases with noncancer chronic lung diseases (pulmonary fibrosis, hamartoma, COPD, inflammatory pseudotumours, sarcoidosis). Of 35 tested samples from patients with noncancer chronic lung diseases only one (2.8%) was KIPyV-positive. It was obtained froma patient with hamartoma. We were not able to detect KIPyV in samples from lung cancer patients.Table 1 showsKIPyV positivity according to the type of respiratory diseases. The difference of KIPyV positivity rates between patients with acute respiratory diseases and patients with chronic lung diseases, including cancer,wasstatistically significant (P=0.0104).

Table 1.KIPyV positivity according to the type of respiratory diseases.

Type of respiratory diseases	β-globin-positive samples (n)	KIPyV-positive samples (n)	KIPyV-negative samples (n)	% KIPyV-positive samples
Acute respiratory diseases	**97**	**10**	**87**	**10.3**
Chronic lung diseases:	**88**	**1**	**87**	**1.1**
of them				
- lung cancer	**53**	**0**	**53**	**0**
- **noncancer chronic lung diseases**	**35**	**1**	**34**	**2.8**
All	**185**	**11**	**174**	**5.9**

DISCUSSION

We analysed 185 β-globine-positive clinical samples (NPS and LBS) from patients with respiratory diseases for the presence of KIPyV and observed a positivity rate of 5.9% on average.We detected highKIPyV prevalence in NPS (10.3%) obtained from patients with acute respiratory diseases of upper and lower respiratory tract. This rate was higher compared to the previously reported data by other authors(7). The virus has been detected by PCR in the respiratory samples, mainly nasopharyngeal specimens,of patients from all continents with reported detection rates ranging from 0.5% to 8%. A higher prevalenceof KIPyV (about 18%)was observed in respiratory specimens from immunocompromised patients (4). TheNPS samples we tested were from immunocompetent patients. The higher virus prevalence observed in NPS in our study might be due to a higher sensitivity of theKIPyV detection test we have used.

We found that KIPyV was more prevalentin NPS from children aged 12 years or less compared to patients older than 12 years (14.8% vs. 8.6%). Thisis in accordance with previous studies, indicating that younger age was associated with higher occurrence of KIPyV infection (8). At the same time, it should be taken into account that in our case, the group of children aged 12 years or less was smaller compared to the group “older than 12 years” (27 vs. 70). We also observed higher KIPyV prevalence among women compared to men (12%vs. 7.7%).However, the difference of the KIPyVstatus according to age and genderwas not significant.

Presence of KIPyV in normal lung tissue was reported by some authors (5, 9). Relatively high frequency of KIPyVdetection (9.2%) was found in transbronchial biopsies from lung transplant recipients but no significant association with a specific clinical and/or histopathological pattern was observed (10). These results suggested that KIPyVcould establish infection in lung tissue. Therefore, we tested for KIPyV lung biopsy specimens, collected from patients with chronic lung diseases. We found low KIPyV positivity among patients with noncancer chronic lung diseases – only one patient diagnosed with hamartoma was KIPyV-positive, suggesting no association between virus infection and these diseases.

Polyomaviruses are suspected to be causative agents of human cancer (11-12). We tested 53 specimens (LBS) from lung cancer cases for KIPyV. All samples were negative for KIPyV, indicating no oncogenic potential of this polyomavirus. Similar results are reported by Teramotoet al.(9) in Japan, although other authors in Italy detected KIPyV in lung cancer samples (5). Further studies are needed for a better clarification of the oncogenic potential of KIPyV for lung cancer development.

In conclusion, our results show that KIPyV is more prevalent among patients with acute respiratory diseases, especially children, indicating that there may be a relationship between the virus and these diseases. The prevalence is very low in chronic lung diseases, suggesting no association with KIPyV infection. We were not able to detect KIPyV in samples from patients with lung cancer, indicating no oncogenic potential of this polyomavirus.

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UV SURFACE DISINFECTION USING GERMICIDAL LAMPS: A STUDY ON THE OPTIMAL UVC INTENSITY AND THE OPTIMAL EXPOSURE TIME

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ABSTRACT

Background: The implementation of ultraviolet light (UV) irradiation has been suggested as a supplement technology to the standard protocols for terminal cleaning and disinfection of room surfaces. Our previously published questionnaire study indicated the widespread use of germicidal lamps in healthcare facilities in Bulgaria, as well as the need for updated recommendations to ensure their effective usage. The aim of the present study was to define the optimal UVC intensity and the optimal exposure time when using germicidal lamps for disinfection of contaminated surfaces.

Material and methods: A test suspension of microorganisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae,* and *Candida albicans*) was spread on a petri dish with nutrient agar. The plates were placed at different distance from the UV lamp, with different UVC intensity reaching the plates: 4 μWs/cm^2 (distance to the germicidal lamp - approximately 3 metres), 8 μWs/cm^2, 15 μWs/cm^2 (2 - 2.5 metres), and 20 μWs/cm^2 (approx. 1 metre). The plates were exposed to varied UVC intensity for varied time periods: 5 min, 15 min, 30 min, 45 min, 60 min, and 90 min.

Results: With the lowest UVC intensity (4 μWs/cm^2), germicidal effect was observed at 90 minutes of exposure. When 8 μWs/cm^2 UVC intensity reached the inoculated plates, germicidal effect was observed at 30 minutes. UV surface disinfection for 15 minutes is adequate when UVC intensity of ≥ 15 μWs/cm^2 is used.

Conclusions: The ceiling-mounted germicidal UV tubes are effective for surface disinfection when exposure time is 15-30 minutes and UVC intensity at each part of the irradiated surface is ≥ 8 μWs/cm^2.

KEYWORDS: germicidal UV lamps, UV intensity, surface disinfection

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INTRODUCTION

Over the past decade, multiple studies have demonstrated that the contaminated surfaces in hospital environment play an important role in the transmission of healthcare-associated pathogens. Although patient-to-patient transmission of nosocomial pathogens most commonly occurs via the hands of healthcare personnel (HCP), contaminated hospital surfaces and medical equipment can also be involved, directly or indirectly, in the transmission pathways (1, 2). According to a recent study, hand contamination with MRSA has been shown to occur with equal frequency after a direct contact of HCP with the colonised/infected patient or by touching only contaminated surfaces (3). The importance of the environmental contamination and the following pathogen transmission has grown with the progressively expanding spread of highly persistent agents like MRSA, VRE, *Acinetobacte*r spp., norovirus, and *Clostridium difficile*, demonstrated to persist in the environment for hours to days and in some cases, for months (4, 5, 6). It is well known that these are the key nosocomial pathogens with a potential to cause hospital outbreaks and responsible for the increasing problem of multidrug resistance both on international and national level (7, 8, 9, 10, 11, 12, 13, 14, 15). Based on these findings it has been concluded that improvements in environmental disinfection may prevent transmission of pathogens and reduce healthcare-associated infections. For this purpose, the implementation of ultraviolet light (UV) irradiation has been suggested as a supplement technology to the standard protocols for terminal cleaning and disinfection of room surfaces (16, 17). The resurgence of the interest in UV disinfection is evidence-based and relies on a number of important advantages: broad-spectrum (killing of bacteria, viruses, and yeasts), absence

of residual effect, “No-touch” method with low maintenance and easy handling, low operating costs and highest reliability (18, 19). A series of currently published studies also demonstrated the effective decontamination of targeted pathogens from patient rooms using automated mobile UV-C-emitting devices (20, 21, 22). The efficacy of UV irradiation is a function of many different parameters such as intensity, exposure time, lamp placement, and air movement patterns. However, in spite of a 50-year experience of its usage, the review of respective sources shows a lack of well-defined guidelines for the hospital practice (19). In our previously published questionnaire study we confirmed the widespread use of germicidal lamps in healthcare facilities in Bulgaria, as well as the need of updated recommendations to ensure their effective usage (23). In order to help resolve this problem we carried out a series of laboratory investigations. The aim of the present study was to define the optimal UVC intensity and optimal exposure time when using germicidal lamps for disinfection of contaminated surfaces.

MATERIAL AND METHODS

Three different germicidal Philips UV tubes with 30 watt power were used in this study.

The organisms we selected for the study were reference strains used in the tests for evaluation of bactericidal and yeasticidal activity of disinfectants - *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, *Enterococcus hirae* ATCC 10541 and *Candida albicans* ATCC 10231 (24, 25). The test microorganisms were subcultured on nutrient agar (ready for use Casein Soya Agar, 450 ml - BulBio) and incubated at 37°C for 24 hours.

Experiments to determine the optimal UVC intensity and the optimal exposure time

To determine the bactericidal effect on surfaces, the experiments were performed in a 35 m^3 room with only one ceiling-mounted UV tube. The UVC intensity, reaching the exposed surface, is determined by the UVC meter.

For each test microorganism a test suspension was prepared with a diluent, containing 1 g tryptone and 8 g NaCl in 1000 ml sterile water. The opacity of test suspensions was determined by WHO International Standard (Opacity 5IRP). The initial density of the suspensions was determined by dilution to obtain numbers of colony-forming units/ml (cfu/ml) and the viable count is represented in ten logs.

Ten microlitres of this suspension were spread on a petri dish with nutrient agar. The plates were placed at different distance from the UV lamp, with different UVC intensity reaching the plates: 4 μWs/cm^2 (distance to the germicidal lamp - approximately 3 metres), 8 μWs/cm^2, 15 μWs/cm^2 (distance to the germicidal lamp - approximately 2 - 2.5 metres), and 20 μWs/cm^2 (distance to the germicidal lamp - approx. 1 metre). The plates were exposed to varied UVC intensity for varied time periods: 5 min, 15 min, 30 min, 45 min, 60 min, and 90 min. Control petri dishes were not irradiated. All plates inoculated with microorganisms were incubated at 37°C for 48 hours.

Reduction of viable count was expressed as a ratio of initial viable count to viable count after exposure to UV light. The results were shown in ten logs.

RESULTS

The reduction of test organisms exposed to varied UVC intensity for varied time is shown in Tables 1-4.

With the lowest UVC intensity (4 μWs/cm^2), germicidal effect was observed at 90 minutes of exposure. For all bacteria the reduction of viable count was > 4 logs and for *C. albicans* > 3 logs. Of all bacteria *P. aeruginosa* was the most sensitive to irradiation, against the higher initial density of the test suspension. When 8 μWs/cm^2 UVC intensity reached the inoculated plates, germicidal effect was observed at 30 minutes, and the reduction of all test microorganisms was > 4 logs. At 15 μWs/cm^2 and 20 μWs/cm^2 UVC intensity the observed results are similar. For all test microorganisms after 15 minutes of exposure to UV light, reduction of the viable count was > 4 logs and for *C. albicans* > 3 logs. Of all bacteria, *E. hirae* was evaluated as the most resistant to UV light and *C. albicans* was assessed as the most resistant test microorganisms in this study (approx. 3 log reduction).

The overall results showed that UVC intensity is an important factor for surface disinfection by germicidal UV light. The optimal intensity of UVC,

we recommend to reach the exposed surfaces, is ≥ 8 μWs/cm^2 and the optimal exposure time is 30 minutes (when distance to the germicidal UV lamps is no more than 2.5 metres). UV surface disinfection for 15 minutes is adequate when UVC intensity of ≥ 15 μWs/cm^2 is used.
All control petri dishes (plates) were recorded as "nc" (not countable).

Table 1. Reduction of test organisms exposed to 4 μWs/cm^2 UVC intensity.

Test organism	Initial density of test suspension		cfu/plate and exposure time for UV surface disinfection						
	cfu/ml	cfu/plate log	5 min	15 min	30 min	4 5 min	60 min	**9 0 min**	**R log**
P. aeruginosa	9.0 x 10^8	6	nc	140	23	23	16	11	**4.96**
S. aureus	6.7 x 10^8	6	nc	nc	30	25	17	3	**5.52**
E. hirae	2.0 x 10^8	6	nc	nc	nc	nc	117	20	**4.7**
C. albicans	1.75 x 10^7	5	nc	nc	240	210	137	77	**3.11**

Table 2. Reduction of test organisms exposed to 8 μWs/cm^2 UVC intensity.

Test organism	Initial density of test suspension		cfu/plate and exposure time for UV surface disinfection					
	cfu/ml	cfu/plate log	5 min	15 min	**30 min**	**R log**	4 5 min	6 0 min
P. aeruginosa	6.0 x 10^8	6	nc	78	14	**4.85**	5	7
S. aureus	9.5 x 10^8	6	nc	70	8	**5.1**	6	8
E. hirae	8.4 x 10^8	6	nc	> 330	35	**4.46**	4	3
C. albicans	3.9 x 10^7	5	nc	> 330	8	**4.1**	1	0

Table 3. Reduction of test organisms exposed to 15 μWs/cm^2 UVC intensity.

Test organism	Initial density of test suspension		cfu/plate and exposure time for UV surface disinfection					
	cfu/ml	cfu/plate log	5 min	**15 min**	**R log**	30 min	45 min	60 min
P. aeruginosa	2.0 x 10^8	6	21	19	**4.72**	6	5	7
S. aureus	6.0 x 10^8	6	185	10	**5.0**	20	5	7
E. hirae	3.4 x 10^8	6	> 330	11	**4.96**	6	1	6
C. albicans	2.9 x 10^7	5	> 330	41	**3.96**	2	1	1

Table 4. Reduction of test organisms exposed to 20 μWs/cm^2 UVC intensity.

Test organism	Initial density of test suspension		cfu/plate and exposure time for UV surface disinfection					
	cfu/ml	cfu/plate log	5 min	**15 min**	**R log**	30 min	45 min	60 min
P. aeruginosa	4.4 x 10^8	6	29	11	**4.96**	8	3	3
S. aureus	9.9 x 10^8	6	157	16	**4.8**	8	2	3
E. hirae	6.5 x 10^8	6	> 300	25	**4.6**	1	1	1
C. albicans	3 x 10^7	5	> 300	43	**3.37**	0	0	0

cfu - colony-forming units; nc - not countable; R – reduction

DISCUSSION

Despite the widespread use of germicidal lamps in healthcare facilities in Bulgaria, the guidelines for their practical usage are not documented. Similar problem in medical practice reported Katara et al., 2008 (19). At the same time, current data demonstrates that UVC intensity is essential for the effectiveness of UV disinfection. Some studies explored surface disinfection with automated, mobile UVC-emitting devices (20, 21, 22). However, research reports with ceiling-mounted ultraviolet germicidal irradiation (UVGI) used for surface disinfection are not common. Because of the widespread use of the ceiling-mounted germicidal UV lamps in healthcare facilities in Bulgaria and with the aim to provide updated national guidelines for their proper use in disinfection practice we decided to undertake a series of experimental studies. The results of this study confirmed the data obtained from Katara et al., 2008 on the exposure time and the maximum distance between the lamps and the surfaces required to achieve effective disinfection with UV light. Their findings for total level of reduction for bacteria and yeasts can also be confirmed based on our results - about 4 logs for bacteria and 3 logs for the yeasts.

In addition, we determined the effective UVC intensity, which should reach the surface to achieve a quality surface disinfection. This dose depends on the distance from the lamp, the type of microorganisms exposed, and the technical specification of the germicidal UV tubes.

Our preliminary study indicated that a germicidal tube may be inefficient even if it irradiates blue-violet light because of its extremely low UVC intensity (26). The intensity of germicidal wavelength light decreases with age, hours of use, and may vary by manufacturer. The authors recommend periodical monitoring (approximately every 6 months) of cleanliness of UV light bulbs and age of UV lamps (27). We also want to emphasise the need for annual control of UVC intensity by UVC meter to ensure effective UV germicidal disinfection in healthcare practice.

In conclusion, our results indicate that ceiling-mounted germicidal UV tubes are effective for surface disinfection when exposure time is 15-30 minutes and UVC intensity at each part of the irradiated surface is ≥ 8 μWs/cm^2. Finally, we accentuate that the germicidal UV light only supports the routine disinfection in medical practice. When ceiling-mounted UV lamps are switched on the room must be emptied of people, and to achieve an effective disinfection UVC intensity should be periodically monitored.

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