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INVESTIGATING THE RELATIONSHIPS BETWEEN ANTIBIOTIC CONSUMPTION AND RESISTANCE (MINIREVIEW)

Bozhana Todorova

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ABSTRACT

All methods for investigating and evaluating the impact of antimicrobial use on resistance pursue the goal of predicting future behaviour of resistance rates, identifying risk factors in the face of available treatment options and developing strategies for combating the emergence and spread of difficult-to-treat pathogens. There is a perception of hospital environment as the main reservoir of antibiotic resistance since patient population is highly exposed to antibiotic pressure. The strongest evidence for the actual relationship between antibiotic consumption and resistance could be drawn from data collected on each individual patient's exposure to antibiotics and subsequent emergence of resistance. Due to different constraints, most hospital researchers have only aggregated microbiology and pharmacy data available and this kind of analysis provides only correlations but not direct causal evidence. However ecological studies allow the formation of an overall view of the total effect of antimicrobials and encompass consequences of both individual antibiotic therapy courses and transmissibility of resistance.

Keywords: *resistance, correlation, antibiotic consumption, ceftriaxone*

INTRODUCTION

Measurements of primary care antibiotic prescriptions for systemic use show much higher rates compared to hospital prescriptions, and most commonly are indicated for respiratory infections (1). Antibiotic use among hospitalized patients accounts for 10-15% of the total consumption, as seen also from analyses of Bulgarian national database (2). Some studies draw several points to the consequences of questionable and not appropriate application of antimicrobials estimated as 20-50%. These include cases of overuse as well as misuse leading to risks of observed adverse effects in the patient, higher economical burden and higher rate of antimicrobial resistance among community pathogens (3, 4).

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IDENTIFYING RELATIONSHIPS BETWEEN ANTIBIOTIC USE AND RESISTANCE

Countries with low or high rates of penicillin resistance having low or high rates of beta-lactam prescriptions, respectively, serve as one of the classic examples for the partially established connection between the prevalence of resistance to antibiotics and their use (5). This trend is followed by another observation of the north-south directed gradient of consumption of antimicrobials in European countries which is parallel to the antibiotic resistance in community prevailing pathogens (6, 7). Through the years of studying, antibiotic consumption gradually gains more recognition as the main factor of the emerging resistance (1). In every ecological level, referred to as an individual patient, community, region or country, efforts succeed to demonstrate a correlation between antibiotic consumption and selection of resistant organisms (6).

However, some studies fail to capture the relationship behind the emerging resistance owing to the fact that antimicrobial use is a major, but not the only factor determining this process. The other factors, or so-called confounding factors, may involve use of unrelated antibiotic groups, noncompliance with infection control practices, and insufficient power or appropriateness of the statistical analysis. A comprehensive research would have a better opportunity to evaluate the impact of antimicrobial use on resistance if not only the trigger in the face of amount and type of prescriptions, but also the transmissibility of resistance among patients is being taken account for. Various studies focused on single hospitals or countries report concomitant changes in antimicrobial use followed by changes in resistance in a consistent direction, and are probably the most convincing. However, most of them employ aggregated or pooled data encompassing a period of one or several years without measuring the necessary delay for the observation of the actual effect of antimicrobial use on resistance. Having this presumption, Goossens et al. 1986 (8) demonstrate a correlation between the percentage of gentamicin-resistant Gram-negative bacilli and gentamicin use during the previous year but not in the same year, i.e. a one-year delay is used as an adjustment (9).

MECHANISM OF SELECTION

As one of the properties of selective pressure, different drugs within an antibiotic class, may have a different selective power for different types of antibiotic resistance (5, 6, 7). One ecological study exemplifies this with the different selective properties of oral aminopenicillins and oral cephalosporins; also of long-acting macrolides and short-acting macrolides. Not only their quantity, but also pharmacological features of the drugs under study, should be included in the final conclusions aiming at the most appropriate model of the ecological impact on bacterial populations. The observed microorganism in this particular study was *Streptococcus pneumoniae* and despite the lower rates of oral cephalosporin consumption compared to oral aminopenicillins, the former were

recognised as better selectors for high level penicillin resistance. A correlation was demonstrated between the decrease in prescriptions of aminopenicillins compared to cephalosporins with the increase in penicillin resistance (5, 6). With regard to macrolide agents, the consumption of long-acting macrolides exerting extended periods of exposure of bacterial populations, in this same model *S.pneumoniiae*, to selective concentrations of antibiotics, was associated as a main factor for developing of erythromycin resistance (6). A clinical trial with healthy volunteers was designed to study the oral carriage of macrolide-resistant streptococci following dosing with azithromycin and clarithromycin. Clarithromycin exposure was related with both, increase in *erm(B)* and *tet(M)* genes carriage, on one hand, and decrease in *mef* carriage, on the other. After azithromycin use a two-fold increase in erythromycin MIC was observed for macrolide-resistant streptococci that carried *mef* (10, 11). If we take into consideration another example of the use of beta-lactam agents, particularly cephalosporins, and a Gram-negative indicator strain, the work of Muller et al. reveals a correlation between ceftriaxone use and the development of resistance in *Enterobacter cloacae* clinical isolates. The authors explain the great impact of this antibiotic on the intestinal flora with the high biliary elimination compared with other extended spectrum agents of this group whose major route of elimination is via the kidney (12).

Due to the horizontal gene transfer of multiple resistance traits, many researchers seek to determine the probability of selection of resistance to a given antibiotic by the consumption of a different one. Many examples of study designs are following various presumptive relationships. A hospital-based ecological study found not only a correlation between imipenem use and resistance in *Pseudomonas aeruginosa* to the drug itself, but data also revealed an association between imipenem consumption and resistance to ceftazidime and piperacillin-tazobactam. The opposite could not be observed and rates of consumption of ceftazidime and piperacillin-tazobactam showed no association with resistance to these two drugs (13). A US hospital investigated the epidemiological association between carbapenem resistance in Enterobacteriaceae and broad spectrum antibiotics. A statistically significant negative correlation was shown for piperacillin-tazobactam use on the incidence of these isolates (14). In Bulgaria for the last two years there is a sequence of reports describing first isolation in the country of carbapenemases in Enterobacteriaceae (15-18). According to IMS data the proportion of carbapenem use in hospitals at national level is very low, for example in 2013 it is roughly 2% of the whole group of cephalosporins, aztreonam and carbapenems, which means that the relationship with emerging carbapenem resistance genes should be investigated also with other broad-spectrum agents and at individual institutions. Strong positive correlations further were found also between the prevalence of MRSA and β-lactam antibiotics and fluoroquinolones (19). One of the comments to these analyses point out the excretion of quinolones in high concentrations in the nares and skin, which could eliminate normal skin flora and promote colonization by MRSA strains that are quinolone-resistant (20). There are also reports on the association of increased prevalence of vancomycin-resisitant enterococci (VRE) in hospitals with the high rates of vancomycin or third-generation cephalosporin use (19).

Antibiotic consumption may have different impact on the prevalence of resistance among different pathogens (7, 21). Each pathogen has its own dynamics determining the emergence and spread of bacterial resistance which should be taken in consideration by subsequent attempts of controlling these processes with the aid of hospital or community based antibiotic use analyses. Unexpected results obtained at a hospital reveal that there was no correlation between fluoroquinolone consumption and resistance among staphylococci and *P.aeruginosa*. On the contrary, fluoroquinolone resistance of *Escherichia coli* correlated well with consumption data. Authors explain these data by linking pathogen-specific patterns of organism–drug interaction with organism prevalence, clonal turnover, and risks to develop resistance in a particular species. It is assumed that a relevant loss of fitness associated with fluoroquinolone-resistance mutations in *Staphylococcus aureus* and *P.aeruginosa* triggers the pathogen-specific distinctions in acquisition of resistance traits (21). There is not always a parallelism in the curves developed when comparing rates of resistance and rates of consumption. Some studies do not reach positive correlations and demonstrate opposite directions of antibiotic use and resistance. A significantly increased use of cefepime failed to result in an increased rate of cefepime resistance in *Acinetobacter baumannii* and even exerted a protective effect against this resistance. Use of piperacillin/tazobactam has been demonstrated to reduce rates of ceftazidime-resistant or ESBL-producing *Klebsiella pneumoniae*. However, this study showed a significantly positive association between piperacillin-tazobactam use and piperacillin-tazobactam resistant *A.baumannii* (19). The application of the principles of population genetics to the study of antibiotic resistance indicates that there is probably a critical level of drug consumption required to trigger the emergence of resistance at significant levels. There may be a threshold of antibiotic exposure that must be reached before resistance increases within the community. The backward process, decline of resistance rates is also not immediately detectable. Despite the fact that high resistance rates have been described for areas whose antibiotic consumption has increased, a given lower level of resistance has not been immediately seen after a decline in antibiotic use (22, 6). Mathematical modeling shows that there is a lag until a decrease is perceived, and the declining rate is lower than the ascending rate. In addition, the new steady state is reached after a series of temporal oscillations whose intensity, duration, and stability depend on how long this biological lag takes (22). In the light of these observations early interventions and policy measures are even more justified and should follow constantly updated detection techniques of new and emerging resistance determinants.

FEATURES OF ANTIBIOTIC USE IN HOSPITAL ENVIRONMENT

There is a perception of the hospital environment as an epicentre of antibiotic resistance. Patient popu-

lation is highly exposed to the antibiotic pressure in terms of number of people over time (23). These high exposure rates are recognized as the most important factor for the selection of antimicrobial resistance. Authors encourage hospital management to perform monitoring and benchmarking of antimicrobial consumption patterns and to address issues concerned with overuse or misuse (24). Patients receiving antibiotic treatment may have an ecological impact on all hospitalized patients (25). There are many factors causing simultaneous effect on the rapid emergence and dissemination of drug-resistant bacteria in hospitals. These include high selective pressure arising from inappropriate and widespread use of antimicrobial agents particularly in intensive care units, patient to patient cross-transmission due to non-adherence to the appropriate infection control measures, transfer of resistance between hospitals, a community contribution to resistance, or the complex impact of the use of a variety of antimicrobials (19).

The strongest evidence for the actual relationship between antibiotic consumption and antibiotic resistance could be drawn from data collected on each individual patient‘s exposure to antibiotics and subsequent emergence of resistance (26). However, a few studies that sampled individual patients before and after treatment have provided the strongest scientific evidence of this correlation (10, 27-30). Due to different constraints, most hospital researchers have only aggregated microbiology and pharmacy data available to study this relationship. One of the common approaches applied to aggregated data is drawing scatterplots and correlations of consumption versus resistance levels for many hospitals or wards at specified time frame for comparison purpose. Nevertheless there are many arising limitations and most important is that this kind of analysis provides only correlations but not direct causal evidence for the emergence of resistance as a result of consumption of antibiotics (26). Statistically significant positive correlation between the use of ceftriaxone and the incidence of ESBL-producing pathogens was demonstrated for Bulgarian national hospital antibiotic consumption. Considering that almost half of the total antibacterial use is dominated by the cephalosporin group which shows increasing ratio of the use of broad-spectrum (particularly ceftriaxone) and narrow-spectrum agents in the last 7 years, a direct association can be investigated for the emerging and spread of different classes of ESBL enzymes (31-37). Ecological studies employing aggregated data do not have individual patient observations and this limitation does not allow us to determine whether patients with resistant isolates were actually exposed to the relevant antibiotic, other antibiotics or other risk factors associated with resistance (38). Desirable innovation for hospitals is the implementation of computerized antibiotic prescriptions and analysis of emergence of resistance in every individual patient as a consequence of antibiotic exposure (26). Keeping in mind the confounding factors involved in the initiation and conducting of an ecological study, the results allow the formation of an overall view and measurement of the total effect of infectious agents. These studies are important from the standpoint of encompassing both direct and indirect effects mediated by individual antibiotic therapy courses and transmissibility of resistance (20). Some authors report that ecologic systems such as that within the hospital tend to react to changes in antimicrobial use much faster than previously thought, that is, within a few months rather than several years. This finding was reported in studies with variations in the percentage of imipenem-resistant *A.baumannii* following changes in carbapenem use in a Spanish hospital, and also changes in *P.aeruginosa* resistance to imipenem following changes in hospital imipenem use (13, 26, 39). Many hospital and outpatient surveys use pooled (or aggregated) data to analyze temporal associations of antibiotic consumption and resistance observed for a certain period. However small variations in antimicrobial resistance and use observed for shorter periods of time, e.g. months, are not taken into account, neither is the quantification of the magnitude of the effect of antimicrobial use on the level of resistance. López-Lozano et al. propose a new approach employing techniques of the time series analysis and eliminating the disadvantages of the usual statistical methods (40).

All methods for investigating and evaluating the impact of antimicrobial use on resistance pursue the goal of predicting the future behaviour of resistance rates, identifying risk factors in the face of available treatment options and developing strategies for combating the emergence and spread of difficult-to-treat pathogens.

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CARBAPENEMASES – TYPES AND DETECTION

Krasimira Ivanova

NRL Control and Monitoring of Antimicrobial Resistance - NCIPD

ABSTRACT

The spread of carbapenemase-producing bacteria is a threat to healthcare and patient safety. In Bulgaria carbapenem resistance is relatively new phenomenon, which has to be studied, especially when it occurs with outbreaks in hospital settings and in the society. The need of such investigation is related to identification of new and circulating carbapenemases and in-depth examination of the phenotype and genetic characteristics of the carbapenemase-producing bacteria detected in our country. Although carbapenemases belonging to Ambler's classes A (KPC-2), B (VIM, NDM-1), and D (OXA-23, OXA-24, OXA-58) have been reported, still there is no full and comprehensive data on the epidemiology of the carbapenemase positive strains in our country. Another issue of major importance is tracking the emergence and the spread of carbapenem-resistant Enterobacteriaceae, which have been increasingly reported worldwide. For a period of 5 months (May-September 2014) at the National Reference Laboratory "Control and Monitoring of Antibiotic Resistance" in the Department of Microbiology at NCIPD were identified several carbapenemase-producing Enterobaceriaceae: five KPC-producing *K.pneumoniae* isolates, one NDM-producing *K.pneumoniae*, and VIM-producing *S.marcescens.*

Keywords: *Carbapenem resistance, carbapenemases, KPC, NDM, K. pneumoniae, Bulgaria*

BACKGROUND

Carbapenem antibiotics as a subclass of β-lactam agents were developed more than 30 years ago and they are among the most broad-spectrum antibiotics in clinical use [1]. These antibiotics become the primary treatment option for multidrug-resistant pathogens such as *Acinetobacter spp*., *Pseudomonas spp.,* and ESBL producing Enterobacteriaceae [1]. The emergence of carbapenem-inactivating β-lactamases in some of the most common bacterial pathogens such as members of the Enterobacteriaceae is about to become an issue of major concern worldwide.

The prime mechanism for β-lactam resistance among Gram-negative bacteria is the production of β-lactamases, capable of inactivating β-lactam antibiotics by hydrolysing the amide bond of the β-lactam ring [2]. These enzymes have been known for more than 70 years and yet some features of their evolution, dissemination and hydrolytic capacity remains unknown [3].

There are two classification systems used to categorise β-lactamases. The Ambler classification system is based on the amino acid sequences; it divides these enzymes into four classes (A, B, C and D). The Bush-Jacoby classification system categorises β-lactamases according to their functional properties [3].

Class A combines β-lactamases that demonstrate broad (TEM-1 and SHV-1), extended-spectrum (ESBLs - CTX-M-15), and carbapenemase activity (KPC, GES) [3]. Resistance to extended-spectrum beta-lactam antibiotics (e.g. third-generation cephalosporins) due to production of extended-spectrum beta-lactamases (ESBLs) continues to increase in Enterobacteriaceae [2]. Clonal dissemination of CTX-M-3 and CTX-M-15 producers was described in four Bulgarian hospitals in Sofia and Pleven [4]. Also evident increase was observed in ESBL producers from 8% in 2007 to 14% in 2013 [5] and significant correlation between the widespread use of broad-spectrum cephalosporins in Bulgarian hospitals [6] and the increase of ESBL-producing bacteria [5]. Considering the fact that the number of these bacteria is growing in our country it can be assumed that in the near future carbapenems will have major importance for handling infections caused by the ESBL-producing bacteria.

Class B includes metallo-β-lactamases (MBLs) capable of hydrolysing all β-lactam antibiotics, except for the monobactam aztreonam [3]. Class C are cephalosporinases that can be chromosomally-encoded (e.g. inducible *Pseudomonas aeruginosa* AmpC β-lactamase) and plasmid-mediated (e.g. CMY-2 in *Escherichia coli*). Generally, class C enzymes contribute to carbapenem resistance if they are hyperproduced or combined with loss of specific outer membrane porins (OMPs), although there are some recently reported cases of β-lactamases, belonging to this class that have potent carbapenemase activity [7, 8]. The enzymes from Class D are named oxacillinases because of their substrate preference for oxacillin. Some of Class D β-lactamases hydrolyse carbapenems and are defined as carbapenem-hydrolysing Class D β-lactamases-CHDLs [9].

One of the unique properties of carbapenem antibiotics was the ability to inhibit Class A and C β-lactamases, but it is no longer so since the emergence and dissemination of enzymes capable of hydrolysing their molecule [10]. During the past few years a significant number of acquired carbapenemases have been identified worldwide and genes encoding these enzymes are associated with mobile genetic elements that facilitate their rapid spread [11]. For this reason, detection and surveillance of carbapenemase-producing organisms have become an issue of major importance for the choice of appropriate therapeutic schemes and the implementation of infection control measures in hospital settings.

CARBAPENEMASES - TYPES AND CHARACTERISTICS

As it was mentioned above carbapenemases are separated in three classes: Class A (serine carbapenemases); Class B (metallo-β-lactamases); and Class D (OXA carbapenemases). Within these classes, further divisions are made and new variants are frequently added [2, 3]. It is a disturbing fact, that carbapenemases belonging to each of these classes have been identified in Bulgaria.

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CLASS A CARBAPENEMASES

Carbapenemases from Class A have the ability to hydrolyse all β-lactam antibiotics [10]. The mechanism of hydrolysis of the carbapenem molecule involves serine at position 70 in the active site of the enzyme [11, 12]. A variety of Class A carbapenemases have been described worldwide: chromosome-encoded (e.g. NmcA, SME, IMI-1) and plasmid-encoded (e.g. KPC types, IMI-2, GES-types) [13, 14, 15, 16]. Since the genes encoding NmcA, SME, and IMI-1 carbapenemases are chromosomally located, these enzymes are not widespread among Gram-negative bacteria and they do not have significant impact on the dissemination of carbapenem resistance. GES β-lactamases (Guiana extended spectrum) were originally considered ESBLs, but now are classified as serine cabrbapenemases [2]. The genes encoding the GES family of enzymes are located in integrons on plasmids [2]. Since 2001, after the discovery of GES-2 in *P.aeruginosa* isolate, their hydrolytic spectrum covers carbapenems [2].

KPC (*K.pneumoniae* carbapenemase) types are the most common Class A enzymes in clinical settings [13]. This carbapenemase was first observed in a *K.pneumoniae* isolate from the US in 1996 [16]. KPC are most often carried and expressed by *K.pneumoniae* isolates, but are no longer confined to this organism. They have been found in *E.coli, Klebsiella oxytoca, Salmonella enterica, Citrobacter freundii, Enterobacter aerogenes, Enterobacter cloacae, Proteus mirabilis, Serratia marcescens,* as well as in non-fermenting Gram-negative bacilli like *P.aeruginosa, Pseudomonas putida*, and *Acinetobacter spp.*[11,15]. Even though these enzymes can hydrolyse carbapenems, the resistance is not apparent and in many cases MIC values are lower than the MIC breakpoints. This can result in under-detection of KPC producers [11, 15]. Boronic acid-based inhibition testing is reported to be sensitive and specific for KPC detection in *K.pneumoniae* [10]. Treatment of infections caused by KPC producers is extremely difficult because of their multidrug resistance, which results in high mortality rates – 50 % or more [13]. The only class A carbapenemases identified so far in Bulgaria are KPC types. Recently *K.pneumoniae* isolate co-producing KPC-2 and the metallo-β-lactamase VIM-1 was described [18].

CLASS B CARBAPENEMASES

Class B metallo-β-lactamases (MBLs) require zinc ion for carbapenemase activity and are capable of conferring resistance to carbapenem antibiotics in a variety of Gram-negative bacteria [2, 19]. MBLs are quite problematic due to: potential for horizontal gene transfer; lack of clinically useful inhibitors; broad hydrolytic profiles that include most β-lactam antibiotics, except aztreonam. However MBL producing isolates often co-express ESBLs and inactivate this monobactam [2, 19]. The mechanism of hydrolysis depends on the interaction of β-lactams with zinc ions in the active site of the enzymes, resulting in their inhibition by EDTA, a chelator of divalent cations [19]. The MBL resistance is usually associated with multidrug resistance, as it was observed in the first in our country prevalence study on the occurrence of carbapenem resistant *P.aeruginosa* [20].

The first described MBLs were chromosomal enzymes present in environmental and opportunistic pathogenic bacteria such as *Bacillus cereus, Aeromonas spp.*, and *Stenotrophomonas maltophilia* [2]. Acquired MBLs have been found in members of the Enterobacteriaceae, *P.aeruginosa, Acinetobacter baumannii*, and other Gram-negative non-fermenters [2].

Among the acquired MBLs, the carbapenemases with the greatest clinical significance are IMP, VIM, SPM, GIM and NDM types. IMP carbapenemase was first detected in Japan in 1990 in *P.aeruginosa* [21], and since then it has been reported mostly in Enterobacteriaceae, *P.aeruginosa*, and *Acinetobacter spp*. [2].

The enzyme SIM-1 (for “Seoul imipenemase”) was identified in *Pseudomonas spp*. and *Acinetobacter spp*., but like GIM and SPM, this carbapenemase has not spread beyond its place of origin [2, 22]. Other representatives of MBL are VIM (Verona integron-encoded metallo-β-lactamase) enzymes. VIM-1 was first isolated in Verona, Italy, in 1997, and since then it has been described as more than 37 variants, mainly identified in *P.aeruginosa* [23]. In Bulgaria VIM-2 was identified for the first time in *P.aeruginosa* isolate form 2006 [24]. For the past two decades VIM and IMP have been detected worldwide, with a trend of moving beyond *P.aeruginosa* and into the Enterobacteriaceae [2]. This tendency was recently observed in Enterobacteriaceae isolates collected in the Cancer hospital in Sofia. The study demonstrated co-existence of VIM-1 encoding genes and ArmA methylase gene in *P.mirabilis* isolates [25]. Carbapenems and aminoglycosides can produce a synergic effect and therefore, dissemination of strains bearing resistance to these antimicrobial drugs would have a considerable clinical impact.

The most recently discovered metallo-β-lactamase is NDM-1 which is spreading rapidly and has been reported worldwide. The gene bla_{NDM-1} has been identified in unrelated Gram-negative bacterial isolates on different plasmid types or as a part of the chromosome (*A.baumannii*) [26]. The Indian subcontinent is considered as the main reservoir for the bla_{NDM-1} genes, since most of the patients infected with NDM-1 producers had traveled to India and Pakistan [27]. Other regions where NDM-1 had been frequently reported are the Balkan states and the Middle East, suggesting that these areas are the second reservoir of NDM-1 producers [27]. NDM-1 producing Enterobacteriaceae have been reported both in hospital and community-acquired infections, including urinary tract infections, septicemia, pulmonary infections, peritonitis, device-associated infections and soft tissue infections [28]. This carbapenemase has been found not only in *K.pneumoniae* and *E.coli*, but also in *K.oxytoca, P. mirabilis, E.cloacae, C.freundii,* and *Providencia spp*. [26].

Most bla_{NDM} plasmids co-harbour multiple but variable resistance determinants encoding various β-lactamases, quinolone resistance, and 16S rRNA methylases that confer resistance to all aminoglycosides [25]. This is also the case with the first NDM-1-producing *E.coli* isolates reported in Bulgaria and co-expressing CTX-M-15 together with the 16S rRNA methylase RmtB. The fact that this was also the very first study

reporting an outbreak of NDM-1-producing *E.coli* in the world is disturbing, considering that *E.coli* is rarely found as a source of nosocomial infections [28].

CLASS D CARBAPENEMASES

The serine β-lactamases from Class D are plasmid encoded and are commonly found in *Enterobacteriaceae*, *P.aeruginosa* and *Acinetobacter spp.* OXA β-lactamases display a wide variety of amino acid sequences. Among those with carbapenem-hydrolysing activity, there is 40 to 70% amino acid identity between groups. Within a group the identity is ≥92.5% [2].

The first OXA β-lactamase with carbapenemase activity was described by Paton in 1993 in a multidrug-resistant *A.baumannii* strain [29]. The majority of OXA carbapenemases have been reported in this opportunistic Gram-negative pathogen, but carbapenemases from this class have shown the potential to spread rapidly among various Gram-negative bacteria [29]. Another disturbing fact about Class D cabrbapenemases is the lack of inhibitor. They are poorly inhibited by boronic acid and EDTA, which compromises their phenotypic detection [30].

There are nine major subgroups of OXA carbapenemases based on amino acid homologies in Gram-negative bacteria. Subgroups 1 (OXA-23-like), 2 (OXA-24-like), 3 (OXA-51-like), and 4 (OXA-58) are frequently detected in *A.baumannii*. The plasmid-mediated OXA-48 enzyme, which forms the sixth subgroup, has been found primarily in *K.pneumoniae, E.coli, C.freundii,* and *E.cloacae* [29, 31]. Among the genes encoding OXA-type carbapenemases, bla_{OXA-23}, bla_{OXA-40}, and bla_{OXA-58} found in *A.baumannii,* and bla_{OXA-48} found in *K.pneumoniae,* are plasmid-borne, while OXA-51 is naturally occurring in *A.baumannii* [32]. Recently OXA-51was reported to be transferred to *K.penumoniae* isolate in Turkey via mobile genetic elements [33].

Among the vast variety of OXA types carbapenemases probably the most clinically important are the OXA-48 CHDLs [29]. The first known producer of OXA-48 was a carbapenem-resistant *K.pneumoniae* isolate that was recovered in Istanbul, Turkey in 2001 [29]. To date, six OXA-48-like variants have been identified, with OXA-48 being the most prevalent [29]. They differ by a few amino acid substitutions. These enzymes inactivate penicillins at a high level and carbapenems at a low level, their activity against broad-spectrum cephalosporins is mild, and they are not susceptible to β-lactamase inhibitors [2]. Since many OXA-48 producers do not show resistance to broad-spectrum cephalosporins or exhibit only decreased susceptibility to carbapenems, their detection is difficult and they can be missed in the routine laboratory practice [29].

At present, the carbapenemase-producing isolates reported in Bulgaria mostly refer to CHDLs. OXA-23 was identified as the key determinant responsible for carbapenem resistance in *A.baumannii* in the University Hospital in Pleven for a period of seven years [34]. Later on both OXA-23 and OXA-58 producing *A.baumannii* were described in the Military Medical Academy in Sofia, confirming the wide geographical distribution of Class D carbapenemases among *A.baumannii* as well as their parallel emergence in outbreak strains [35]. The first OXA-24 carbapenemase-producing *A.baumannii* isolates from Bulgaria were recently identified at the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance [36]. The same study revealed the co-existence of OXA-24 and OXA-23 in one *A.baumannii* isolate [36].

CLASS C B-LACTAMASES WITH CARBAPENEMASE ACTIVITY

There are several reported Class C β-lactamases with potent carbapenem hydrolysing activity, including CMY-2, CMY-10, ACT-1 and DHA-1 [8]. They all have catalytic activity for imipenem, which may not depend on porin loss or hyper efflux. These plasmid-mediated Class C enzymes were identified in Enterobacteriaceae [8] and they might lead to significant prevalence of carbapenem resistance in the near future because of their potential to dessiminate by mobile genetic elements. Most recently another class C β-lactamase was isolated from carbapenem-resistant *A.baumannii* - ADC-68 (*Acinetobacter*-derived cephalosporinase ADC-type). It was found that the gene encoding ADC-68 is a part of the chromosome [8].

DETECTION OF CARBAPENEMASE PRODUCERS

Detection of carbapenemase-producing pathogens is a matter of major importance. It is the main factor determining the choice of appropriate therapeutic schemes and the implementation of infection control measures. The detection strategy for carbapenemases should include a screening step followed by a phenotypic and genotypic confirmation step.

PHENOTYPIC METHODS FOR CARBAPENEMASE DETECTION

The initial steps in carbapenemase screening are based on analysis of susceptibility testing and evaluation of carbapenem minimum inhibitory concentrations [37]. Detection of carbapenemase producers based only on MIC values may lack sensitivity. MICs observed in carbapenemase-producing microorganisms can exhibit considerable variation depending on the type and expression of carbapenemase enzyme, the bacterial species and the presence of other resistance mechanisms such as cephalosporinases, reduced permeability and/or efflux pumps [11, 38]. Because of these possibilities phenotypic confirmation is required. The phenotypic methods useful for detecting carbapenemase-producing bacteria are: the modified Hodge test, the inhibitor -based synergy test, the Carba NP test, spectrophotometric analysis and isoelectric focusing (IEF).

The modified Hodge test is used for detection of diffusible carbapenemases [38]. The test is based on the inactivation of a carbapenem by the cells of the carbapenem-producing organisms, which enables a carbapenem-susceptible indicator strain to grow towards a carbapenem disk, along the band of inoculum of the test strain [37]. Specificity of the modified Hodge test can be low if the tested microorganisms are CTX-M ESBL- or AmpC-producers, with reduced or absent porin expression and may give false-positive results [11, 37].

The next step is distinguishing between the different classes of carbapenemases that the tested isolate may produce. This step is based on *in vitro* inhibition of carbapenemase activity by adding specific inhibitor

for each carbapenemase class. This phenomenon is called synergy between the carbapenem and the inhibitor [37]. The inhibitor used for detection of Class A carbapenemases is boronic acid. For detection of Class B ethylene diamine tetra-acetic acid (EDTA) or dipicolinic acid can be used [39]. A group of experts from EUCAST and the ESCMID Study Group for Antibiotic Resistance Surveillance (EARSS) have recommended the following procedures for detection of Class A and B enzymes in Enterobacteriaceae: for Class A enzymes (KPC or other enzymes), production is suspected when a difference of ≥4 mm in the zone diameter is observed between meropenem (10 µg) and meropenem plus boronic acid (600 µg) [40]. As boronic acid can also inhibit class C enzymes, comparison between the zone diameters of meropenem and meropenem plus cloxacillin (750 µg) disks suggests the presence of a strain hyperproducing the chromosomal AmpC or producing a plasmid-encoded AmpC when the latter diameter is increased by ≥5 mm [2, 38]. The detection of class B enzymes is based on a disk combination test using meropenem and meropenem plus EDTA or dipicolinic acid and the test is considered to be positive when there is an increase in the zone diameter of ≥5 mm. [2, 11]. There is no inhibitor for Class D carbapenemases. With Enterobacteriaceae temocillin can be used as an indicator of OXA-48. If there is no zone of inhibition around the temocillin disk and there are negative results in all the synergy tests the isolate is presumptive for production of OXA-48.

The Carba NP test is a biochemical test, based on detection of the acidification, resulting from imipenem hydrolysis [41]. It is also useful for identification of the different types of carbapenemases (Classes A, B and D) in Enterobacteriaceae and *Pseudomonas spp.,* coupled with tazobactam and EDTA as inhibitors. This relatively new technique has 100% sensitivity and specificity [41].

Spectrophotometric measurement of carbapenem hydrolysis is considered to be the reference standard method for detection of carbapenemase production. β-lactamase activities can be determined by measuring the change in absorbance for the antibiotic molecules at indicated wave lengths for each carbapenem antibiotic [2].

Another biochemical test for carbapenemase detection is isoelectric focusing (IEF) [2]. This method is used for separation of proteins by their charge and detection of β-lactamases is accomplished with the chromogenic cephalosporin nitrocefin. IEF gives information about isoelectric point and inhibition characteristics of the enzyme. IEF is especially valuable for the detection of multiple β-lactamases present in an isolate [2].

GENETIC METHODS FOR CARBAPENEMASE DETECTION

Genetic methods for detection of resistance genes are based on nucleic acid hybridisation and amplification [38]. Therefore, the knowledge of specific primers (amplification nucleotides) and probes (labeled single-stranded oligonucleotides) is necessary in order to detect the genetic target of interest. There are list of primers designed to amplify the most prevalent carbapenemase genes identified in Gram-negative bacteria such as bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, $bla_{SIM}$$bla_{KPC}$, bla_{OXA-48}, bla_{GES} among others [42]. DNA hybridisation is among the oldest methods applied in molecular technology for detection of genes conferring bacterial drug resistance [47]. This method can give information about the degree of sequence identity, and specific sequences can be detected and located on a given chromosome [47].

PCR is a rapid and reliable technique for screening of all clinically relevant carbapenemase genes [46]. There are several variations of the basic PCR technique but the most widely used for detection of carbapenemase genes is the Taq Man technology [47]. The main feature of Taq Man technology is that the amplified DNA is detected as the reaction progresses in “real time”. In cases in which resistance depends upon the expression level (over expression or down regulation) of the gene, Real-Time Reverse Transcriptase-PCR (rt RT-PCR) is used to detect not only the presence, but also the mRNA expression of the gene [47]. The main disadvantages of PCR-based technologies for detection of carbapenemases are their cost, and the absence of detection of any novel carbapenemase gene [42].

Another method for examining genes encoding antibiotic resistance is DNA-sequencing [43]. The process is automated and the method is useful for comparing and analysing the variations among genes conferring carbapenem resistance [43].

In the past few years the development of the microarray analysis allowed detection and determination of the relative abundance of diverse individual sequences in complex DNA samples [44]. There are commercially available hybridisation microarray systems for Gram-negative bacteria allowing the detection of a large number of resistance genes and up to 96 samples to be tested simultaneously.

CONCLUSION

As it was highlighted in the present review, carbapenemases belonging to Ambles Classes A (KPC-2), B (VIM-2,VIM-1, NDM-1), and D (OXA-23, OXA-24, OXA-58), have been reported in Bulgaria. This suggests heterogeneous distribution of the β-lactamases with carbapenemase activity in a country with still low rates of carbapenem consumption and represents a strong argument for the need of further investigation of the carbapenemase-mediated antimicrobial resistance in Bulgaria.

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RAPIDLY GROWING MYCOBACTERIA IN SUSPECTED FOR TUBERCULOSIS PATIENTS IN BULGARIA

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ABSTRACT

The aim of this study was to describe Nontuberculous Mycobacteria (NTM), in particular Rapidly Growing Mycobacteria (RGM), isolated from clinical specimens of suspected for tuberculosis patients in Bulgaria. Notification of NTM is not mandatory in Bulgaria, according to Regulation 21 of the MoH (in contrast to registration of TB infections) (2). The survey was conducted at the National Reference Laboratory of Tuberculosis, National Centre of Infectious and Parasitic Diseases (NRL-TB, NCIPD) and covers the five year period – from 2009 to 2013. NTM were identified by conventional phenotypic methods and line probe assays. In total 245 NTM strains were identified to species level. More than one third of them were RGM - 32.65% (n = 80) including the clinically significant species: *M. abscessus, M. chelonae* and *M. fortuitum* - 73.75% (n = 59).

KEYWORDS: *Tuberculosis, NTM, RGM, line probe assays*

INTRODUCTION

More than 150 species belong to the genus *Mycobacterium,* including *M. tuberculosis* complex, *M. leprae* and NTM. In the last years NTM became a rapidly increasing group and new species have been added as a result of the usage of highly discriminative molecular methods for species identification. NTM are widespread in the environment: soil, rocks, and water. Biofilm formation is a successful survival strategy of these very hydrophobic microorganisms and allows them to persist in water distribution systems (10, 11). According to the classification of E. Runyion the species of NTM are divided in two groups: slow-growing and rapidly-growing mycobacteria (17). Isolates of NTM that require about 7 days to form mature colonies on subculture are termed as “rapidly growing mycobacteria” or RGM. They represent approximately a half of NTM (70 species) and form 6 groups based on pigmentation and genetic relatedness, presented in *Table 1* (6).

***Table 1*: Rapidly growing mycobacteria (RGM) groups and their clinical association**

Groups of RGM	Species in the groups	Clinical relevance
***M. fortuitum* group** (Nonpigmented)	*M. fortuitum, M. peregrinum, M. senegalense, M. brisbanense, M. septicum, M. porcinum, M. houstonense, M. boenickei, M. neworleaneansense, M. setense,*	post-traumatic wound infections, localised cutaneous infections, cardiac surgery, catheter sepsis
***M. chelonae/ M. abscessus* group** (Nonpigmented)	*M. chelonae, M. immunogenicum, M. abscessus subsp. abscessus, M. abscessus subsp. bolletii, M. salmoniphilum*	localised post-traumatic wound infections, keratitis, catheter sepsis, chronic lung diseases, chronic otitis media, disseminated cutaneous diseases in immunocompromised patients on long-term corticosteroids and/or chemotherapy
***M. mucogenicum* group** (Nonpigmented)	*M. mucogenicum, M. aubagnense, M. phocaicum*	usually contaminants in sputum/ water, catheter sepsis in immunocompromised patients
***M. smegmatis* group** (Late-pigmented)	*M. smegmatis, M. goodii*	localised post-traumatic wound infections, postsurgical wound infections, pulmonary infections, osteomyelitis
Early-pigmented RGM	*M. neoaurum, M. canariasense, M. cosmeticum, M. monacense*	pulmonary infections, healthcare associated infections including hemodialysis, central-catheter associated infections
***M. mageritense/ M.wolinskyi* group** (Genetically very closely related)	*M. mageritense, M. wolinskyi*	osteomyelitis, face-lift plastic surgery

Usually the RGM infection depends on the close interaction between man and environment. It is a result of geographical location, demographic changes such as aging of the population in a certain area, including increasing numbers of immune suppressed due to HIV, cancer or medical interventions (5, 9). There is no clear evidence of man to man transmission or from animal to

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human one (13). It is necessary to differentiate RGM infection from pseudo outbreak. Laboratory cross-contamination, contaminated instruments (bronchoscopes, needles, etc.) and solutions, could result as RGM isolation from clinical material without causing infection (*M. abscessus, M. fortuitum* and slow-growing *M. avium* complex, *M. gordonae, M. xenopi* (16). If therapy is indicated, especially in immunosuppressed patients with pulmonary disease, it is recommended to consider the diagnostic criteria of the American Thoracic Society (3). They include clinical, roentgen and microbiological aspects. Microbiological criteria are developed according to the type of clinical material and the required repeated isolation of NTM. Infections caused by RGM can be presented in several groups. **Pulmonary diseases** are the most common manifestations: infections range from asymptomatic to severe bronchiectasis and lethal cavitation (*M. abscessus, M. immunogenicum, M. mucogenicum, M. goodii*) (15). **Skin and soft tissue infections** are associated with contamination of injuries from the environment by gardening, operations in aesthetic surgery, tattooing, cosmetic treatments and more (*M. fortuitum, M. mageritense, M. chelonae, M. abscessus*) (16). **Eye infections** are as a result of LASIK (Laser Assisted In-situ Keratomileusis) surgery (7). **Catheter infections** due to catheterisation, vascular shunt contamination of medical instruments (bronchoscopes), dialysis machines and others (*M. fortuitum, M. chelonae, M. abscessus; M. smegmatis, M. mucogenicum*) (15). **Disseminated forms** in immunosuppressed patients with/without HIV/AIDS - *M. abscessus* (15).

AIM:

To describe the species of RGM isolated from clinical specimens of suspected tuberculosis patients in Bulgaria for the period 2009 - 2013.

MATERIALS AND METHODS:

The study included patients isolates from TB laboratory network in Bulgaria and NRL TB, NCIPD. The species identification of RGM strains was carried out in NRL-TB only (4). The good laboratory practice was guaranteed by External Quality Assessment at sub and supranational level.

245 strains were specified as NTM, out of them 32.65% (n = 80) were RGM. For the purpose of the study one patient was represented by a single strain. Strains were isolated from various clinical specimens of suspected TB patients: sputum, bronchoalveolar lavage (BAL), gastric lavage (from children) and cerebrospinal fluid (CSF) following the recommendations provided by national and international guidelines (1, 4, 12). The main methods were: Ziehl – Neelsen staining; culture on liquid media MGIT (BACTEC® MGIT 960 System, BD, NJ, USA) and on solid media Löwenstein-Jensen (BD) for visualisation of colony's morphology, pigmentation and photoreactivity; phenotypic markers as temperature dependence and growth speed; sensitivity/resistance to the p-nitro benzoic acid (pNBA); presence/absence of MRT64 antigen by BD MGIT TBc Identification Test and Line Probe Assays Geno Type® Mycobacterium CM and AS, HAIN, Lifescience (17). LPA are commercial PCR tests designed to identify the most common and relevant to human pathology NTM. After DNA isolation from pure culture with subsequent amplification and reverse hybridisation to specific oligonucleotides, immobilised on a membrane strip, species identification is performed. These two LPAs can be performed independently of each other and complement one another. Isolates defined as genus *Mycobacterium* by GenoType® CM were tested on GenoType® AS.

GenoType® Mycobacterium CM (Common Mycobacteria) allows simultaneously molecular identification of *M. tuberculosis* complex and the following most common NTM: *M. kansasii, M. xenopi, M. gordonae, M. marinum/M. ulcerans, M. avium, M. intracelulare, M. scrofulaceum, M. malmoense, M. chelonae, M. fortuitum, M. abscessus, M. interjectum, M. peregrinum.*

Geno Type® Mycobacterium AS (Additional Species) allows independent molecular identification of additional types of NTM, relevant to human pathology: *M. genavense, M. simiae, M. ulcerans, M. celatum, M. haemophilum, M. smegmatis, M. mucogenicum, M. goodii, M. lentiflavum, M. heckeshomense, M. szulgai, M. flei, M. gastrii, M. asiatikum, M. shimoidei.*

RESULTS AND DISCUSSIONS:

We observed a trend of gradual increasing of the number of RGM strains isolated from the beginning to the end of the study, respectively: in 2009 – 7 strains; in 2010 – 15 strains; in 2011 – 19 strains; in 2012 – 20 strains and in 2013 – 19 strains. The RGM strains distribution is presented on *Figure 1.* We found higher prevalence of the isolated strains in the more urban areas in Bulgaria like Sofia, Plovdiv and Varna

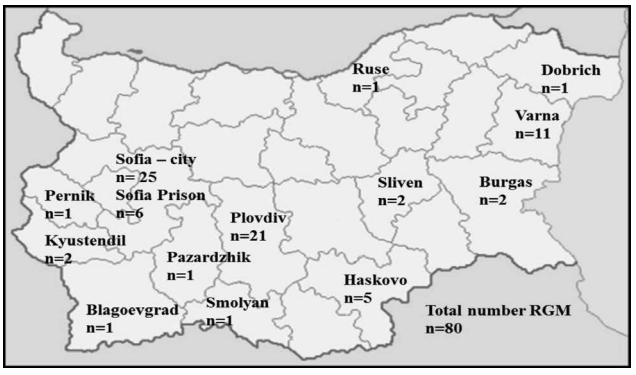


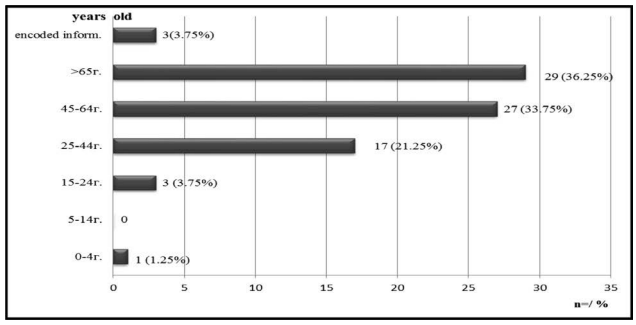
Figure 1: **Distribution of RGM strains by the patients' residence, 2009 – 2013**

The most common specimens were sputa – 91.3% (n = 73), BAL - 6.3% (n = 5), gastric lavage - 1.3% (n = 1) and CSF - 1.3% (n = 1). Smear microscopy positive were 10% (n = 8), only. The majority of them were sputa 87.5% (n = 7) and 12.5% (n = 1) was a CSF. The gender distribution, as well as in TB patients, showed that males were - 67.5% (n = 54) and females - 31.25% (n = 25). Regarding gender, personal data of one patient was encrypted - 1.25% (n = 1).

Patient age structure is presented in *Figure 2*. The most frequently RGM were isolated from population

in the age groups over 45 years old 70% (n = 56). Children were 1.25%.

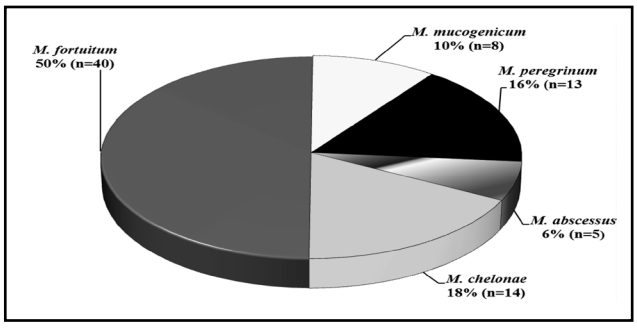
***Figure 2*: Age structure of patients with RGM isolates, 2009 – 2013**



Species structure of the RGM was quite varied in the five-year period in Bulgaria (*Figure 3*).

M. fortuitum and *M. chelonae* were the most common species.

***Figure 3*: Distribution by species identification of RGM, 2009 – 2013**



It should be noted that ***M. fortuitum*** is a frequently isolated organism from respiratory specimens in many countries and rarely causes lung infections (usually in cases of chronic aspiration disease) (3, 18). Most often *M. fortuitum* is associated with post-traumatic wound infections; infections associated with health care; operative wounds and osteomyelitis after open fracture. According to the results of the study *M. fortuitum* was the most frequently identified RGM species in Bulgaria - 50% (n = 40). It was confirmed in 39 cases in sputum and in one CSF. Mixed bacterial culture was found in one patient: *M. fortuitum* and *M. tuberculosis* complex. One of the patients was drug addicted and four patients were previously treated for tuberculosis.

We identified ***M. chelonae*** in 18% (n = 14) of the RGM strains. They were found in 13 sputa and one gastric lavage. Two of these patients had an evidence of chronic lung disease and in one of the patients we identified mixed mycobacterial culture of *M. chelonae* and *M. tuberculosis* complex. In an other case – three-month-old child, we found *M. chelonae*, but the previous isolate was the slow growing *M. lentiflavum.* We considered *M. lentiflavum* as a contamination associated with healthcare in the hospital, according to the anamnesis data.

We identified ***M. abscessus*** in five cases, out of them four were from sputa and one from BAL. Smear microscopy was positive in 60% (n = 3) of the specimens. ***M. mucogenicum*** and ***M. peregrinum*** are considered as a common contaminant. The percentage of these isolates in the study were 26.25% (n = 21), and it was difficult to define them as etiological agents of the disease, according to ATS recommendations (3).

CONCLUSION:

In this microbiological study of RGM species from TB suspected patients in Bulgaria, we observed a trend of gradual increasing of the number of RGM strains for the period 2009 – 2013. *M. fortuitum, M. chelonae* and *M. abscessus* were the most common RGM, associated with pulmonary symptoms. The identification of NTM to the species level is very important for determining patient's treatment, because there are predictable antimicrobial susceptibility patterns (8). The correct species identification of NTM directs clinicians` attention to appropriate therapy and in this way they are able to monitor the diseases.

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DEVELOPMENT AND PRELIMINARY EVALUATION OF A NOVEL METHOD FOR DNA EXTRACTION FROM SERUM

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ABSTRACT

Molecular methods became indispensable tool for diagnosis of various infectious diseases. In cases of bacteremia and sepsis, DNA detection assays provide the speed and sensitivity required for initiation of an early appropriate antimicrobial treatment. While blood is still the preferred diagnostic specimen, a number of studies brought evidence for the value of serum as an alternative sample for PCR-based diagnosis of sepsis. Thus in this study our efforts were focused on developing a rapid and efficient single tube (ST) method for DNA isolation from simulated serum samples. Evaluation of the method was accomplished by two separate Real-time PCR assays on simulated serum samples. In a side by side comparison, the optimized DNA extraction procedure performed equally or with better efficiency than a commercial DNA extraction kit.

INTRODUCTION

Small amounts of cellfree human DNA and RNA (also micro RNA) are found in peripheral blood. These nucleic acids are currently successfully beingtargeted for early detection of cancer, prenatal diagnostics etc. The potential of these nucleic acids for bacterial and fungal infections diagnostics have been described. Circulating DNA concentration in blood is usually low (ng/ml) and is represented by relatively short fragments (<1 000 bp), rendering DNA isolation complicated[1]. Although commercially available kits for circulating DNA extraction are available (e.gQIAamp Circulating Nucleic Acid Kit, Qiagen), they are still costly, lack capacity for scalability andthe data for microbial DNA applicationsare inconsistent.

Recently Vu et al. published a simple, convenient and sensitive procedure for isolation of small DNA fragments from serum (~150bp), based on the selective DNA compaction precipitation by spermine[1]. In this study we have tested this method for extraction of fungal DNA from simulated serum samples and propose further modifications with respect to its efficiency.

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MATERIALS AND METHODS

The method consists of three basic steps: a) protein purification; b) selective DNA compaction precipitation byspermine,and c) DNA purification and elution;

Reagents used:10MUrea(Sigma-Aldrich) dissolved in TE buffer (10mM Tris-HCl, 1mM EDTA pH=8), 1M Sperminetetrahydrochloride in nuclease-free ddH_2O (AlfaAesar), Washing buffer 1 (50% isopropanol, 300mMNaCl, 10mMMgCl_2), Washing buffer 2 (75% ethanol), Elution buffer – TE buffer, proteinase K (AppliChem, Germany)

Briefly, **the original protocol (OP)** of Vu et al., involves dilution of serum 2.5x with water, heating 95°C for 15 min., followed by centrifugation at 16000g for removal of coagulated protein. Spermine is then added to the supernatant at 2.5-5mM and the samples are precipitated after 15min incubation at room temperature. Two washing steps are performed for removal of spermine and the dried DNA is resuspended in TE.

We have tested several modifications of this protocol and finally established a single tube protocol as follows:

Modified protocol (MP): 800µlhuman serumin 2ml-tube was diluted with 1200 µl of 10M ureato 6M final concentration. 10µl of1M spermine was then added (5 mM final concentration). After 15min incubation at room temperature with regular mixing, samples were centrifuged for 15 min at maximum speed (17000g). The supernatant was discarded and DNA was washed sequentially in200µl of washing buffer 1 and 2, followed by 10 min centrifugations at 17000g. After careful removal of WB with pipette, DNA is dried at 55°C for 5 min and resuspended in 25µl TE.

Control samples were extracted by Purelink DNA mini kit (Life Technologies) following manufacturer instructions.

For evaluation of the method, various amounts of fungal DNA (*Aspergillusniger* and *Candida parapsilosis*) were spiked in serum obtained from healthy individual. EvaGreen Real-time PCR assays targeted at fungal ITS2 and human β-globin respectively were performedwith DNA extracted by various procedures.

RESULTS AND DISCUSSION:

OPTIMIZATION OF METHOD FOR DNA EXTRACTION FROM SERUM.

Initially we have tested the originalprotocol of Vu et al.for DNA isolation by compact precipitation publishedrecently [1] The method involves serum dilution with water, 95°C heating and centrifugation, inducing nonspecific protein coagulation and removing a major part of the serum proteins, also lowering the salt concentration to levels not inhibiting compact precipitation (according to the authors). By adding spermine

to the supernatant,the authors accomplish effective precipitation of 151bp λ-bacteriophage DNA fragment at even0,01ng/ml which is difficult to achieve with standard methods.

Our experiments have shown that after dilution, heat treatmentandcentrifugation of serum, large amount of protein coagulates was formed reaching almost half of the sample volumeand leading to a major reduction in DNA yield. By incubating serum at different temperatures (60-90°C) it was confirmed that heat treate ment has negative impact on the DNA yield probably by binding or entrapment of DNA within the protein coagulates (Figure 1).

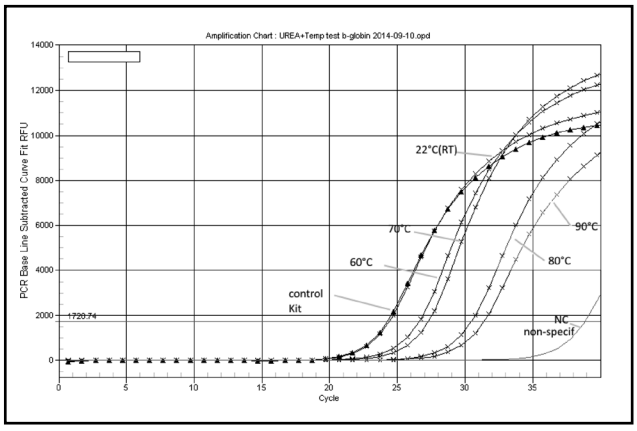

Figure 1. Heat treatment of 2.5x diluted serum at various temperatures (60-90°C) for 10 min and DNA extraction by spermine precipitation. Control DNA was extracted with PureLinkDNA mini kit (Life Technologies). EvaGreen Real-time PCR with human β-globin primers shows Ct delay with increasing temperature. Only the incubation at room temperature resulted in amplification efficiency (Ct=24.38) comparable to that of the control extracted by commercial DNA kit (Ct=24.24).

To eliminate these obstacles we have tested series of modifications comprising:***a)*** 3 fold sample dilution with waterinstead of 2.5x; ***b)*** 2mg/ml proteinase K treatment; ***c)***treatment with urea(either added directly as dry substance to8M final concentration or dilution 2.5x fold with 10M urea). All experiments were performed in triplicate. Results are presented in Table 1.

Table 1.Results of various treatments of serum samples for DNA - protein separation.

Sample Treatment	Incubation 95°C, 15 min	Protein precipitates
2.5x dilution withddH_2O (original protocol)	+	+++
3x dilution withddH_2O	+	++
2mg/ml Proteinase K	+	+
dryurea added to 8M (Choe et al.)	-	++
2.5x dilution with 10M Urea (modified protocol)	-	-

As shown in **Table 1.**protein precipitates were fully removed only with 10M urea treatment. Urea is a denaturing chaotropic agent, successfully applied in proteomics and recombinant DNA technology for solubilization of hydrophobic proteins and inclusion bodies. The idea about using urea was inspired by an article dealing with purificationof recombinant proteinfrom DNA with spermine[2]. While authors add urea to the bacterial lysates in a dry form, in case of serum this results in a significant protein precipiptation (Table 1). Therefore we have attempted to dilute the serum with saturated solution of urea and found that it effectively dissolves all proteins without any heat treatment. However since urea has negative effect on spermine-DNA binding[2], at the next stage we have used fungal DNA to test the optimalspermine concentration for DNA extractionin presence of urea (Figure 2).

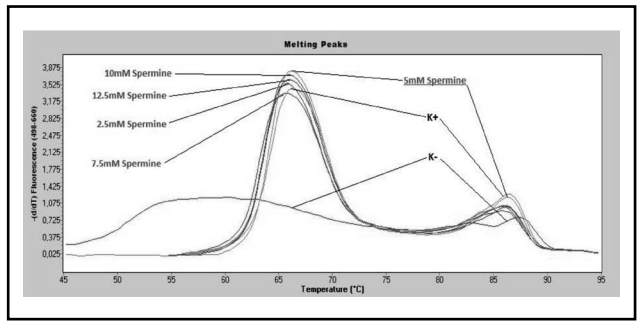

Fig.2 Optimization of spermine concentration in the presence of 6M urea.Fungal DNA (100 copies of *A. niger*)was spiked in serum and DNA was extracted by the modified method with varying spermine concentrations (0-12.5mM). DNA yield was monitored by probe-basedReal-time PCR. Positive control (K+) was extracted by commercial kit, while negative control (K-) had ddH_2O instead of spermine.

Although no significant differences found, 5mM spermine appeared to be the optimal concentration for DNA extraction in presence of 6M urea and 0.6mM EDTA (from TE buffer used to dissolve urea). In a similar experiment with bacterial lysates and using 8M urea, Choeet al.[2] reported10 mM as the optimal sperminеconcentration, which in the current experiment resulted in a lower DNA yeald. Taken into consideration that the authors used higher percentage of urea and EDTA this might be explained. Both substances reduce efficiency of the precipitation, which in turn requires more spermine.

In an additional experiment we have compared different modifications of the original method with the commercial kit PureLink mini DNA (Life Technologies)with respect to the DNA integrity and size. Results on Figure 3 show that the different methods preferably isolate high (DNA kit) or low (proteinase K) molecular DNA. Only the urea - modified method provided both high as well as low molecular DNA.The protein precipitates encountered with the original method probably entrapa major part of the serum circulating DNA which leads to suchlowerDNAyield that could not be visualized by electrophoresis.

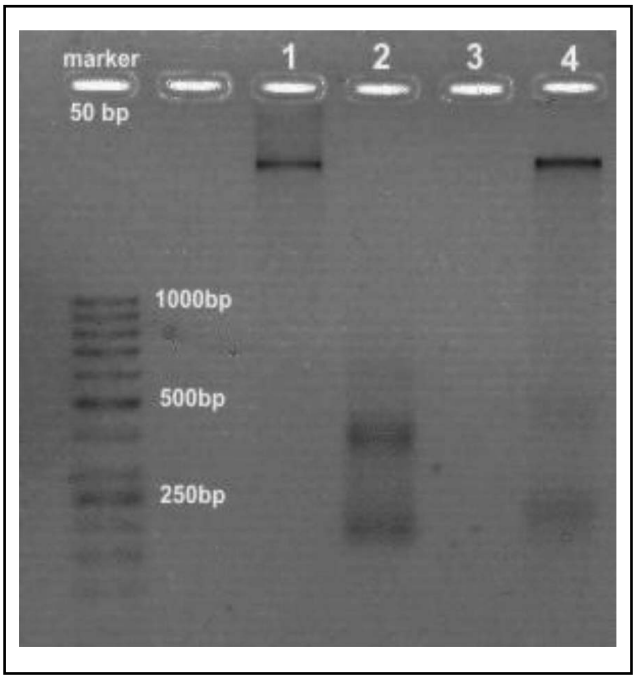

Figure 3. Agarose gel-electrophoresis of genomic DNA from serum isolated by different modifications of the original method (Vu et al.) and a commercial DNA isolation kit.**DNA ladder 50bp (NEB);1.**PureLink mini DNA kit (Life Technologies), **2.**Proteinase K+ heat treatment; **3**.Original method (water dilution 2,5x); **4.** Urea modified (10M Urea dilution 2,5x)

Invasive fungal infections (IFI) are a serious problem. In the last two decades there have been a significant rise in IFI caused by conditionallypathogenic fungal species. Most of the cases are observed in patients that are immuno-compromised or hospitalized. The fungi that are most commonly causing opportunistic infection are *Candida sp*. , *Aspergillus sp*., and *Cryptoccocusneoformans(Candidiasis* - 49% lethality,*Aspergillosis* - 90% lethality [3]. Highest risk patients are those with continues neutropenia, immunosuppressed, prolonged antibiotic treatment, surgery, HIV infected or undergone solid organ transplant [4,]. Central nervous system cryptococcosis(*C. neoformans)* is one of the most common severe fungal brain infection in our geographic region [5].Common bacterial co-infections can complicate the clinical picture. It is established that almost 23% of episodic IFI (candidemia) can be accompanied bacteremia [6].

Molecular methods for diagnostics and detection of IFI are rapid and very sensitive.These methods are mainly PCR-based anddespite their great potential, several technical issues associated with their use for fungal DNA isolation create significant discrepancies between different assays and impede efforts towards standardization. The main problems are associated with the fact that fungi are ubiquitously distributed in the environment and all assays are prone to contamination by fungal DNA. Therefore false-positive results are occasionally obtained, complicating diagnostic interpretations[7].

With this regard our method provides significant advantage over commercially available ones being single tube-based and hence much less prone to contamination. Another advantage is that it is not restricted by the sample volume like most kits, provided that a high-speed benchtop centrifuge is available.

CONCLUSION

A novel simple, rapid, single tube and low-cost method for DNA extraction from serum has been developed with potential application to diagnosis of bacteremia, fungiemia and sepsis. The protocol efficiency is at least comparable or better than a commercial kit.

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WEST NILE VIRUS INFECTION IN A BULGARIAN MAN: A CASE REPORT

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ABSTRACT
West Nile virus belongs to the family *Flaviviridae*. Birds are the natural reservoir, whereas humans and horses are dead-end hosts. The transmission is maintained by mosquito biting. The enzootic/epizootic cycle is linked to the activity of mosquito vectors of the genus *Culex*. The clinical presentation varies from subclinical to acute febrile illness and/or neurological manifestation. We present a case report of a young man with acute febrile syndrome and CNS disorder. Testing of acute and convalescent serum samples showed high level of IgM antibodies against West Nile virus.
Key words: *West Nile virus, neurological manifestation, serological data.*

ABBREVIATIONS
WNV – West Nile Virus
WNF – West Nile fever
UK – United Kingdom
WNE – West Nile Encephalitis
CNS – Central Nervous System
ECG – Electrocardiogram
CT – Computed Tomography
MRI – Magnetic Resonance Imaging
EEG – Electroencephalography
CSF – Cerebrospinal Fluid
HIV – Human Immunodeficiency Virus
HBV – Hepatitis B Virus
HCV – Hepatitis C Virus
HSV – Herpes Simplex Virus
ID – Infectious Diseases

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INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus within the family *Flaviviridae* [1, 2]. In 1937, the virus was first isolated from a febrile woman in the West Nile district in the Northern Province of Uganda [3]. Birds are the natural reservoir and the virus transmission cycle involves *Culex* spp. mosquitoes [4]. In Europe, the seasonal activity is from mid-June to mid-November [5]. Most human infections are subclinical [4] and the vast majority of clinical cases develop an acute, systemic febrile illness ("West Nile fever", WNF) [6]. Less than 1% of infected people develop neurologic illness: meningitis, encephalitis, acute poliomyelitis-like syndrome (flaccid paralysis) [1, 2, 6, 7]. The incubation period is approximately 2-14 days [4]. The leading symptoms are fever, headache, gastrointestinal symptoms, sometimes rash and arthralgia [4]. In case of neurological illness, there is neurological manifestation. Sporadic cases and outbreaks are reported in Middle East, Africa, Asia, Australia, America, Canada, the Caribbean and Europe [7]. In Europe, the largest outbreaks are in Romania and the Russian Federation [5]. Sporadic cases are reported in Italy, Spain, Portugal and Tunisia [5]. In the United Kingdom, circulation of the virus was found among birds [5]. Increasing antibody titres were documented in Czech Republic, Austria and Germany [5]. In the Balkan area, human epidemics were reported in Greece, Romania, Croatia, Macedonia, Kosovo, Montenegro and Serbia [5].
Here we describe a case report of a young man with West Nile encephalitis (WNE) in Bulgaria. Only one other case of WN fever virus infection is reported in Bulgaria (8).

CASE PRESENTATION

On 17th August 2014, a 24-year-old man was hospitalised at the Department of Intensive Care, Military Medical Academy (Sofia, Bulgaria). He had medical history of one week`s illness involving fever, fatigue, headache, abdominal pain, nausea and vomiting. He had been assigned to antibiotic treatment for five days by the family physician with partial effect. A day before hospitalization, he had headache, dizziness, vomiting, and generalised seizure. A team from the emergency centre transported the man to the Military Medical Academy. In the emergency room, he was intubated and hospitalised at the Department of Intensive Care. He had medical history of pneumonia the year before and epilepsy during his childhood. The patient lives in Croatia and came to Bulgaria, Varna for the summer holiday. At the time of admission he was in poor general condition with mental disturbances and fever. Blood pressure – 156/98, heart rate – 95/min, the remaining indicators of physical status were normal. Neurological examination revealed the need for lumbar puncture. Lumbar puncture was performed on the second day of his hospitalisation. CNS findings: normal opening pressure; clear colour; cells: 2 x 10^6/L; protein: 0.70 g/L; glucose: 4.3 mmol/L; chlorine: 126 mmol/L. Imaging

investigations: ECG, X-ray, thransthoracic echocardiography, abdominal ultrasound and CT scan – normal. MRI scan – normal, evidence of sinusitis. EEG: evidence of encephalopathy, without focal or paroxysmal manifestations, no periodical activity. Microbiological investigations: blood cultures, CSF, urine, and throat swab cultures were without growth. *P. aeruginosa* was identified from tracheobronchial aspirate. Serological tests for HIV, HBV, HCV, syphilis, influenza A and B, HSV 1, *C. pneumoniae*, *M. pneumoniae*, Q-fever and *R. conorii* were negative. Serum sample for WNV, sent to the National Reference Laboratory, was found positive for IgM and negative for IgG antibodies. Second serum sample was taken 14 days later. The result was the same as the first one. One month after the onset of the illness, third sample analysis was performed. Slight decrease in IgM titres against WNV was observed, and again IgG was negative.

The patient was consulted by ophthalmologist, neurologist, toxicologist, endocrinologist, infectious diseases specialist and psychiatrist. He was assessed as status epilepticus and specific therapy was started while the result for WNV was pending. During the hospital stay supportive, symptomatic, empirical antibiotic and antiviral therapies were performed. Owing to the adequate intensive care, the man recovered and the intubation tube was removed. On the 14th day, he was discharged afebrile, with normal mental status, epileptic prophylaxis and chemoprophylaxis.

DISCUSSION

In this report, we presented a case of a young man with fever, headache, gastrointestinal disorders, generalised seizure and mental disturbances. Imaging investigations did not establish pathological process. The examination of CSF showed discrete changes indicative of viral encephalitis: slight proteinorachia with normal cell count. The medical course of the disease was developing in the background of epilepsy during childhood and epidemiological data on travelling from Croatia to a summer visit in a sea city in Bulgaria. The microbiological examination did not establish any bacterial pathogen, but serological data revealed high titres of specific antibodies against WNV. Considering the clinical manifestation of encephalitis, laboratory findings and epidemiological data, we concluded that this was an infection with WNV, presented as WNE. We can explain the good outcome of the disease as attributed to the clinical course of the illness, young age of the patient and absence of co-morbidity. The man recovered without any neurological disturbances.

Knowing the epidemiological characteristics of WNV, we have to be more active and search for this disease as a cause of acute febrile syndrome or/and neurological manifestation. Referring to publications from European countries, especially from the Balkan area, we have to expect larger number of WNV infection in Bulgaria. The clinicians should take into consideration WNV and improve the collaboration with laboratory specialists.

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DETECTION AND PHYLOGENETIC ANALYSIS OF ERYTHROVIRUS B19 (EVB19) IN PATIENTS WITH HaEMATOLOGIC DISORDERS (PRELIMINARY RESULTS)

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ABSTRACT
Erythrovirus B19 (EVB19) is a pathogen associated with a wide range of disease manifestations, syndromes and pathological conditions with acute or chronic course, some of which depend largely on the condition of the immune system and the haematological status of the patient.
The **aim** of this study is to prove the involvement of EVB19 as the etiological and/or persistent agent in patients with haematological disorders using different laboratory diagnostic tools.
Materials and Methods: The present study included 33 serum samples from patients with haematological diseases. *Group 1* included the following diagnoses: aplastic anemia, erythroblastopenia, thrombocytopenia and pancytopenia (n=10); *group 2* included transfusion-dependent anaemic patients (thalassemia major and others haemolytic anemia) and haemorrhagic diathesis (n=21), and g*roup 3* included patients with unidentified fever (n=2). Etiological presence of EVB19 was demonstrated by serological, specific B19-IgM/IgG antibodies (indirect ELISA assay), and molecular (extraction and amplification of viral DNA, sequencing and phylogenetic analysis) methods.
Results: The presence of acute infection (B19-IgM marker) was demonstrated in 4/33 (12.12%) of the tested patients. Specific B19-IgG were found in 18/33 (54.55%) as evidence of past/ persistent infection or obtained from donor blood. Positive PCR signal of B19 -DNA was reported in 14/33 (42.42%) patients. The combination of B19-IgG positive diagnostic marker and B19 DNA positive result was detected in 12/33 (36.36%) patients. 14 PCR products were subjected to sequencing and determination of EVB19 genotype. Phylogenetic analysis showed dominance of genotype 1, subgenotype 1a.
Conclusion: EVB19 is proved as a pathological factor in a number of haematological diseases for which frequent blood transfusions are indicated. This pilot study focuses on testing of donor blood for this blood-transmissible agent (demonstrated by serological and molecular markers), which is especially necessary due to asymptomatic infection with erytrovirus B19 in about 30-40% of cases.
Key words: *erythrovirus B19, B19-IgM/IgG antibodies, B19-NS1 region, genotyping*

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INTRODUCTION

The B19 virus was discovered by chance in 1975 by the Australian virologist Y. Cossart. The virus gained its name because it was discovered in well B19 of a large series of microtiter plates labelled in this way [1]. Since 1995 B19 virus has been classified in the family Parvoviridae, subfamily Parvovirinae, genus Erythrovirus, due to its tropism for erythroid progenitor cells in the bone marrow. Erythrovirus B19 (EVB19) is a ssDNA virus, 18–26 nm in diameter without supercapsid [2]. The lack of an envelope makes the virus resistant to physical and chemical inactivation, treatment with detergents and it often survives in blood products [3, 4].
The viral genome consists of 5596 nt sequence encoding: conservative (NS1), variable region (VP1) and a small connecting area between them (NS1/VP1u) as a junction region.
Based on phylogenetic analysis of the DNA sequences, EVB19 is subdivided into three genotypes with no impact on the clinical course [5]. Viral replication takes place in human bone marrow cells and shows a pronounced tropism for erythroid precursors. The main virus receptor is the blood group P antigen found on the surface of a wide range of cells (erythroblasts, megakaryocytes, endothelial cells, fetal myocytes, hepatocytes, placental trophoblast and others).
Clinically, EVB19 is a pathogen associated with a wide range of disease manifestations, syndromes and pathological conditions with acute or chronic course, some of which depend largely on the condition of the immune system and the haematological status of the patient.
In immunologically healthy hosts, B19 may cause a number of acute, generally self-limited diseases, namely, fifth disease or erythema infectiosum in children, acute polyarthritis in adults and transient aplastic crisis [6, 7]. In pregnant women, B19 infection may result in fetal loss, congenital anaemia, non-immune fetal hydrops and fetal death [8, 9].
In immunocompromised hosts, B19 infection may persist and lead to chronic anaemia (pure red cell aplasia) and less frequently, thrombocytopenia, neutropenia, and pancytopenia [10, 11].

The **aim** of this study is to prove the involvement of EVB19 as the etiological and/or persistent agent in patients with haematological disorders using different laboratory diagnostic tools.

MATERIALS AND METHODS

Serum samples were collected from 33 patients (n = 33) at the age of 13-52 years with haematological diagnoses grouped as:
- *Group 1*: aplastic anaemia, erythroblastopenia, thrombocytopenia and pancytopenia (n=10);
- *Group 2*: transfusion-dependent anaemia (thalassemia major and others haemolytic anaemia) and haemorrhagic diathesis (n=21);
- *Group 3*: unidentified fever and impaired haematological parameters (n=2)

All serum samples were stored at -20ºC until tested for B19 immunoglobulin M (IgM) antibody, IgG antibody and B19 DNA.

Serological method - Enzyme immunoassay (indirect ELISA test) was used for determining the presence of specific parvovirus B19 IgM/IgG antibodies (***recomWell Parvovirus B19 IgM/IgG***, according to the manufacturer`s instructions).

MOLECULAR METHODS

- **Extraction of viral DNA** from starting specimen (serum) with commercial test *NucleoSpin Blood*
- **PCR technique** *(KAPA Taq PCR Kits)* with consensus primers (work concentration 20 pmol)

*Forward Primer (**e1905f**): 5' TGCAGATGCCCTCCACCCA 3'*

*Reverse Primer (**e1987r**): 5' GCTGCTTTCACTGAGTTCTTC 3'*

Cycling parameters of NS1 erythrovirus region:

1 cycle at 94°C for 6 min; 5 cycles at 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min; 45 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec; and a final elongation step at 72°C for 7 min. Final: hold at 4°C.

- **Electrophoresis in 2% agarose gel** for visualising the PCR products (103 bp).
- **Purification of PCR products** with *QIAquick PCR purification Kit*
- **DNA sequencing assay**, with commercial kit *BigDye Terminator v3.1 cycle sequencing kit (Life Technologies)* and primers: *e1905f* and *e1987r* (concentration 5 µmol).

Cycling parameters of DNA sequencing:

96°C for 2 min, 96°C for 1 min, 96°C for 1 sec, 50°C for 5 sec, 60°C for 4 min; Go to step 4 repeat 29 x. Final: hold at 10°C

- **Bioinformatic processing of results -** *SeqScape v2.7, BioEdit v7.1 (Sequence Alignment Editor), MEGA v5.05*

RESULTS AND DISCUSSION

SEROLOGICAL RESULTS

The presence of a marker for acute infection (B19-IgM) was established in 4/33 (12.12%) of the studied patients (Table 1) - two positive samples from group 1 and all patients with unidentified fever (group 3).

Table 1: Results from serological testing by ELISA, Erythrovirus B19 IgM/IgG

TESTED SAMPLES	B19-IgM marker		B19-IgG marker	
	(+) IgM	(-) IgM	(+) IgG	(-) IgG
Group 1 **(n=10)**	2/10	8/10	5/10	5/10
Group 2 **(n=21)**	0/21	21/21	11/21	10/21
Group 3 **(n=2)**	2/2	0/2	2/2	0/2
Total number (n=33)	**4/33 (12.12%)**	**29/33 (87.88%)**	**18/33 (54.55%)**	**15/33 (45.45%)**

Specific B19-IgG were found in 18/33 (54.55%). They are evidence for past/persistent infection or obtained from donor blood. Most of them (n = 11) were identified in the group of transfusion-dependent anaemia. Tercan et al. [12] had similar results in a study of a patient group that had received multiple blood transfusions or blood products before collecting serum samples. A prospective study by Heegaard et al. found evidence of B19 infection in 13/43 (30%) patients with chronic anaemia [13]. Our results are in agreement with these studies, suggesting that B19 infection may be a relatively common finding in patients suffering from chronic anaemia in predisposed subjects.

MOLECULAR RESULTS

In our study, we conducted PCR analysis to demonstrate the conservative region (NS1 region) of the erythrovirus B19 genome (Figure 1).

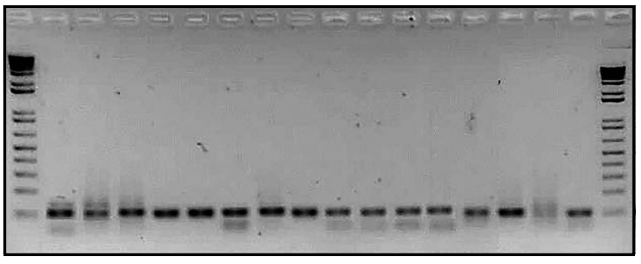

Figure 1: Electrophoretic analysis (2% agarose gel) of B19-NS1 PCR products (103 bp)
Lanes 1 and 18 - MM (100bp); lanes 2-15 - positive samples; lane 16 - negative PCR control; 17 - positive PCR control

Positive PCR signal was detected in 14/33 (42.42%) samples (Table 2) and with higher degree in group 2.

Table 2: PCR results for B19 DNA

Tested samples	B19-DNA marker	
	(+) DNA	(-) DNA
Group 1 **(n=10)**	5/10	5/10
Group 2 **(n=21)**	7/21	14/21
Group 3 **(n=2)**	2/2	0/2
Total number (n=33)	**14/33**	**19/33**

Table 3: Combination of diagnostic markers, which confirm the presence of B19 virus in the tested samples

(+) IgM; (+) IgG; (+) B19-NS1 region	(+) IgM; (+) B19-NS1 region	(+) IgG; (+) B19-NS1 region
2/33 (6.06%)	**3/33 (9.09%)**	**12/33 (36.36%)**

Evidence of B19 infection was found in 3 (9.09%) out of 33 patients by identifying viral DNA and specific IgM antibody (Table 3). We detected the combination of positive diagnostic markers B19-IgG and B19-DNA in 12/33 (36.36%) patients. Chronic persistence of B19 infection plays an important clinical role in patients with haematological diseases [12]. 2/33 (6.06%) of tested samples showed positive results for all EVB19 diagnostic markers (B19-IgM, B19-IgG and B19-DNA). Those samples were from group 3 patients.

DNA SEQUENCING

Each sample positive for B19-NS1 region was subjected to sequence analysis and genotyping. This analysis showed dominance of genotype 1, subgenotype 1a (Figure 2). One variant of genotype 1 was found.

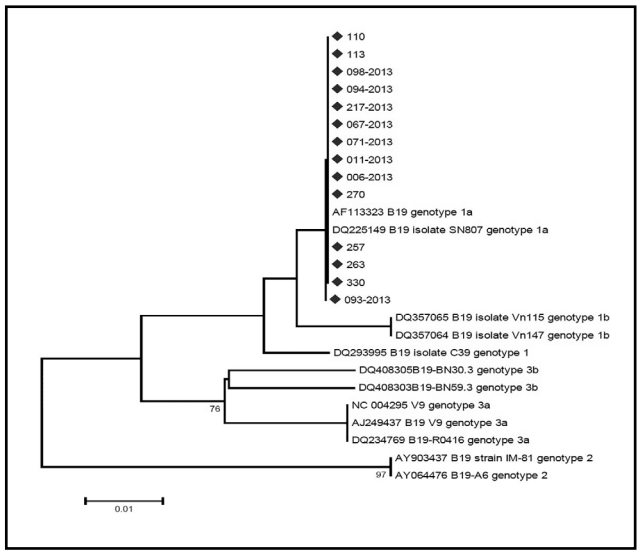

Figure 2: Phylogenetic analysis of B19-NS1 positive samples. The phylogenetic analysis is based on the NS1 region (103 nt) and Neighbour-joining algorithm, Kimura two-parameter model, and included 26 sequences (14 from this study and 12 downloaded from GenBank). Only bootstrap values ≥70% (1000 replicates) are shown.The Bulgarian isolates are marked with the symbol

Other authors [14] report similar findings in a survey of B19 viral spread in Europe and the dominance of genotype 1 in this region. Because of the relatively small number of test samples we cannot link the relevant EVB19 genotype and haematological manifestations of infection.

Important clinical aspect to consider is the risk of infection through B19-contaminated blood products. Recent advances in diagnosis and pathogenesis form a platform for the development of modern therapeutic and prophylactic alternatives for EVB19 [15].

CONCLUSION

For countries that have entered the elimination phase for vaccine-preventable infections such as measles, rubella [16] and poliovirus [17], a better knowledge on the epidemiology of parvovirus B19 may help clinicians in the differential diagnosis of B19 clinical manifestations. This conclusion is particularly adequate during inter-epidemic period when the majority of notified cases need a correct diagnostic approach and surveillance [18].

EVB19 is proved as a pathological factor in a number of haematological diseases for which frequent blood transfusions are indicated. This pilot study focuses on testing of donor blood for this blood-transmissible agent (demonstrated by serological and molecular markers), which is especially necessary due to asymptomatic infection with erytrovirus B19 in about 30-40% of cases.

In summary, for accurate diagnosis we recommend that all cases of haematological disorders should be examined for specific B19 antibodies and tested for the presence of B19 DNA.

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HEPATOTROPIC CO-INFECTIONS IN HIV-POSITIVE PATIENTS: IMPACT ON CELLULAR IMMUNITY PARAMETERS

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ABSTRACT

Background: Due to shared transmission routes, the prevalence of co-infections with HIV and hepatotropic viruses increases worldwide, leading to complications, unpredictable response to combined antiretroviral therapy (cART) and increased mortality.

We evaluated the impact of hepatotropic infections in HIV+ patients subjected to cART, using routine immune monitoring data. Our aim was to identify phenotypic indicators of impaired cellular immunity and therapy response in co-infection.

Materials and methods: Three patients groups were studied: **A.** Monoinfected (HIV+, n=15), **B.** HCV-coinfected (HIV+HCV+, n=15), **C.** HCV and HBV co-infected (HIV+HCV+HBV+, n=13). Peripheral blood samples were obtained at baseline, and after 12 months of cART. Absolute counts (AC) and percentage (%) of lymphocyte (Ly) subsets were determined by flow cytometry using Multitest6-ColorTBNK reagent and TRUCountTubes (BDBiosciences, FACSCantoII).

Results: Basic immunologic parameters and HIV viral load (VL) did not differ significantly between groups at baseline. Co-infected patients were distinguished with poor immunologic response to cART, and specific NK subset dynamics. While progression of HIV monoinfection (**A**) was associated with decreased levels of cytotoxic $CD56^{lo}$ subset, the latter was stable and correlated with viral supression in co-infected patients (**B**, **C**). Hepatotropic co-infection affected significantly the regulatory $CD56^{hi}$ subset: it increased in HIV+HCV+ group (**B**) and lastingly decreased in double co-infection (**C**), regardless of cART.

Conclusions: Hepatotropic co-infection significantly affects cART mediated immune recovery. This effect is independent from influence on viral load, and is mediated at least in part through disturbed differentiaton and balance of $CD56^{hi}$/$CD56^{lo}$NK subsets. Extending the routine immune monitoring to peripheral blood NK subsets is instrumental in HIV/hepatotropic co-infection.

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INTRODUCTION:

Human Immunodeficiency Virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) are persistent pathogens causing global epidemics with major social impact [1 – 3]. Presently, over 35 million people are living with HIV/AIDS. The introduction of cART in 1996 led to an increased life expectancy and reduced number of AIDS-related deaths. In the settings of cART and recovering immunity, HIV-related pathology is mostly associated with the side effects of long-term treatment, combined with particular manifestations of concomitant infections [3]. Due to shared transmission routes, co-infections with HIV and hepatotropic B and/or C viruses are very common, affecting over 30% of the HIV+ individuals and over 50% of specific risk groups as intravenous drug users (IDUs) [4 – 6]. For the period 1987 - 2013, 16.3% of the studied HIV+ patients in Bulgaria were HCV+; 2.7 %-HBV+, and 2.6%-with double HCV/HBV infection For the period 2010 - 2013 these proportions have increased significantly to 29.4%, 11%, and 4.3%, respectively [7], reflecting the increasing share of IDUs among the new HIV-1 diagnoses in the last years [8]. This considerable population remains poorly studied worldwide. The lack of specific monitoring and treatment approaches in the settings of co-infection results in restrain from timely application of available therapy, frequent complications and poor prognosis [5, 9 – 12].

Control of viral replication is associated with the cellular component of host immune response, and requires efficient elimination of pathogen, development of immune memory, and limitation of non-specific inflammation. Chronic infections are characterized by lack of protective immunity associated with misbalance of effector and regulatory signals. Chronic infections are a classical example of disturbed immune homeostasis that is further complicated in case of concomitant persistent stimulation of different etiology [13].

The mutual interactions between HIV and hepatotropic infections are still poorly understood. Recent studies have suggested increased risk of faster progression to AIDS and slow CD4 recovery among co-infected individuals. On the other hand, HIV decreases the chances of spontaneous HCV clearance, increases HCV viral load and accelerates the course of HCV-associated liver disease leading to increase in morbidity and mortality among co-infected individuals [6, 9, 14, 15]. HIV infection is a risk factor for reactivation of HBV infection and progression to cirrhosis and aggressive hepatocellular carcinoma, while cART is associated with increased risk of hepatic flares in both HCV and HBV co-infected patients [14]. Current-

ly published data have demonstrated that the clinical course and prognosis of chronic viral infections depend on the complex cross-talk between effector and regulatory subsets of natural and adaptive immunity [13]. The contribution of innate effector and regulatory subsets as NK, NKT, TCRγδ and DC to the immunopathogenesis of chronic uncontrolled viral infections is just starting to be unveiled. The natural enrichment of NK cells in the liver (30% vs. 5-20% in peripheral blood), and their ability to eliminate virally infected hepatocytes, imply a key effector position in acute and chronic HCV infection, and even more so in HCV/HIV co-infection [16, 17]. The intensity of CD56 expression differentiates between immature, cytokine secreting NK cells ($CD56^{high}$), and differentiated readily cytotoxic cells migrating to sites of acute inflammation ($CD56^{low}$). The major cytokines released by NK $CD56^{hi}$ cells depending on the precise conditions of stimulation include TNF-a, GM-CSF, IL-10 and IL-13. Therefore, in addition to potentiating an IFNg-mediated adaptive response, $CD56^{hi}$ cells have well recognized regulatory effects [18]. Further on, liver contains the highest ratio of NKT/conventional T cells compared to other organs. Activated NKT can secrete both cytokines with strong antiviral activity (IFNg, IL-17), and large amounts of the anti-inflammatory cytokines IL-4 and IL-10 [19]. The γδ T cells are known to have a wide range of functions, including immune protection against pathogens in the early γδ T-cell response and immunoregulation in some late γδ T-cell responses [20]. Both quantitative imbalance and functional impairment of NK, NKT and γδ T cells have been documented in persistent HIV and hepatotropic infection [21 – 27]. However, the precise mechanisms and consequences of impaired NK cell differentiation remain poorly elucidated in the settings of coinfection. The aim of the present study was to evaluate the impact of concomitant hepatotropic HCV or HBV/HCV co-infection on the dynamics of $CD56^{lo}$NK, $CD56^{hi}$NK, NKT and TCRgδ subsets in HIV+ patients before and in the course of 12-month cART, and to identify possible phenotypic indicators of impaired cellular immunity and poor therapy response in the settings of co-infection.

MATERIALS AND METHODS:

Study design and patients groups. The present study is a retrospective analysis of immunophenotyping data obtained during the routine immune monitoring of HIV+ patients registered at the Speciliazed Hospital for Active Treatment of Infectious and Parasitic Diseases "Professor Ivan Kirov", Sofia. Included in the study were only treatment-naïve patients that have been on follow-up during the period Nov 2007 – Aug 2013. Two types of patients entered the study: those with **"early"** HIV infection (CD4AC > 350 cells/ul, untreated), and those with **"advanced"** HIV infection (CD4AC < 350 cells/ul), that have started cART, and were still alive and on cART 12 months later. Three groups of treated HIV+ patients were defined: **A.** monoinfected, (HIV+, n=15), **B.** with HCV co-infection, (HIV+HCV+, n=15), **C.** with double HCV and HBV co-infection (HIV+HCV+HBV+, n=13). Patient groups were sex and age-matched. The evidence of hepatotropic (HBV and/or HCV) co-infection was based on a positive serological result (HBsAg and/or anti-HCV), confirmed with at least one positive result for the presence of viral nucleic acid during the period of follow-up.

Virological tests. HIV infection has been confirmed by the National Reference Laboratory of HIV/AIDS in the National Centre of Infectious and Parasitic Diseases (NCIPD) according to the current guidelines [28]. All patients have been examined for the presence of concomitant hepatotropic infection at the time of registration, using relevant ELISA kits for qualitative detection of HBsAg (SURASE B-96) and antibodies to core, NS3, NS4, and NS5 HCV antigens (NANBASE C-96N, GBC, Taiwan), and/or RT-PCR for quantitative viral nucleic acid detection (COBAS, AmpliPrep/COBAS TaqMan HBV and HCV tests, Roche Molecular Systems, Branchburg, NJ, USA) at the National Reference Laboratory of Viral Hepatitis, NCIPD.

Immunophenotyping. Samples for routine immune monitorring (5 ml peripheral blood in BD Na Heparin Vacutainer tubes) were obtained in the course of routine follow-up of HIV+ patients at the National Reference Laboratory of Immunology, NCIPD. The study included samples obtained before (baseline), and 12 months after the start of cART. Absolute counts and percentage of lymphocyte subsets were determined with a BDMultitest 6-Color TBNK reagent (CD45/CD56+16/CD4/CD8/CD19) using standard lysis–no–wash procedure and TRUCount tubes. (BD Biosciences). At least 5000 events in the lymphocyte gate were collected and analyzed using BD FACSCanto II flow cytometer and DIVA 1.2.1 software. In addition to standard monitoring data, additional information for NK, NKT, TCRγδ, and immature CD4+CD8+ T Ly was obtained after reanalysis of registered data. (**Table 1, Fig.1**).

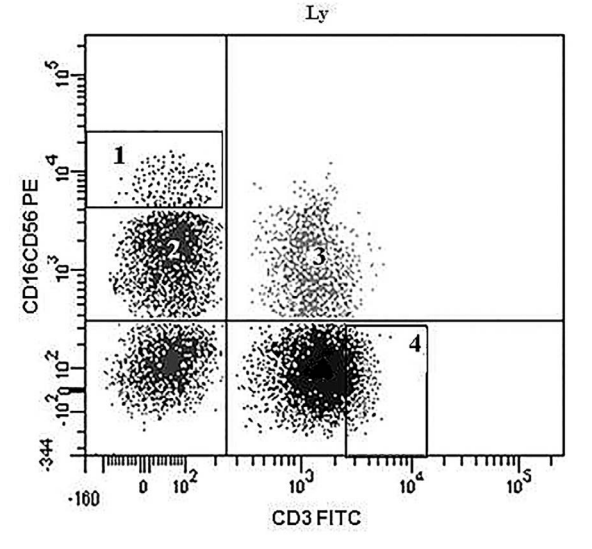

Fig.1 Lymphocyte subsets determined by flow cytometry. A typical dot-plot generated from peripheral blood analysis with BDMultitest 6-Color TBNK reagent. In addition to routinely defined total NK (CD3-CD56+/CD16+), total T (CD3+), and total B cells (CD19+CD3-), the following subsets were determined: 1. $CD56^{hi}$ NK ($CD3\text{-}CD56^{hi}$); 2. $CD56^{lo}$NK ($CD3\text{-}CD56^{lo}$); 3. NKT (CD3+CD56/ CD16+); 4. TCRγδ T ($CD3^{hi}CD56$-).

Statistical evaluation Between-group significant differences were calculated using the non-parametric Mann-Whitney or Kruskal-Wallis test for more than two groups; intergroup significant differences at different time points were evaluated by Wilcoxon non-parametric test, and correlations - with Spearman rank order correlation test. P-values equal or less than 0.05, were considered significant (GraphPad Prism 4.0 – 6.0).

Table 1. Lymphocyte subsets determined by flow cytometry.

ROUTINE IMMUNOPHENOTYPING DATA		ADDITIONAL IMMUNOPHENOTYPING DATA	
Phenotype	**Ly subset**	**Phenotype**	**Ly subset**
CD3+CD19-	Total T	**CD3+CD56+**	NKT
CD3+CD4+	T helper/ inducer	**CD3^{hi}CD56-**	TCRγδ T
CD3+CD8+	T cytotoxic	**CD3-CD56 ^{hi}**	Cytokine-secreting NK
CD3-CD19+	Total B	**CD3-CD56 ^{lo}**	Cytotoxic NK
CD3- CD56/ CD16+	Total NK	**CD4+CD8+CD3+**	Immature T

RESULTS:

1. Baseline characteristics of monoinfected and co-infected patients groups. The demographic and laboratory characteristics of studied patients group are given in **Table 2**. The plasma HIV VL and the absolute count of CD4 T cells (CD4 AC) are the principal indicators for the activity and progression of HIV infection, and therefore - for the start of cART. Monoinfected (**A**) and co-infected (**B**, **C**) patients did not differ significantly according to the average plasma HIV VL before the start of cART (*mean log RNA copies/ml*: 4.8, 4.5, 4.1, respectively, p > 0.05) No significant differences existed between the baseline CD4 AC, either (*CD4 AC mean /SEM:* 275 /16, 244 /31, 248 /41 for groups **A**, **B** and **C** respectively; p > 0.05, **Fig.2A).** Further on, the ratio between CD4 and CD8 T Ly (CD4/CD8) related to virus-driven immune activation was also comparable between the three groups (*CD4/CD8 T mean /SEM:* 0.3 /0.04, 0.4 /0.06, 0.3 /0.03 respectively; p > 0.05, **Fig.2B).** Finally, the total T and B Ly counts did not differ significantly, either (data not shown).

Table 2. Demographic and laboratory characteristics of cART-treated HIV+ patients, included in the study.

Characteristics		Group A (n=15)	Group B (n=15)	Group C (n=13)
Gender (%)	Male Female	100 0	100 0	100 0
Age (years)	Mean Min-max	27.6 17 - 38	27.3 17 - 35	26.8 17 - 32
Infection duration[1] (months)	Mean Min-max	12.5 10 - 14	11.7 10 - 16	12.1 6 - 16
Baseline CD4 AC (cells/μl)	Mean Min-max	275 209 - 435	244 44 - 516	248 82 - 538
Endpoint CD4 AC (cells/μl)	Mean Min-max	553 191 - 1349	402 114 - 717	365 86 - 881
Baseline HIV VL (log RNA copies/ml)	Mean Min-max	4.8 2.2 - 6.7	4.5 1.7 - 5.9	4.1 2 - 5.6
Endpoint HIV VL (log RNA copies/ml)	Mean Min-max	2 1.3 - 4.9	2.3 1.3 - 5.9	2.7 1.3 - 5.1

[1] Time since diagnosis of HIV-1 infection

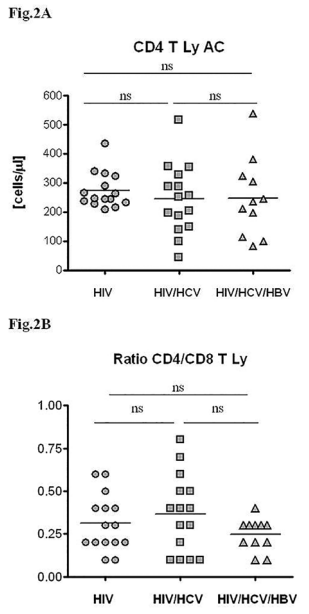

Fig.2 Baseline immunologic characteristics of patients groups: A/ CD4 AC (cells/ml); **B/** CD4/CD8 ratio.

2. Concomitant hepatotropic infection affects the response to cART. Supression of viral replication (log VL < 1.6 copies/ml) and reconstitution of CD4 T Ly pool are the well accepted indicators for cART efficiency. According to our results, immune recovery in response to 12-month cART was significantly affected by the presence of hepatotropic co-infection. The increase of CD4 AC (ΔCD4 AC) was significantly higher in monoinfected patients (**A**): ΔCD4 AC mean */SEM:* 278 /66, as compared to patients with concomitant HCV (**B**), 157 /37, and even more so - to patients with HCV/HBV co-infection (**C**) 113 /41; p < 0.05 (**Fig.3A**). The poor quantitative restoration of the CD4 pool in group **C** was combined with increased share of immature CD4+CD8+ T Ly, unlike the groups **A** and **B** (Δ*CD4+CD8+ % mean /SEM:* 0.4 /0.2 vs. -0.06 /0.03 and -0.07 /0.09, p < 0.01 and p < 0.05, respectively), (**Fig.3B**).

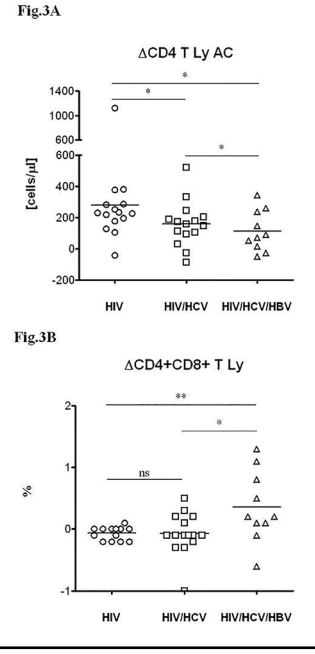

Fig.3 Immunologic response to 12-month cART in patient groups A, B and C:. **A/** ΔCD4AC (cells/ml); **B/** ΔCD4+CD8+ (%). Δ was defined as the difference between the corresponding values determined after 12 months of cART and just before the start of therapy (baseline).

3. Hepatotropic co-infection affects selectively NK subset dynamics.

To evaluate the relative impact of HIV and HCV infection on the studied innate immunity parameters, we have first compared patients with early and advanced HIV infection, and then – the 12-month and baseline points in both the mono- and co-infected groups.

Our results show that in the HIV+ monoinfected group (**A**), the share of cytotoxic CD56^{lo}NK decreases with infection progression and is restored in the course of cART: *% CD56^{lo} mean /SEM:* advanced, baseline 7.2 /1.6 vs. early 13 /2.6, $p < 0.05$ and vs. advanced, 12mo cART 11.3 /1.8, $p < 0.001$ (**Fig.4 A**). Interestingly in the settings of HCV co-infection (**B**), the share of CD56^{lo}NK was not affected by HIV infection progression, and remained stable in the course of cART: *% CD56^{lo} mean /SEM:* advanced baseline 13.6 /2.5 vs. early 8.3 /1.1, $p > 0.05$; and vs. advanced, 12mo cART 13.8 /2.3, $p > 0.05$, **(Fig.4 B**). Similar trends were observed for group **C** (data not shown). Importantly the baseline share of CD56^{lo}NK correlated inversely with HIV VL after 12-month cART ($p < 0.01$, $R = - 0.51$, **Fig.4 C**).

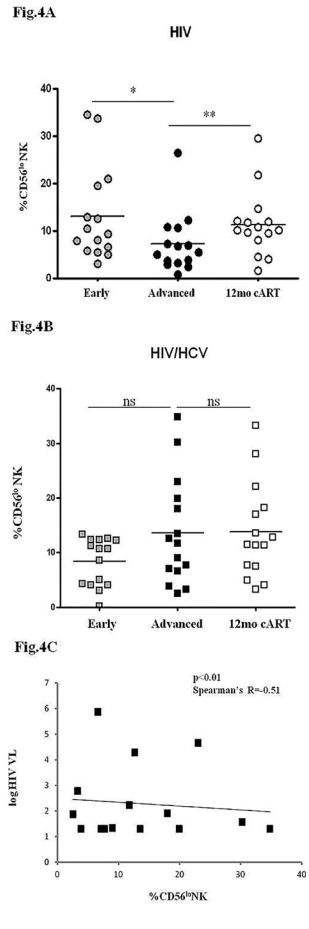

Fig.4 Dynamics of CD56^{lo} NK subset in the settings of HIV monoinfection, and HIV/HCV co-infection: A/ CD56^{lo}NK levels in: HIV+patients with early infection (grey); advanced infection (black); the latter after 12 months of cART (white); **B/** CD56^{lo}NK levels in HIV+HCV+ patients with early (grey) and advanced (black) infection; the latter after 12 months of cART (white); **C/** Correlation between the baseline share of CD56^{lo}NK and plasma HIV VL after 12 months of cART.

Further on, in monoinfected HIV+ patients (**A**), the cytokine-secreting CD56^{hi}NK subset was not affected by infection progression or cART: *% CD56^{hi} mean / SEM:* advanced, baseline 1.0 /0.2 vs. early 0.7 /0.1, $p > 0.05$; and vs. advanced, 12mo cART 0.9 /0.2, $p > 0.05$, (**Fig.5 A**). However, in conditions of HCV co-infection (**B**), the level of CD56^{hi}NK progressively increased, and was not modified by cART: *% CD56^{hi} mean /SEM:* advanced baseline 1.0 /0.2 vs. early 0.4 /0.1, $p < 0.01$, and vs. advanced, 12mo cART 1.23 /0.2, $p > 0.05$, (**Fig.5 B**). Importantly the baseline level of CD56^{hi} subset was significantly reduced in group **C** as compared to group **B**: *% CD56^{hi} mean /SEM:* 0.5 /0.1 vs. 1.0 /0.2, $p < 0.05$, and this difference further increased after 12mo cART**:** 0.5 /0.1 vs. 1.2 /0.2; $p < 0.01$ (**Fig.5 C**)

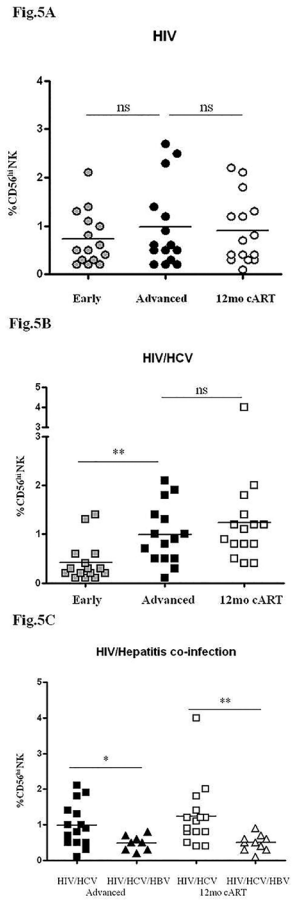

Fig.5 Dynamics of CD56^{hi}NK subset in the settings of HIV monoinfection, and HIV/HCV co-infection. CD56^{lo}NK levels were compared in: **A/** HIV+patients with early (grey) and advanced (black) infection; the latter after 12 months of cART (white); **B/** in HIV+HCV+ patients with early (grey) and advanced (black) infection; the latter after 12 months of cART (white); **C/** groups B (HIV+HCV+) and C (HIV+HCV+ HBV+) before and after 12 months of cART.

No significant differences were established for the regulatory NKT and TCRγδ+ T subsets: between the mono- (**A**) and co-infected patients groups (**B**, **C**), regardless of HIV infection progression or cART (data not shown).

DISCUSSION

Immune monitoring is an integral part of the follow-up of HIV+ patients. CD4AC remains the principal surrogate marker used to estimate disease progression, for treatment decisions, and measurement of cART efficacy [28]. Although a number of cellular immunity parameters have been shown to correlate with viral activity and predict cART outcome, none has been routinely, adopted [29 – 31]. Even more so, an appeal for simplified immune-monitoring protocols has been recently launched [32]. Thus, to date no specific immune parameters have been recommended

for the evaluation and follow-up of HIV patients with concomitant hepatotropic infection. We have extended routine immunophenotyping analysis in co-infected patients on innate immunity subsets with effector / regulatory function, and provided new evidence on the association of $CD56^{hi}$ and $CD56^{lo}$NK subsets with HIV infection progression and cART efficiency, without adding to the costs of monitoring.

First of all, our results clearly show that standard immunophenotyping parameters including percentages and numbers of CD4, CD8, total T and B cells cannot differentiate between mono- and co-infected patients groups. Second, we have confirmed that a concomitant hepatotropic infection significantly affects immune response to cART, both in terms of CD4 numbers, and of CD4 functional maturation. This was observed in spite of comparable HIV viral loads in the three patients groups after 12-month cART, meaning that hepatotropic infection may specifically affect the restoration of the CD4 pool, independently of HIV viral load.

A third important observation was that unlike NKT and TCRgδ T, $CD56^{hi}$ and $CD56^{lo}$NK subsets display different dynamics in the settings of mono- vs. co-infection. NK cells comprise 20% – 30% of all liver lymphocytes. NK cell function is determined by the balance of activating and inhibitory-receptor expression, which in turn may be modified by the liver microenvironment [17] Therefore, NK subset composition is of particular interest in HIV patients with hepatotropic infection. In fact, it is debatable whether $CD56^{hi}$ and $CD56^{neg}$ NK subsets are involved in CD4 T Ly destruction and disease progression, or rather in viral control [16, 22].

In line with others, we observed progressive depletion of the cytotoxic $CD56^{hi}$ subset in the course of HIV infection that was not easily reversible by cART. A decreasing share of $CD56^{lo}$ NK in the course of progressive HIV or HCV monoinfection has already been reported [25, 33]. However, we report for the first time that concomitant hepatotropic infection maintains the level of NK $CD56^{lo}$ within the reference ranges observed in the early stages of HIV infection, not significantly different from healthy controls.

In addition, we demonstrate a direct relationship between the levels of this subset and HIV replication control after 12-month cART. A plausible explanation for these observations could be an accelerated rate of NK differentiation within the liver under the pressure of hepatotropic infection, compensating for their HIV-mediated destruction. At the same time, the superior HIV suppression observed in the settings of co-infection was compromised by a poor immune restoration. As already shown by others, on-going low level immune activation associated either wih latent HIV reservoirs or non-HIV latent or activated pathogens, as in the case of HCV/HBV, is the major problem in the settings of life-long cART [6]. The most important message of this observation is that HIV VL monitoring in patients with concomitant HCV and/or HBV infection should be obligatorily combined with, at least, basic immunophenotyping.

If $CD56^{lo}$ appears a significant factor for HIV control, $CD56^{hi}$ subset in our hands was better related to hepatotropic viral activity as it showed no specific dynamics in HIV monoinfection while progressively increasing in untreated HCV and/or HBV co-infection. Logically, a Th1-mediated supression of viral replication should be accompanied by an increasing share of immature $CD56^{hi}$NK, performing a negative feed-back down-regulation. In line with our results, $CD56^{hi}$NK cells were shown to predominate in HBV patients after pegylated IFN-γ therapy [34]. Recently, Eisenhardt M et al. have shown that CD27(+)$CD56^{hi}$NK display strong anti-HCV activity and may be involved in the spontaneous clearance of acute hepatitis C in HIV-positive patients [35]. $CD56^{hi}$ NK were further demonstrated to protect a HIV/HCV co-infected hemophiliac A patient from opportunistic infections and virus-related cancers despite very low CD4+ cell counts [36]. Elevated activity of $CD56^{hi}$ NK cells was also observed in HIV-1 clade A or clade D HIV+ patients with untreated infection and severe CD4 loss [37]. On the other hand, the increased proportion of $CD56^{hi}$ cells in chronically infected HCV patients as compared with HCV resolvers and uninfected controls has been interpreted as the result of decreased differentiation rate, contributing to T-cell polarization and liver damage [25]. Still another point of view, based on the fact that the immature NK subset was protective against liver metastasis, is that $CD56^{hi}$ are part of the liver immune tolerogenic microenvironment [19].

We have also demonstrated that double hepatotropic co-infection was characterized with a significant and lasting decrease of the immature NK subset. A plausible explanation would be that the high HCV viral load in the settings of HIV co-infection is associated with profound defects in the differentiation of both innate and adaptive responses, leading to altered cytokine environment in liver, reduced regulatory response and increased pathology.

Our hypothesis is that a balanced generation of immature tolerogenic $CD56^{hi}$ and their differentiation to cytotoxic $CD56^{lo}$ NK cells drives a protective cellular immune response, combining viral clearance with minimal tissue damage. Persistent infections may specifically affect this balance. Progressive untreated HIV infection is characterized with depletion of cytototxic NK^{lo} subset, and domination of tolerogenic NK^{hi} that favors unrestrained viral replication. On the other hand, NK^{hi} are beneficial in the context of liver microenvironment where they are able to clear hepatotropic infection without extensive liver damage. Concomitant HIV/hepatotropic infection violates the balance, depending on the quantitative and temporal dominance of the individual pathogens. While contributing to viral clearance in HIV monoinfection, abundant NK^{lo} may bring damage to liver in case of secondary hepatotropic co-infection. On the other hand, HIV co-infection of HCV/HBV+ patients might drive to differentiation and depletion the $CD56^{hi}$ subset with anti-HCV activity.

In conclusion, peripheral blood levels of $CD56^{hi/lo}$ subsets provide accessible and important information in

case of concomitant HIV/hepatotropic infection. Larger prospective case-control studies, completed with NK subset functional assessment in relation to viral (HIV, HCV, HBV) activity are needed to implement this information in the monitoring and therapeutic decisions concerning co-infected patients.

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SUITABILITY OF WEEKLY CASE DATA ON TOTAL ACUTE HEPATITIS FOR THE DETECTION OF VARIATIONS IN HEPATITIS A INCIDENCE

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ABSTRACT

The development of a model for early detection of unexpected increases in hepatitis A incidence can facilitate timely risk communication and other public health measures, aimed at reducing further infection transmission. In Bulgaria, the absence of weekly reporting on hepatitis A cases complicates the design of this model. Due to the technical time it takes to identify the causative agent of an acute hepatitis case, weekly reporting is aimed at providing only the total number of acute hepatitis cases, without specifying the virus type. Acute hepatitis cases are then broken down by causative agent in the monthly and annual reports. This study evaluates whether acute hepatitis case numbers in weekly reports can be used to estimate hepatitis A case numbers. The reliability of weekly and monthly reports is assessed through careful consideration of error rates. The relationship between weekly acute hepatitis and monthly hepatitis A case numbers at national and regional levels is assessed through correlation. The analysis demonstrates that weekly operational data on acute hepatitis cases is suitable for further modelling of variations in hepatitis A virus incidence.

Keywords: *hepatitis A, incidence, reliability, alert threshold*

INTRODUCTION

Hepatitis A is an infectious liver disease, caused by the hepatitis A virus (HAV). It is transmitted via the fecal-oral route through person-to person contact or by ingestion of contaminated food or water (1). Hepatitis A infection in young children is usually asymptomatic, whereas adults may have mild disease or develop serious complications (1). It is estimated that 80-95% of children less than 5 years old have asymptomatic infections, compared to only 10 to 25% of adults (2, 3). Mortality from hepatitis A is low, and the virus does not cause chronic liver disease (1). However, the infection is expensive in terms of direct medical costs and lost productivity (1). There are an estimated 1.4 million cases of hepatitis A worldwide every year (4).

Bulgaria is a country with intermediate hepatitis A endemicity (5) and substantial increases in incidence every 5-6 years. Studies in other countries have revealed that areas of intermediate endemicity have larger adolescent and adult populations, susceptible to the disease, in comparison to areas of high endemicity, where most people get infected early in life, develop mild or no symptoms, and acquire immunity (1). In 2013, 46% of all hepatitis A cases in Bulgaria were aged over 20 years which is evidence of the accumulation of a large number of susceptible individuals in the adult population (6). Immunisation against hepatitis A is recommended for people with chronic liver disease, travellers to areas with high hepatitis A endemicity and other risk groups, but is not mandatory (7).

With an increasing proportion of susceptible individuals in the adult population, and the risk of more serious manifestations and lethality from the disease at a later age, the ongoing monitoring and systematic analysis of HAV infection in Bulgaria has become more important for the epidemiological control of hepatitis A.

An alert threshold detection model for HAV infection that works with ongoing weekly data can be a useful tool, aiding the timely reaction of the public health system. Working on a weekly basis is important, as a number of studies and guidelines on hepatitis A outbreak management underscore that measures to prevent further infection are most efficient, if taken within 2 weeks of the beginning of outbreaks (8). However, in Bulgaria weekly reports provide an aggregate number of all cases of acute hepatitis. Identification of the causative agents depends on immunological assays (9), and hence differentiation of acute hepatitis cases by virus type only takes place in monthly and annual reports.

The aim of this study is to assess the reliability of weekly acute hepatitis cases and to test whether these data can be used to estimate hepatitis A incidence rates at national and regional level.

MATERIALS AND METHODS

Data on acute hepatitis for the period 2003-2013 were obtained from the National Communicable Disease Surveillance System. In accordance with national legislation (10,11), all medical professionals are obliged to send a rapid notification for each suspected case of acute hepatitis to the Regional Health Inspectorates (RHI) which collect the data and report the aggregated information to the National Center of Public Health and Analyses (NCPHA). The NCPHA processes the data from the 28 RHI and sends summarized data to the National Centre of Infectious and Parasitic diseases (NCIPD) and Ministry of Health on a daily, weekly, monthly and annual basis. Information about acute hepatitis cases divided by the type of the virus is available only on a monthly and annual basis. The EU case definitions and case classifications for viral hepatitis were adopted in 2005 (10). The EU case definitions from 2008 are applied since 8 July 2011 (11). In this study, all reported cases (respectively both the probable and confirmed cases after 2005) were

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used in the analysis. We have used the operational weekly reports on acute hepatitis cases, annual reports on acute hepatitis cases, as well as monthly and annual reports on cases of HAV infection. Population data for the same period was obtained from the National Statistical Institute.

In the first part of the study, reliability of weekly reports on acute hepatitis and monthly reports on hepatitis A was assessed. This was done at the regional and national level, through adding up the weekly acute hepatitis (respectively the monthly hepatitis A) cases to obtain yearly estimates, and then comparing these estimates to the accurate (validated) numbers, presented in annual reports.

In order to assess the applicability of weekly acute hepatitis data for the assessment of variations in HAV infection cases, the operational weekly data was added up to obtain monthly and annual case numbers based on weekly reports. Weeks were sorted into months on the basis of the date on which the median day of each week (Thursday) fell.

Descriptive statistics and correlation were used to analyze the data. As data is highly positively skewed and normality of the distribution could not be achieved through transformations, Spearman's rank correlation coefficient (Rho, ρ) was used for correlation.

RESULTS

Tendencies in acute hepatitis cumulative incidence (2003-2013)

The annual incidence of hepatitis A in Bulgaria between 2003 and 2013 varies in a cyclical fashion with cycles of 5-6 years. Total reported acute hepatitis incidence combine all cases of acute hepatitis A, B, C and D, as well as hepatitis cases with unspecified causative agents. It varies in the same fashion as hepatitis A. The incidence of all other acute hepatitis types is significantly lower and relatively stable, with a visible decrease in hepatitis B incidence through the years, resulting from the introduction of mandatory vaccination of newborns against hepatitis B in Bulgaria in 1992 (Fig. 1)

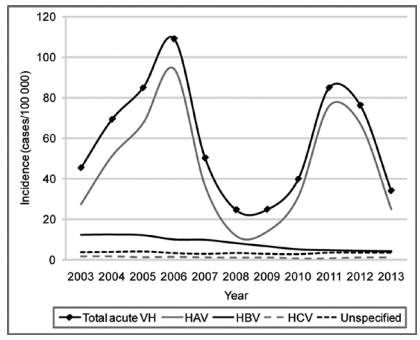

Fig. 1. Annual incidence of acute hepatitis in Bulgaria, 2003-2013. Note: Hepatitis D incidence is negligible and not presented here.

While hepatitis A cases comprise a consistently high proportion of acute hepatitis cases, this proportion increases further in years of high total incidence. For the period under study hepatitis A has comprised between 48% (2008) and 89% (2011) of all hepatitis cases. The very strong linear relationship between acute hepatitis and hepatitis A annual incidence, demonstrated in Fig. 2 (Spearman's Rho of 0.99, $p<0.01$), indicates that acute hepatitis cases may be useful in estimating hepatitis A cases.

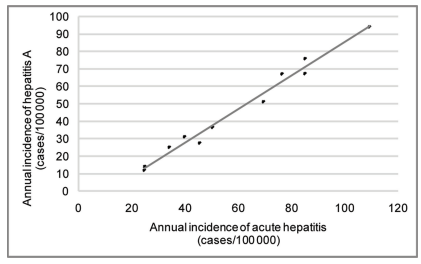

Fig. 2. Relationship between annual acute hepatitis and hepatitis A incidence in Bulgaria, 2003-2013

Reliability of weekly acute hepatitis reports

The relationship described above is very strong and promising, yet it is based on annual reports of acute hepatitis and hepatitis A. In practice, however, ongoing monitoring can only be based on weekly acute hepati-

Table 1: Over and underreporting in weekly reports of acute hepatitis in Bulgaria and in Silistra region (2003-2013)

	Annual number of acute hepatitis cases Bulgaria			Annual number of acute hepatitis cases in Silistra		
	A	B	(A/B)*100	C	D	(C/D)*100
Year	Sum of weekly reports	Case number in corrected annual reports	Weekly over/ underreporting (% of number in annual report)	Sum of weekly reports	Case number in corrected annual reports	Weekly over/ underreporting (% of number in annual report)
2003	3640	3561	102.22	129	121	106.61
2004	5661	5412	104.60	147	125	117.60
2005	6850	6595	103.87	73	55	132.73
2006	8495	8421	100.88	124	71	174.65
2007	4119	3878	106.21	164	78	210.26
2008	2146	1883	113.97	131	64	204.69
2009	2222	1887	117.75	109	55	198.18
2010	3192	3007	106.15	46	24	191.67
2011	6427	6258	102.70	24	13	184.62
2012	5773	5590	103.27	12	13	92.31
2013	2559	2479	103.23	10	12	83.33

tis reports. Case numbers, reported on a weekly basis are prone to error, and are then corrected in the monthly and annual reports, with annual reports considered to be the most accurate sources of information. The reliability of weekly operational acute hepatitis case data was assessed through comparing weekly with annual report numbers. For each year, all cases from weekly reports were summed up to get annual numbers of acute hepatitis cases, based on weekly data. The sums were then compared to the numbers in corrected annual reports. Errors are expressed as a percentage of the case number from the annual report (Table 1).

For total acute hepatitis case numbers in Bulgaria, overreporting was observed in the weekly reports throughout the period (Fig. 3A, Table 1), with an average overreporting of 6%. The overreporting percentage was negatively correlated to case numbers (Fig. 3C, Spearman's rho $\rho = -0.63$, $p<0.05$). This reflects the principle that error rates increase when numbers are lower.

The same analysis was performed on all 28 regions with varying results. Many regions have a general tendency to slightly or strongly overreport, but it is not consistent through the years, and underreporting does occur (see Table 1, Silistra, Years 2012 and 2013). Six regions stand out with highly unreliable weekly acute hepatitis reporting, as measured through the average absolute error (difference from annual report, expressed as percentage of annual report). These regions are Silistra, with an average absolute error of 59 % (Figure 3B and D, Table 1), Pernik (54%), Blagoevgrad (50%), Kustendil (33%), and Sofia-Region (26%). For six other regions (Vidin, Montana, Varna, Stara Zagora, Razgrad, and Haskovo), reporting was better for this period, but still with an average absolute error between 10 and 20%. For all other regions, this percentage was at or below 10%, down to 1% for Sofia-City. The average error percentage is not significantly higher for regions which report less number of cases throughout the period. To demonstrate how reporting quality may differ significantly in time, Silistra is given as an example in Fig. 3B and D.

Error rates by year relate to case number only in some regions. Significant ($p<0.05$) correlations between error and case number were found for Blagoevgrad ($\rho = -0.7$), Sofia-Region ($\rho = -0.70$), Veliko Tarnovo ($\rho = -0.68$), Varna ($\rho = -0.82$), Stara Zagora ($\rho = -0.70$) and Kardzhali ($\rho = -0.65$).

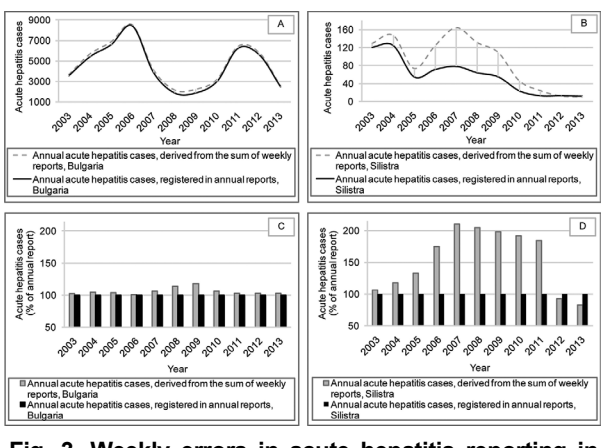

Fig. 3. Weekly errors in acute hepatitis reporting in Bulgaria and Silistra, 2003-2013. In A (Bulgaria) and B (Silistra) the errors are visualized in terms of absolute reported case numbers. In C (Bulgaria) and D (Silistra), errors are represented as percent of the acute hepatitis case numbers in corrected annual reports.

Reliability of monthly hepatitis A reports

Hepatitis A reports will be an integral part of further regression analysis, aimed at estimating hepatitis A incidence from weekly acute hepatitis case numbers. Annual hepatitis A reports are the most accurate, but using monthly reports in developing the model is a better choice in terms of a vastly increased number of time-points, which will also capture seasonal fluctuations in hepatitis A incidence.

Unlike acute hepatitis weekly reports, monthly hepatitis A case reports are characterized with good reliability, both at a national and regional level. Average percent errors for most regions range between 0% and 6%, which leads to an average percent error for Bulgaria of <1%.

Suitability of weekly reported acute hepatitis data for the timely detection HAV outbreaks

To assess whether acute hepatitis cases, reported on a weekly basis, may be used in regression modelling, aimed at estimating hepatitis A incidence, correlation analysis was performed. In order to correlate the two datasets, weekly acute hepatitis cases were summed up for each month, in order to achieve a monthly acute hepatitis estimate. This number was then correlated with the monthly hepatitis A case numbers, derived from monthly reports. Spearman's Rho for Bulgaria was 0.97 ($p<0.01$), which demonstrates a very strong correlation. For the regions, the Spearman's rank coefficients are presented in Table 3. They range from 0.97 for Pazardzhik to 0.66 for Veliko Tarnovo. Significance for all coefficients is $p<0.01$.

DISCUSSION

Hepatitis A cases comprise the highest proportion of acute hepatitis cases in Bulgaria. There is a very strong linear correlation between acute hepatitis and hepatitis A annual incidence between 2003 and 2013. Thus, acute hepatitis cases may be useful for estimating hepatitis A cases. However, a regression model for estimating weekly hepatitis A cases will have to be developed, based on weekly acute hepatitis and monthly hepatitis A reports, as the first will be the actual source of information in practice, and the second provide a sufficient number of time points and make modelling sensitive to seasonal hepatitis A incidence fluctuations.

The analysis demonstrated problems with the reliability of weekly acute hepatitis reporting at the regional level, with a general tendency to overreport and some regions overreporting heavily over a number of years. Error rates may increase as a function of lower overall case numbers. Alternatively, it may be that overreporting increases during outbreaks. To test whether there is a relationship between error rates and overall case number, correlation analysis was performed in space (looking at the median case number and average error rate in each region), and in time (looking at variations in error rates and case numbers through the years). The results revealed that errors do increase as a function of lower case numbers, but that this relationship is only valid for Bulgaria as a whole and for some regions.

Careful analysis of over/underreporting through time has revealed that, for many regions, the error in weekly acute hepatitis reporting is either low or relatively consistent, and the time-series graphs of annu-

Table 3: Spearman correlation coefficients, characterizing the relationship between weekly acute hepatitis case numbers and monthly HAV case numbers, 2003-2013

Region	Spearman's rho	Region	Spearman's rho	Region	Spearman's rho	Region	Spearman's rho
Pazardzhik	0.97	Targovishte	0.88	Pleven	0.85	Pernik	0.81
Plovdiv	0.94	Vratsa	0.88	Smolyan	0.85	Gabrovo	0.81
Sliven	0.94	Schumen	0.87	Sofia – region	0.85	Kustendil	0.78
Burgas	0.93	Kardzhali	0.87	Dobrich	0.84	Razgrad	0.77
Sofia – city	0.92	Montana	0.87	Silistra	0.84	Ruse	0.75
Haskovo	0.90	Varna	0.87	Lovech	0.83	Blagoevgrad	0.66
Yambol	0.90	Stara Zagora	0.86	Vidin	0.82	Veliko Tarnovo	0.66

al numbers of acute hepatitis cases, calculated from weekly reports, are parallel to, even if not completely aligned with, the time-series graphs of annual numbers of acute hepatitis cases from corrected final reports (situation similar to that for the whole of Bulgaria (Fig. 3A). This means that correlation and further regression analysis between weekly acute hepatitis and monthly hepatitis A numbers will not likely be weakened substantially by the errors in weekly reporting. For several regions, however, the weekly reporting errors are expected to weaken the correlation and regression analyses. These are Silistra (Fig. 3B), Blagoevgrad, Kustendil, Montana, Varna, and Razgrad. This does not mean that the model will necessarily be weak, but that it could be substantially improved through improved quality of reporting. In most regions with higher weekly acute error rates, there is a general tendency towards increased reliability in the last years. These regions may benefit from modelling, based on the second half of the 2003-2013 period, rather than on the whole period.

Hepatitis A monthly reporting is way more reliable, both at the regional and at the national level.

While taking the problems with reliability into account, correlation analysis still demonstrates good linear relationships between weekly acute case numbers and monthly hepatitis A case numbers. This means that further regression analysis and modelling can provide a successful mechanism to estimate hepatitis A numbers from acute hepatitis data with relatively good confidence.

CONCLUSION

The strong correlation between acute hepatitis and hepatitis A annual incidence indicates that acute hepatitis cases may be useful in estimating hepatitis A cases at the national level. This is further supported by the relatively good reliability of reporting, and the strong correlation between weekly acute hepatitis reports and monthly hepatitis A reports at the national level. As the regression model, facilitating ongoing estimation of hepatitis A incidence, will be based on weekly acute hepatitis reports and monthly hepatitis A reports, this analysis demonstrates that modelling will be strong and successful at the national level.

Lower reporting quality with regard to acute hepatitis in some regions over the last 11 years will affect the quality of further modelling attempts. Still, most of the regions with higher hepatitis A incidence rates, which tend to have more serious hepatitis A outbreaks, performed well on the reporting quality assessment. These are the regions which will benefit most from further modelling attempts.

Overall, the weekly acute hepatitis and monthly hepatitis A data are suitable for modelling, aimed at ongoing monitoring and alert threshold determination of hepatitis A incidence – important tools to support the timely reaction of the public health system in preventing further spread of the disease.

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STRIVING FOR BETTER COMMUNICATION WITH UNDERSERVED COMMUNITIES IN BULGARIA - A STEP TOWARDS IMPROVING IMMUNISATION COVERAGE

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ABSTRACT

With regard to the immunisation uptake, Roma communities in Bulgaria are still underserved. Evidence of the inadequate immunisation coverage in this population is the large measles outbreak occurred in the country in 2009-2011 which affected predominantly Roma people who were about 89% from all measles cases. The international project "Let's talk about protection" was developed with the aim to identify a proper way for communication with parents, especially from so called "hard-to-reach" groups, to motivate them to vaccinate their children and thus to increase the immunisation uptake. It started in 2012 and finished in 2014. Experts from the National Centre of Infectious and Parasitic Disease in collaboration with specialists from other institutions (European Centre for Disease Prevention and Control, World Health Communication Associates and Bulgarian Association "National Network of Health Mediators") adapted a Practical Guide, created and issued a Flipbook on vaccines and immunisations with focus on the communication between health care providers and parents. As a result of the project, it is expected that there will be increased awareness among parents with regard to benefits from timely vaccination of their children.

Key words: *Immunisation coverage, underserved groups, vaccine-preventable diseases, measles*

INTRODUCTION

During the years, the national immunisation coverage with first and second dose of measles-mumps-rubella (MMR) vaccine was not satisfactory. At regional level the coverage was even lower, especially amongst the Roma population (1, 2, 3).

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The most serious evidence of the inadequate coverage in this population is the large measles outbreak occurred in the country in 2009-2011 when the disease affected predominantly the Roma minorities.

The lower vaccination uptake in so called "hard-to-reach" groups is a result of a number of reasons. And maybe the crucial one is that there is not enough proper communication between health care providers (HCPs) and this particular population.

AIM

The aim of the international collaborative project "Let's talk about protection" was identification of a proper way for a better communication between HCPs and parents, especially from "hard-to-reach" groups, with respect to motivating them to get their children vaccinated against measles and other serious diseases as a mean of protecting their health.

MATERIALS AND METHODS

Representing the National Centre of Infectious and Parasitic Diseases as part of the team of the collaborative project "Let's talk about protection", 2012-2014 (in co-operation with European Centre for Disease Prevention and Control, World Health Communication Associates and Bulgarian Association "National Network of Health Mediators"), we adapted and implemented a Practical guide about vaccines and immunisations for HCPs - general practitioners (GPs), pediatricians, physicians, infectious diseases specialists, school nurses. A Flipbook addressed to the GPs and Roma health mediators (RHMs) with focus on how to communicate properly with hard-to-reach groups when explaining to them the benefits of vaccination, was also created and issued.

RESULTS

In the Guide are presented key messages of different society groups (parents, "hard-to-reach" groups, experts on immunisations, etc.) and addressed to HCPs. The parents' advices imply their willingness to enrich their awareness of the risks and benefits of vaccinations and also concerns on the possible adverse event following immunisation. Roma people advise on more respect and acquaintance with their traditions and way of life, so that the communication will become easier. Experts on immunisations share their experience and say that the continuous education of HCPs is a very important issue because the level of vaccination coverage is very much depending on the level of knowledge of health care workers.

Scientific information about vaccine preventable diseases (VPDs) in Bulgaria over the years is also presented in the Guide and in the Flipbook. Thus, the health care providers could obtain additional facts on the prevalence and severity of the various VPDs in the country. For example, they could enrich their knowledge about the burden of some VPDs in Bulgaria in pre- and post-vaccination era (Tables 1 and 2).

Table 1. Burden of some VPDs in Bulgaria in pre- and post-vaccination era and in 2011

Disease	Disease burden (Average number of deaths related to some VPDs)		
	Before the introduction of the vaccine	After the introduction of the vaccine	2011
Diphtheria	365	3	0 no death cases since 1994
Tetanus	36	12	1
Pertussis	207	6	0
Poliomyelitis	20	0	0 no death cases since 1994

Table 2. Bulgarian data on VPDs' cases reported in pre-vaccination period and in 2011

Disease	Pre-vaccine era (Estimated Annual Morbidity)	Cases reported in 2011	Rate of decrease
Diphtheria	3659	0	100%
H. influenzae	16	0	100%
Hepatitis B (acute)	2708	344	87%
Measles	17262	157	99%
Mumps	17034	139	99%
Pertussis	6062	46	99%
Poliomyelitis	219	0	100%
Rubella	10813	41	100%
Smallpox	eradicated since 1927†	0	100%
Tetanus	288	0	100%

†Bulgaria is one of the first countries in the world where smallpox has been eradicated

Table 3. National immunisation coverage (%) with MMR1 and MMR2 in Bulgaria, 2001-2013

Year	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
MMR1	90,1	92,1	95,5	94,7	96,2	95,7	96,0	95,9	96,1	96,5	94,5	93,7	95,1
MMR2	68,8	79,2	89,4	90,8	92,4	93,3	94,0	94,3	92,8	95,7	93,9	94,0	93,5

In the Flipbook an extensive research on the incidence of some serious infectious diseases and how the vaccination affects the diseases' spread is graphically presented. The health care professionals can use these data when visually explain the benefits of vaccination. Examples of the impact of immunisation against measles, diphtheria, hepatitis B (Figure 1, 2, 3), etc. are presented. Useful information for the relevant vaccines and immunisation schemes are also included in the printed materials.

DISCUSSION

Despite the reported high national immunisation coverage with MMR1 and MMR2 (Table 3), a nationwide measles outbreak occurred in 2009–2011. During the outbreak a total of 24365 cases (incidence: 322 per 100,000) and 24 deaths are reported (mortality: 0.32 per 100,000). Most cases (73%) are children under 15 years of age. Only 5% of the total cases are with complete immunisation status (2 doses of measles-mumps-rubella vaccine (MMR) received). The remaining cases are either not vaccinated (22%) or not fully vaccinated (25%), or they are of unknown vaccination status (48%).

On average 89.6% of all infected persons are Roma, which clearly shows that the majority of the Roma population was still susceptible to measles (3).

The insufficient vaccination coverage of underserved Roma communities is a challenge not only for Bulgaria (1, 2) but also for most of the EU member states (4, 5, 6). One of the key messages arising from this outbreak is that the approach to a better control of VPDs should be focused on improvement of the routine immunisation activities amongst Roma communities in the country (7). This could be achieved only by better communication with health care providers and the supportive partnership of the Roma health mediators. A step towards this direction is the realised project.

As a result of the project, it is expected that there will be increased awareness among parents with regard to benefits from timely vaccination of their children.

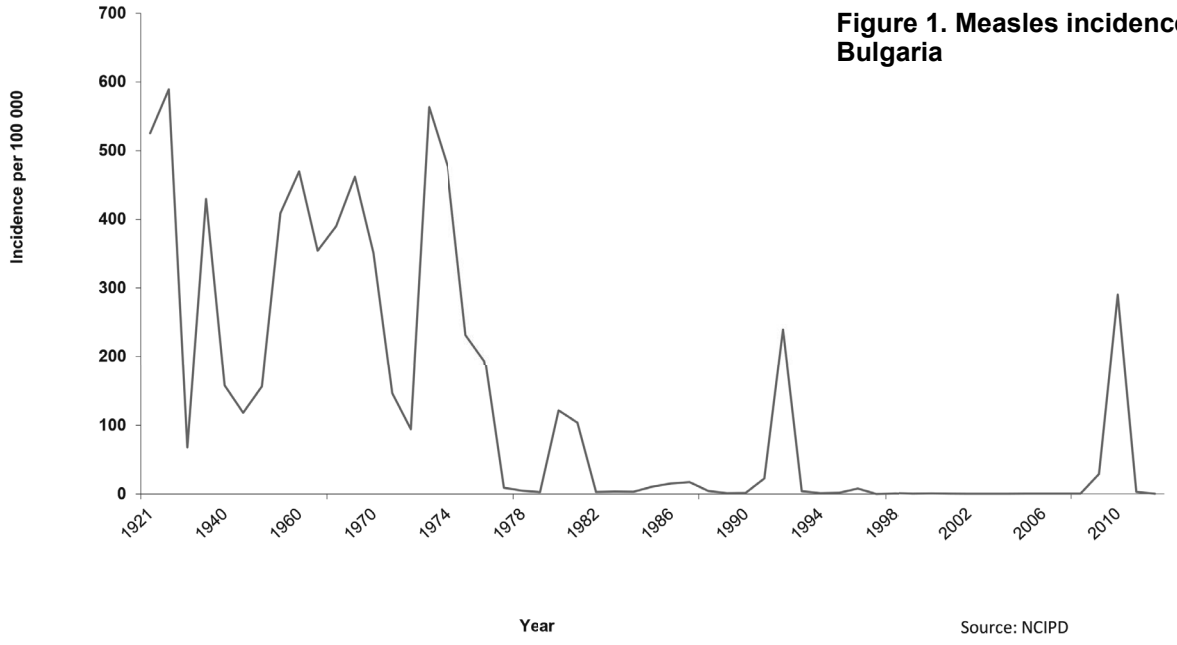

Figure 1. Measles incidence in Bulgaria

Source: NCIPD

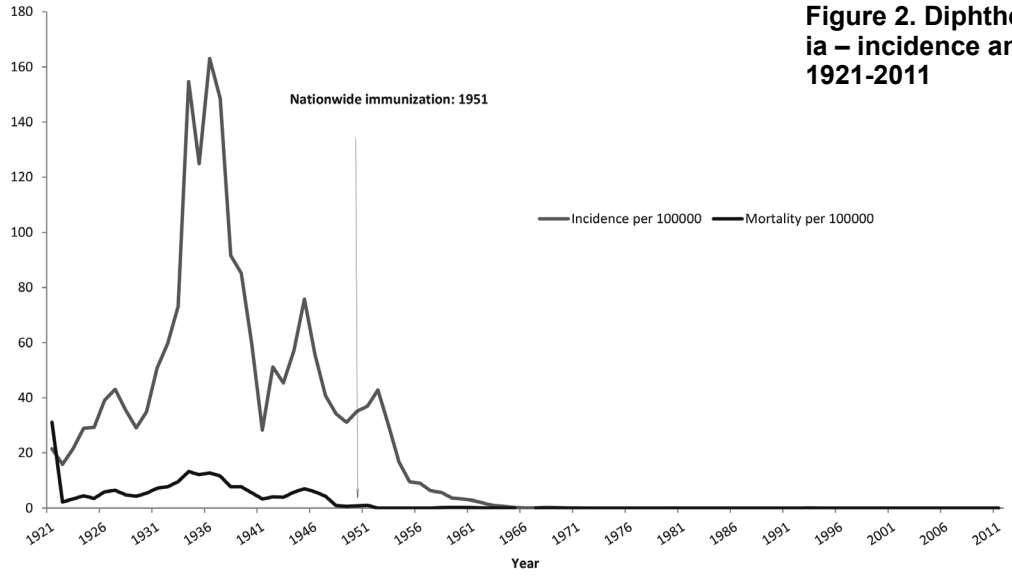

Figure 2. Diphtheria in Bulgaria – incidence and mortality, 1921-2011

Source: NCIPD

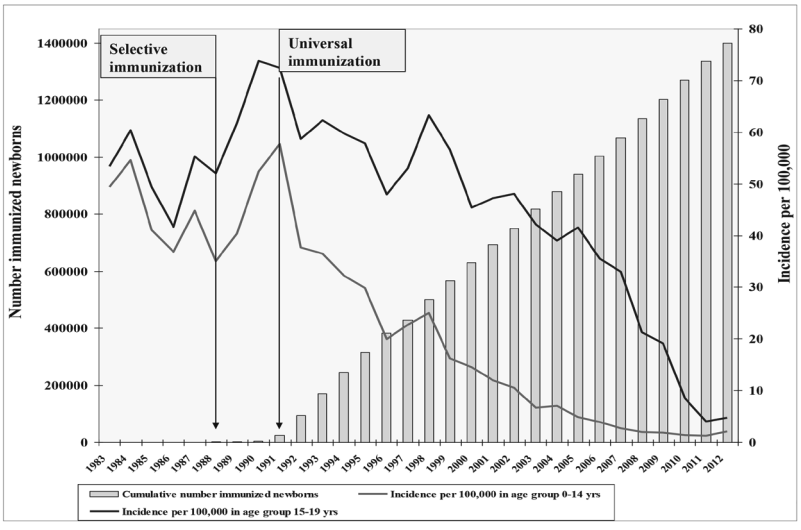

Figure 3. Cumulative number of immunised with HBV vaccine newborns and Hepatitis B incidence in children 0-14 and 15-9 years of age in Bulgaria in 2012

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