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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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ACKNOWLEDGEMENTS

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ROUTINE LABORATORY DETECTION OF *KLEBSIELLA PNEUMONIAE* CARBAPENEMASE-PRODUCING *ENTEROBACTERIACEAE*

S. Sabtcheva[1], B. Todorova[1], I. N. Ivanov[2], E. Dobreva[2], K. Ivanova[2], V. Dobrinov[2], T. Kantardjiev[2]

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ABSTRACT

The prompt and accurate detection of carbapenemase-producing *Enterobacteriaceae* is essential for patient care and infection control procedures. That is why, the introduction of standardised method for routine carbapenemase detection in clinical diagnostic laboratories seems to be mandatory. According to EUCAST methodology carbapenemase inhibition tests with boronic acid derivatives provide a reliable phenotypic confirmation for class A enzymes. We evaluated the performance of a combined disc test, the KPC&MBL&OXA-48 disc kit, for detection of *Klebsiella pneumoniae* carbapenemase (KPC) production in clinical *K. pneumoniae* strains recovered at Bulgarian hospitals. Our results indicated that the KPC&MBL&OXA-48 combined disc test is rapid, easy to perform, simple to interpret, and cost-effective method for routine laboratory detection of KPC production with 100% sensitivity and specificity.

Keywords: *Enterobacteriaceae*, *Klebsiella pneumoniae* carbapenemase, detection

INTRODUCTION

Carbapenem resistance in *Enterobacteriaceae* can arise by production of carbapenemases belonging to Ambler class A, B and D or production of AmpC cephalosporinase and extended-spectrum β-lactamase (ESBL) combined with decreased outer membrane permeability due to loss or alteration of porins. Carbapenemase genes are often associated with additional antibiotic resistance determinants, limiting therapeutic options and are mainly carried on transferable plasmids, facilitating their spread (1). Therefore, it is crucial for patient care and infection control procedures that any routine diagnostic laboratory could perform rapid and reliable detection of carbapenemase-producing pathogens. Recently, phenotypic confirmation methods based on the specific inhibition of class A and B carbapenemases have been described (2) and subsequently, standardised combined disc tests have been developed commercially.

In this study, we evaluated the performance of a commercially available KPC&MBL&OXA-48 combined disc kit for presumptive identification of class A carbapenemases in meropenem non-susceptible *Klebsiella pneumoniae* isolates.

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MATERIAL AND METHODS

Thirty two clinical isolates of *K. pneumoniae* non-susceptible to meropenem (disc diffusion zone diameter ≤ 21 mm) were studied. The tested strains included a panel of seventeen *K. pneumoniae*, which were sent to the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance and confirmed to harbour the bla_{KPC} genotype (3). No additional carbapenemase genes were detected. Furthermore, fifteen meropenem non-susceptible and carbapenemase-non-producing *K. pneumoniae* from the laboratory collection of the cancer hospital were also included. The lack of carbapenemase activity in these isolates was confirmed biochemically by the Carba NP test (4). Susceptibility to β-lactams was determined by disc diffusion method on Mueller-Hinton II agar according to EUCAST (the European Committee on Antimicrobial Susceptibility Testing) recommendations with discs supplied by Becton Dickinson (Sparks, MD). The results were interpreted in accordance with EUCAST clinical breakpoints (5). *Escherichia coli* ATCC 25922 was used as antibiotic-susceptible control. Species identification was carried out by using GNI cards on the VITEK 2 system (bioMerieux Vitek Inc., Hazelwood, MO).

Phenotypic detection and confirmation of *Klebsiella pneumoniae* carbapenemase (KPC) production was performed with the KPC&MBL&OXA-48 combined disc kit (Liofilchem, Roseto degli Abruzzi, Italy) following manufacturer's instructions. Briefly, Mueller-Hinton II agar plate was inoculated with overnight culture suspension of the test organism equal to 0.5 McFarland standard. Disc MRP (meropenem 10 μg), disc MR+BO (meropenem 10 μg and phenylboronic acid as a KPC inhibitor), disc MR+CL (meropenem 10 μg and cloxacillin as an AmpC inhibitor), disc MR+DP (meropenem 10 μg and dipicolinic acid as a MBL inhibitor) and disc TMO (temocillin 30 μg) were placed on inoculated agar plate. Following incubation at 36°C for 18–24 hours, the differences between the zones of inhibition of the meropenem discs compared to the meropenem discs combined with inhibitors were determined in mm. The test was considered positive for KPC production when the diameter of the growth-inhibitory zone around the MR+BO disc was ≥4 mm larger than that around the meropenem-alone disc. Moreover, a zone difference of both ≥4 mm for disc MR+BO and ≥5 mm for disc MR+CL compared to disc meropenem-alone was inter-

preted as positive for AmpC production with porin loss. Finally, the concomitant absence of synergy with any inhibitors and zone diameter ≥11 mm with temocillin disc was interpreted as positive for ESBL production with porin loss. The discs for MBL detection were not analyzed, since the study was designed to evaluate the detection of KPC production. Phenotypic confirmation of ESBL and AmpC production were conducted following EUCAST guidelines for ESBL- and acquired AmpC β-lactamase-producing *Enterobacteriaceae* with the ESBL+AmpC screen disc kit (Liofilchem, Roseto degli Abruzzi, Italy) as recommended by the manufacturer.

RESULTS

Among the *K. pneumoniae* harbouring $bla_{KPC,}$ 94% (16/17) were resistant to meropenem and imipenem. These isolates were also ESBL producers. The seventeenth isolate was of intermediate susceptibility to both carbapenems and did not co-produce neither ESBL nor AmpC. All isolates were resistant to piperacillin/tazobactam (zone diameter 6-10 mm) and showed zone diameter of temocillin above 11 mm (13-23 mm). Among the fifteen carbapenemase non-producers, 40% (6/15) were resistant to meropenem while the remaining nine isolates showed intermediate susceptibility. All carbapenemase non-producers were susceptible to imipenem and were ESBL-positives.

Performance results for the combined disc test with MR-BO were easy to interpret and correctly classified all KPC producers to Ambler class A of enzymes, showing significant increase of the growth-inhibitory zone around the MR+BO discs compared to that around the meropenem-alone disc (Fig.1). At the same time, no synergy with any of the inhibitors was observed among the fifteen carbapenemase non-producers and the zone diameter of temocillin was above 11 mm for all of them. These results together with the susceptibility to imipenem incriminated ESBL plus porin loss as the probable cause of meropenem non-susceptibility in carbapenemase non-producers (Fig.2). Overall, the MR-BO combined disc test appeared to be accurate method for KPC detection with 100% sensitivity and specificity.

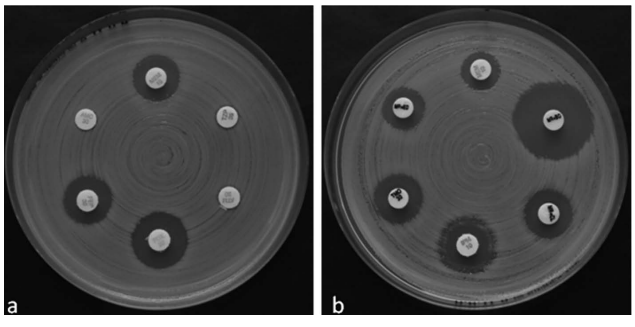

Figure 1. Antibiogram (a) and combined disc test (b) for KPC-positive *Klebsiella pneumoniae* PR2791. (a) Clockwise: MEM, meropenem; TZP, piperacillin/tazobactam; ATM, aztreonam; FOX, cefoxitin; FEP, cefepime; AMC, amoxicillin/clavulanic acid. (b) Clockwise: MEM, meropenem; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin; IPM, imipenem; TMO, temocillin; MR+DP, meropenem with dipicolinic acid. Resistance or reduced susceptibility to carbapenems and inhibition of carbapenemase activity by phenylboronic acid indicated the presence of a class A carbapenemase.

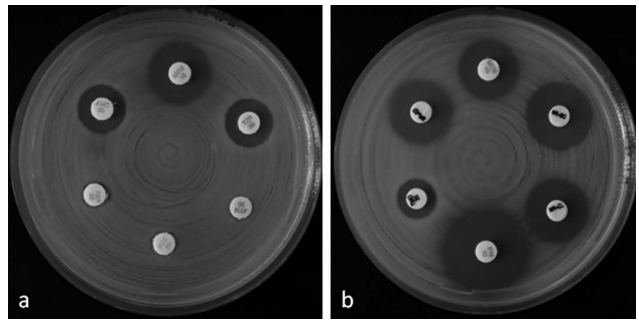

Figure 2. Antibiogram (a) and combined disc test (b) for KPC-negative *Klebsiella pneumoniae* 5032. (a) Clockwise: MEM, meropenem; TZP, piperacillin/tazobactam; ATM, aztreonam; FOX, cefoxitin; FEP, cefepime; AMC, amoxicillin/clavulanic acid. (b) Clockwise: MEM, meropenem; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin; IPM, imipenem; TMO, temocillin; MR+DP, meropenem with dipicolinic acid. Resistance or reduced susceptibility to meropenem together with concomitant absence of synergy with any of the inhibitors and zone diameter of temocillin ≥ 11 mm suggested the presence of ESBL with porin loss in *K. pneumoniae* 5032.

DISCUSSION

The prompt and accurate detection of carbapenemase-producing *Enterobacteriaceae* (CPE) is essential for patient care and infection control procedures. That is why, the introduction of standardised, reliable, cost-effective and easy to perform method for routine carbapenemase detection in clinical diagnostic laboratories seems to be mandatory. In our previous studies, it has been shown that concomitant resistance to penicillin/inhibitor combinations and temocillin together with reduced susceptibility to carbapenems but susceptibility to oxyimino-cephalosporins could be a sensitive screening tool for difficult-to-detect OXA-48-positive/ESBL-negative *Enterobacteriaceae* (6, 7). In the present study of clinical *K. pneumoniae* isolates recovered at Bulgarian hospitals, we evaluated the performance of the commercially available KPC&MBL&OXA-48 combined disc kit for presumptive identification of class A carbapenemases. Consistent with previous works utilising meropenem as an indicator antibiotic and its combination with boronic acid derivatives for the detection of KPCs (8, 9), our results showed that the MR-BO combined disc test ensured accurate KPC detection among the studied collection. Although this study may be limited by the number of strains tested and the fact that the majority of KPC isolates were carbapenem resistant, our results indicated that KPC&MBL&OXA-48 combined disc method is rapid, easy to perform, simple to interpret and highly sensitive for the discrimination of ESBL/AmpC with porin loss from CPE and the differentiation of class A enzymes.

In conclusion, the KPC&MBL&OXA-48 combined disc kit was a sensitive and specific phenotypic screening method for the detection of KPC-producing isolates. Further evaluation of this method in a larger collection of more diverse isolates appears suitable. Accurate and rapid identification of carbapenem resistance mechanisms in the clinical laboratory remains important for infection control procedures and initiation

of adequate antibiotic therapy against these multi-drug-resistant pathogens.

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MDR-TB WITH ADDITIONAL FLUOROQUINOLONE RESISTANCE IN BULGARIA

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ABSTRACT

The aim of this retrospective study was to examine the multidrug-resistant tuberculosis with additional fluoroquinolone resistance in Bulgaria, its distribution in the country and further evolution. The study covered eight-year period of time from 2007 to 2014.

Clinical isolates from 32 patients were confirmed at the NRL TB, NCIPD, as MDR-TB with additional resistance to ofloxacin. Conventional drug susceptibility testing was performed by BACTEC MGIT 960 System to first- and second-line drugs using testing concentrations of 0.1 µg/ml for isoniazid, 1.0 µg/ml for rifampicin, and 2.0 µg/ml for ofloxacin. Mutations in the relevant genes were detected by GenoType® MTBDR*plus*/*sl*. Genotyping was performed by 24 loci MIRU-VNTR and spacer olygonucleotide typing (spoligotyping).

The most common mutation in the *rpo*B gene was S531L (96.15%). Isoniazid resistance was attributed to the C15T mutation in the regulatory region of the *inh*A gene in 80.76% of cases and the A90V mutation in the *gyr*A gene was found in 70% of cases.

The clustering rate for MIRU-VNTR was 0.73, and for spoligotyping – 0.78.

The highest prevalence of fluoroquinolone-resistant MDR-TB cases was in Dobrich District.

Further evolution of the resistance to XDR-TB was identified in four cases.

Timely detection of fluoroquinolone resistance in MDR-TB is essential for the treatment regimen and its effectiveness.

LIST OF ABREVIATIONS

DST Drug susceptibly testing
FLD First-line drug
FQ-R Fluoroquinolone-resistant
INH Isoniazid
LPA Line probe assay
MDR-TB Multidrug-resistant tuberculosis
MGIT Mycobacteria Growth Indicator Tube
MIRU Mycobacterium Interspersed Repetitive Units
NCIPD National Centre of Infectious and Parasitic Diseases
NRL TB National Reference Laboratory of Tuberculosis
OFL Ofloxacin
RMP Rifampicin
SIT Spoligo International Type
SLD Second-line drug
VNTR Variable Number of Tandem Repeats
XDR-TB Extensively drug-resistant tuberculosis

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INTRODUCTION

Fluoroquinolones (ofloxacin, moxifloxacin, levofloxacin, gatifloxacin) are among the most effective second-line drugs used in the treatment of MDR-TB infections (1, 2) and timely detection of resistance is essential for the treatment regimen and its effectiveness (3).

Fluoroquinolone resistance found in MDR-TB lies in the middle of definitions for MDR and XDR tuberculosis. Many authors define this condition as pre-XDR tuberculosis (4, 5, 6).

The target of fluoroquinolones is the DNA gyrase, a type II topoisomerase with A and B subunits, encoded by *gyr*A and *gyr*B genes. Resistance to FQ is attributed to mutations occurring mainly in the *gyr*A gene, in the quinolone resistance-determining region (codons from 74 to 113). Mutations in the *gyr*B gene are less common and usually in addition to *gyr*A mutations (7).

The aim of this retrospective study was to examine the MDR-TB with additional fluoroquinolone resistance in Bulgaria, its distribution in the country and further progression. The study covered eight-year period of time from 2007 to 2014.

MATERIALS AND METHODS

For the period 2007-2014 in the NRL TB, NCIPD, 253 MDR-TB patients were tested with DST for SLDs. Among them 32 cases were found resistant to ofloxacin and susceptible to the injectable drugs (amikacin, kanamycin, capreomycin). In the study 87.5% (n=28) of the patients were males, 12.5% (n=4) were females and the age range was between 22 and 74 years. All cases were mapped according to the official resident registration. Every patient was represented by a single strain.

Identification of *M.tuberculosis* complex isolates was done by BD MGIT TBc Identification Test, a chromatographic test for detection of MPT64 in liquid cultures.

The conventional DST was performed by BACTEC MGIT 960 System to FLDs and SLDs as follows: INH – 0.1 µg/ml; RMP – 1.0 µg/ml; OFL – 2.0 µg/ml.

The LPA GenoType® MTBDR*plus* and GenoType® MTBDR*sl* were also performed in order to detect mutations in the target genes.

Genotyping methods included 24 loci MIRU-VNTR and spacer olygonucleotide typing (spoligotyping) (8). The MIRU-VNTR website (www.MIRU-VNTRplus.org) was used to compare the strains and determine the clusters. The 24 VNTR loci set were represented in ascending order. A cluster was defined as isolates from two or more patients that have the same spoligotype or VNTR pattern, or both.

RESULTS AND DISCUSSION

The molecular tests GenoType® MTBDR*plus* and GenoType® MTBDR*sl* can detect *M.tuberculosis* complex from acid fast bacilli positive sputa or culture and find the most frequent mutations associated with resistance to the main anti-TB drugs. Out of 32 cases 26 were tested by GenoType® MTBDR*plus*. The results showed more frequent occurrence of the S531L mutation in *rpo*B (96.15%) compared to the Bulgarian MDR-TB isolates as a whole (88.06%) (9).

In the study C15T in the *inh*A gene was far more frequent than the S315T1 mutation in *kat*G, 80.77% and 19.23%, respectively, while in 2012 the occurrence of C15T in the MDR-TB was 43.28% (9).

Summarised results from molecular testing are shown in Table 1 and Table 2.

Table 1*:* GenoType® MTBDR*plus* results for rifampicin and isoniazid resistance of FQ-R MDR-TB clinical isolates, 2007-2014.

n (%)	Nucleotide/ aminoacid substitution *rpo*B	Nucleotide/ aminoacid substitution *kat*G	Nucleotide/ aminoacid substitution *inh*A	MTBDR*plus* interpretation	BACTEC MGIT 960 System
21 (80.77%)	**S531L** ΔWT8, MUT3	-	**C15T** ΔWT1, MUT1	MDR	MDR
3 (11.55%)	**S531L** ΔWT8, MUT3	**S315T1** ΔWT1, MUT1	-	MDR	MDR
1 (3.84%)	**S531L** ΔWT8, MUT3	* ΔWT1	-	MDR	MDR
1 (3.84%)	* ΔWT7	* ΔWT1	-	MDR	MDR

* *To specify the nucleotide/aminoacid substitution in the corresponding codons, sequencing of the gene is required*

Δ – deletion; WT – wild type; MUT – mutation; RMP – rifampicin; INH – isoniazid

GenoType® MTBDR*sl* was performed for 20 of the cases. The most common *gyr*A mutation was A90V (GCG → GTG) found in 70 % of cases (Table 2). The rest of the strains harboured variable mutations.

Table 2*:* GenoType® MTBDR*sl* results for the *gyr*A gene in FQ-R MDR-TB, 2007-2014.

n (%)	*gyr*A	Nucleotide/aminoacid substitution	MTBDR*sl* Interpretation	BACTEC DST result
14 (70%)	ΔWT2, MUT1	A90V	FQ – R	OFL - R
3 (15%)	ΔWT3, MUT3A	D94A	FQ – R	OFL - R
1 (5%)	ΔWT3, MUT3C	D94G	FQ – R	OFL - R
1 (5%)	ΔWT2, MUT2	S91P	FQ – R	OFL - R
1 (5%)	ΔWT1	*	FQ – R	OFL – R

* *To specify the nucleotide/aminoacid substitution in the corresponding codons, sequencing of the gene is required*

Δ – deletion; WT – wild type; MUT – mutation; FQ – fluoroquinolones; OFL – ofloxacin; R - resistant; S - susceptible

Spolygotyping was performed for 20 strains (Fig. 1) and MIRU-VNTR for 26 strains (Fig. 2).

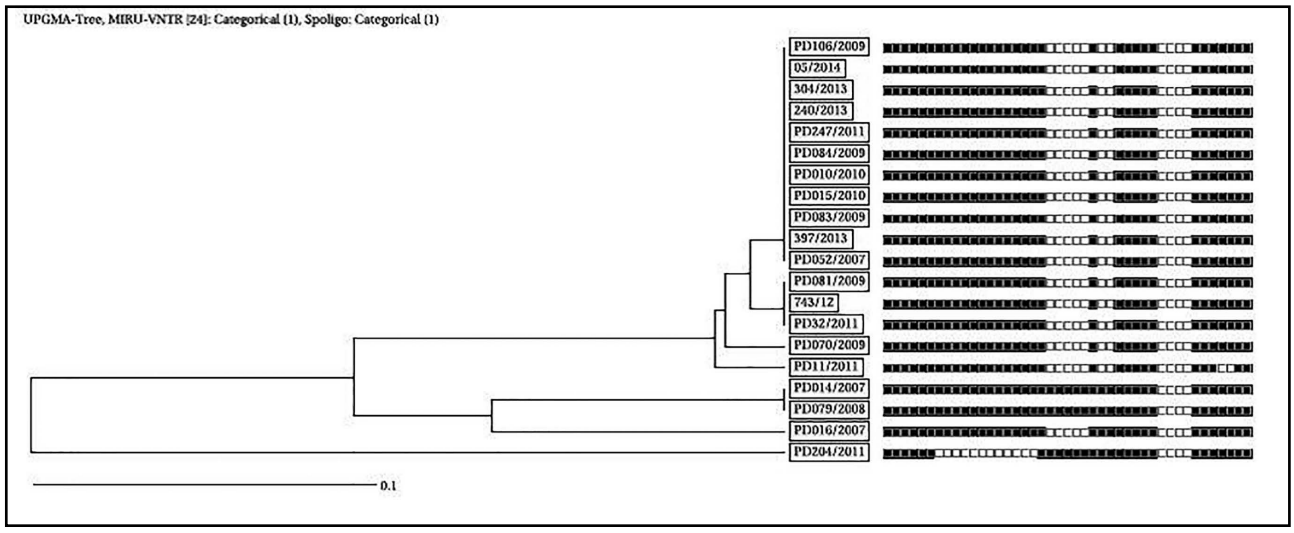

Figure 1: Spoligotyping results, FQ-R MDR-TB, 2007-2014.

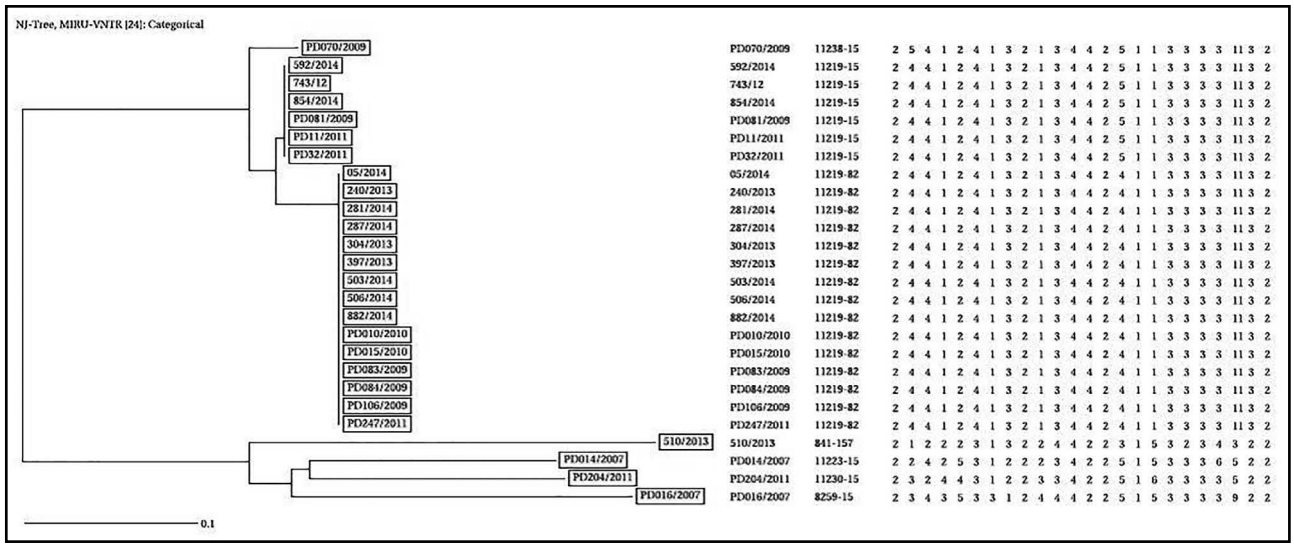

Figure 2: MIRU-VNTR 24 loci typing results, FQ-R MDR-TB, 2007-2014. The 24 VNTR loci set were represented in ascending order.

The clustering rate for MIRU-VNTR was 0.73, and for spoligotyping – 0.78. The SIT 41 was represented by two big clusters (n=15 and n=7), differing only in VNTR 2531 (MIRU23) allelic copy number (4 and 5). The Beijing lineage was not found among the typed FQ-R strains although it was detected among the MDR-TB in Bulgaria (10). Comparable results were obtained in a recent seven-year study on MDR-TB strains from all over the country where SIT41 (TUR lineage) had prevalence of 47.5% (11).

The high clustering rate is either because of recent transmission or because of the dominance of highly conservative ancestral strain in the population.

Distribution of FQ-R MDR-TB in Bulgaria by districts is shown in Fig. 3 and each spot represents a single case. We observed a trend of higher occurrence of the resistant forms in Northern Bulgaria. The highest detected level of FQ-R MDR-TB was in Dobrich District with n = 8, two of which were from the State Psychiatric Hospital.

Some of the high burden MDR-TB districts such as Montana, Vidin, and Sofia city were not represented by fluoroquinolone-resistant cases.

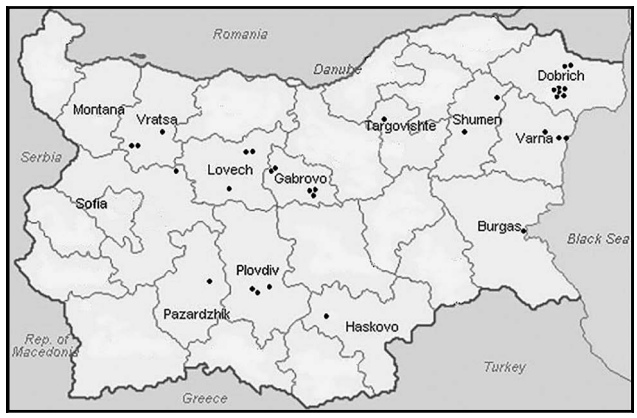

Figure 3: Distribution of the FQ-R MDR-TB cases by place of residence, 2007 – 2014.

The FQ-R MDR-TB appeared to be in a sustainable condition. Further evolution of the resistance to XDR–TB was found in four patients during their second-line treatment. The rest of the patients (n=28) had no detected resistance to amikacin, kanamycin and/or ca-

preomycin until their treatment outcome occurred.

CONCLUSIONS

The prevalence of FQ-R MDR-TB was mainly in the Northern part of Bulgaria. Clustering rate for MIRU-VNTR was 0.73, and for spoligotyping – 0.78. There were two clusters formed with distinguishable mutation pattern: S531L in the *rpo*B gene, C15T in the *inh*A gene, and A90V in the *gyr*A gene. Out of 32 cases resistance progressed to XDR-TB in four patients.

ACKOWLEDGEMENTS

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EVALUATION OF THE CARBA NP TEST FOR DETECTION OF CARBAPENEM-PRODUCING *ENTEROBACTERIACEAE*: PRELIMINARY RESULTS

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ABSTRACT
Rapid and accurate detection of carbapenemase-producing *Enterobacteriaceae* (CPE) is of critical important for patient care and for preventing their further dissemination. Although molecular detection of carbapenemase genes is considered the gold standard, no commercial PCR assay has the ability to detect all CPE since the existing phenotypes mutate and new ones emerge. Recently the biochemical Carba NP test has been developed for rapid identification of carbapenemase production in *Enterobacteriaceae*. The objective of this study was to evaluate the performance of an updated version of the Carba NP procedure for detection of carbapenemase production in *Enterobacteriaceae* isolated in Bulgaria. Among the 51 isolates tested, 36 produced carbapenemase according to PCR and sequencing. The Carba NP test detected all carbapenemase producers of KPC-type, NDM-type and VIM-type except the sixVIM-1-producing *Proteus mirabillis* (17% of all carbapenemase-producing *Enterobacteriaceae* in this study). The carbapenemase negative isolates were correctly identified as negative. The Carba NP test had an overall 83% sensitivity and 100% specificity. The false negative test results with VIM-1-positive *P. mirabilis* need further investigations.
Keywords: Carba NP test, *Enterobacteriaceae*

INTRODUCTION

Resistance to carbapenems in *Enterobacteriaceae* involves multiple mechanisms including production of carbapenemases (e.g. KPC, NDM, VIM, IMP and OXA-48-like), alterations of outer membrane permeability due to loss of specific porins and up-regulation of efflux pumps combined with hyper production of certain types β-lactamases (e.g. AmpC) (1). It is critical to distinguish the carbapenem resistance due to carbapenemase production since genes encoding these enzymes are often associated with mobile genetic elements which facilitate their rapid spread and the number of carbapenemase-producing *Enterobacteriaceae* (CPE) continues to increase globally (2, 3). Rapid and accurate CPE detection is very important for patient care and it is the first step to limit dissemination in an environment with rising incidence of nosocomial infections associated with carbapenemase-producing organisms (4, 5). Although molecular detection of carbapenemase genes is considered the gold standard, no commercial PCR assay has the ability to detect all CPE since the existing phenotypes mutate and new ones emerge. Another limitation is the cost of the genetic assays. Phenotypic recognition of the carbapenemase producers is thus more reliable as screening tool and it is the main factor for determining appropriate therapeutic options and the implementation of infection control measures (5).
Recently the Carba NP test has been developed for rapid identification of carbapenemase production in *Enterobacteriaceae*. This phenotypic method is a biochemical test based on the detection of acidification by the change of the color of a pH indicator resulting from imipenem hydrolysis. The Carba NP was reported to be 100 % specific for carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas aeruginosa* (6). Although the details about this phenotypic method were published three years ago, there are several developments of the Carba NP procedure. The original Carba NP test is using phenol red solution as a pH indicator and a bacterial lysis buffer (B-PERII, Bacterial Protein Extraction Reagent, Thermo Scientific, Pierce). Another modification of the method is the Blue-Carba NP test using bacterial cultures without lysis step and the colonies are directly resuspended in a solution with 0.04 % bromtymol blue as pH indicator (7). For less than 2 hours these two modifications of the method allow carbapenemase detection. The Blue-Carba is even faster as the cell lysis step is omitted (7). The CarbAcineto NP test is a newly-designed modification that allows detection of carbapenemase production in *Acinetobacter* spp. Here the lysis buffer is replaced by NaCl 5 M and the bacterial inoculum is increased compared to the original Carba NP test. Unlike the original Carba NP that fails to detect class D carbapenemases in *Acinetobacter* spp., the CarbAcineto NP has been reported with sensitivity of 94.7% and specificity of 100% for all carbapenemases with the exception of some GES types (8). In order to apply a single protocol for rapid detection of all carbapenemases (KPC, MBL and OXA types) in *Enterobacteriaceae, Pseudomonas* and *Acinetobacter* spp., a modified Carba NP (MCNP) test has been described this year (9). In this method, results are obtained more quickly with cetyl trimethyl ammonium bromide (CTAB) 0.02% used as lysis buffer for *Enterobacteriaceae, Pseudomonas* and *Acinetobacter*. According to the published data this new method detected all carbapenemases with 100% sensitivity and 100% specificity (9). The advantages of the MCNP test are the detection of carbapenemases from Am-

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bler's Classes A, B, and D from different *Enterobacteriaceae, Pseudomonas* and *Acinetobacter* species by using single protocol as well as the short time to observe results, especially in the case of Class B carbapenemase-producing bacteria. In addition, the use of MCNP contributed for the identification of a new type Class A carbapenemase as cloning and sequencing experiments revealed. The producer of this carbapenemase, named FRI-1, was *Enterobacter cloacae* isolate, resistant to penicillins, narrow and broad-spectrum cephalosporins, aztreonam, and carbapenems but susceptible to expanded spectrum cephalosporins (10).

There are several steps in the Carba NP methods and each of them has important impact on obtaining the results: the lysis step, the quantity of the bacterial inoculum, the growth conditions, and the concentration of the antibiotic used in the solution. All of the Carba NP modifications affect some of these steps and they are still subject of experimental work.

In this study, we evaluated the performance of an updated version of the Carba NP procedure (7) for detection of carbapenemase production in *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains

A total of 51 carbapenem non-susceptible *Enterobacteriaceae* isolates recovered from clinical specimens and sent to the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance between 2011 and 2015 were investigated. All isolates were characterised for their carbapenemase genetic content by using multiplex Real-time PCR targeting bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{SME}, bla_{KPC}, bla_{SPM}, bla_{GES}, and bla_{OXA-48} as previously described (2).

Prerequisites and Procedure

Eppendorf tubes of 1.5 ml; Imecitin (Imipenem + cilastatin, Actavis group PCT); B-PERII, Bacterial Protein Extraction Reagent (Thermo Scientific, Pierce); Solution A (10 mM Zinc sulfate heptahydrate solution, 0.5% phenol red solution and 0.1 N sodium hydroxide solution were used for the procedures. We applied the protocol of the Carba NP test using bacterial colonies. This version of the method has several modifications that simplified the lysis step eliminating the need of centrifugation step and shortening the time of the test procedure. Improvements included the use of 1.5 ml Eppendorf tubes instead of 96-well microplates and reduced amount of the tested bacteria.

The Carba NP test was performed as follows. By using 1/3 to 1/4 of 10 µl calibrated loop the bacteria obtained from 24h cultures grown on Muller-Hinton agar at 37°C were resuspended in 100 µl 20 mM Tris-HCl lysis buffer (B-PERII, Bacterial Protein Extraction Reagent, Thermo Scientific, Pierce) in each of two 1.5 ml Eppendorf tubes. The samples were vortexed for one or two minutes to ensure that bacterial colonies are correctly resuspended and 100 µl of Solution A was added in the first Eppendorf tube used as negative control. In the second tube (the test tube) 100 µl Solution A and 6 mg/ml imipenem (extemporaneously prepared) were added. Afterward, the tubes were vortexed and then incubated at 37°C for a maximum of 2 hours. Carbapenemase activity was revealed when the color in the tube containing imipenem changed to yellow while the negative control tube remained red. If change in color was not observed in both tubes, the result was interpreted as negative. A change in color to yellow in the negative control and in the tube with the antibiotic was assumed as non interpretable result.

RESULTS

Thirty-six of the 51 carbapenem non-susceptible *Enterobacteriaceae* expressed carbapenemase genes. Of these, 24 were *Klebsiella pneumoniae* (17 bla_{K-PC}-positive and 7 bla_{NDM}-positive), 4 were bla_{NDM}-positive *Escherichia coli*, 6 were bla_{VIM}-positive *Proteus mirabillis* and 2 *Serratia marcescens* carried VIM carbapenemase gene. The 15 non-carbapenemase-producing *Enterobacteriaceae*, that were included as negative controls, harboured genes encoding CTX-M-, TEM-, and CMY-type beta-lactamases. Thirty of the 36 carbapenemase-positive strains gave positive results with the exception of the 6 *P.mirabilis* isolates possessing genes encoding VIM-type carbapenemase (Table 1). All of the non-carbapenemase producing strains gave negative result. The sensitivity of the Carba NP test for KPC- and NDM-producing *K.pneumoniae* and NDM-producing *E.coli* was 100%, while the overall sensitivity was 83%. No false positive results were detected in the carbapenemase non-producing isolates, thus the specificity of the method was 100% (Table 1). Positive results were observed at different times for the different carbapenemase types. Twelve out of 17 KPC producers gave positive result within 15 minutes and 5/17 within 30 minutes. Most of the NDM-producing isolates (8/11) showed positive results after15 minutes of incubation and the remaining 3/11 - after 30 minutes. Both VIM-producing *S.marcescens* gave a positive reaction for the presence of a carbapenemase within 30 minutes of incubation. None of the tested strains gave a non-interpretable result.

Table 1. Results of the Carba NP test performed on a collection of carbapenemase-producing and carbapenemase-non-producing *Enterobacteriaceae*

Species	n	Carbapenemases	Carba NP test	
			Negative	Positive
K. pneumoniae	17	KPC	0	17
	7	NDM-1	0	7
	7	Negative	7	0
E. coli	4	NDM-1	0	4
	3	Negative	3	0
P. mirabilis	6	VIM-1	6	0
M. morganii	2	Negative	2	0
S. marcescens	2	VIM	0	2
	3	Negative	3	0

Table 2. Time required for obtaining positive results for different carbapenemase producers

Carbapenemase class	Carbapenemase type	Species	n	15´	30´	60´	120´
Class A	KPC	*K. pneumoniae*	17	12	5		
Class B	NDM-1	*K. pneumoniae*	7	4	3		
		E.coli	4	4			
	VIM-1	*P. mirabilis*	6	0	0	0	0
	VIM	*S. marcescens*	2		2		

DISCUSSION

The study results confirmed that the Carba NP test has excellent sensitivity for KPC- and NDM-producing *Enterobacteriaceae,* as previously reported (5, 6). The method can be used successfully for recognition of carbapenemase activity in clinical isolates showing slight decreasing in susceptibility or resistance to carbapenems (imipenem, meropenem, ertapenem).

Weak expression of VIM-1-encoding genes or their chromosome location may explain the negative results that we observed in all *P.mirabilis* isolates. The lack of detection in these cases may be related to the growth conditions or incomplete lysis step. According to the authors of the updated version of the Carba NP test the zinc concentration in the medium is crucial for detection of VIM and NDM carbapenemases and this may have had an impact on our negative results regarding VIM-1-producing *P.mirabillis.* In our study, data was obtained by using bacterial isolates grown only on one type MH agar although the Carba NP test might be performed on blood agar and trypticase soy agar as recently demonstrated (5). Lower catalytic activity of VIM-1 can also play role in this case. The false negative results will be a subject of further investigations.

CONCLUSION

The Carba NP test is easy to perform and easy to interpret. It can be used in any laboratory, although difficulties may occur with the standardisation of the procedure because the antibiotic solution should be prepared in-house. The Carba NP test demonstrated to be highly sensitive for detection of CPE and the introduction of this relatively new technique in the routine laboratory practice will facilitate the recognition of the carbapenemase-producing *Enterobacteriaceae* and prevent their spread.

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IMPLEMENTATION OF THE MULTILOCUS SEQUENCE TYPING METHOD FOR TYPING OF CARBAPENEMASE-PRODUCING *KLEBSIELLA PNEUMONIAE* IN BULGARIA

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ABSTRACT
With the global increase of infections caused by multidrug resistant organisms, there is a growing need for high resolution and reliable typing method able to track those strains. Multilocus Sequence Typing (MLST) is an internationally recognised high-resolution typing method based on DNA sequencing of house-keeping genes providing data that are readily exportable between laboratories. In the recent years, multidrug high-risk MLST clones are considered one of the main vehicles for spreading antimicrobial resistance. These clones have acquired numerous adaptive determinants accompanied by plasmids encoding multidrug resistance and their tracking is vital for preventing further dissemination. In this pilot study, we report the implementation of the MLST technique for Bulgarian *Klebsiella pneumoniae* strains producing various types of carbapenemases. The results revealed that NDM-1 producers belonged to sequence type ST11, VIM-1 producer was classified as ST147, KPC-2 producers linked to both ST15 and ST258, while OXA-48 producer was determined as ST530. The detection of exclusively high-risk international clones such as ST11, ST15, ST147 and ST258 is a worrisome finding that needs further investigations.
Key words: *Klebsiella pneumoniae*, carbapenemase, MLST, Bulgaria

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INTRODUCTION

The Multilocus Sequence Typing (MLST) is a method principally based on DNA sequencing of several house-keeping genes that are required for the maintenance of basic cellular functions and are expressed in all strains of a species under normal and pathophysiological conditions. It is a high-resolution molecular typing method able to identify species and strains of pathogens (1). MLST has several advantages over the other typing methods such as MLVA, PFGE, AFLP: (i) the method uses sequence data and therefore can detect changes at bacterial DNA level that are not apparent by phenotypic approaches; (ii) it can be performed easily at any DNA sequencing facility and there is no need for specialised reagents; (iii) it does not require use of living bacteria or high-quality genomic DNA and can be performed on killed cell suspensions, thus avoiding all the difficulties associated with the transport and manipulation of pathogens or clinical samples; (iv) the data generated are fully and readily portable for exchange among laboratories and can be shared throughout the world via Internet. In addition, the Internet can also be used to disseminate MLST methods providing standardisation of approaches (2). Data processing and comparisons are performed within global online databases that encompass information of a large number of isolates.

In the recent years, multidrug high-risk MLST clones are considered one of the main vehicles for spreading antimicrobial resistance. These clones have acquired numerous adaptive determinants accompanied by plasmids encoding multidrug resistance and their tracking is vital for preventing further spread.

Klebsiella pneumoniae is a Gram-negative nosocomial pathogen responsible for an important proportion (4 to 8%) of the healthcare associated infections worldwide (3, 4). Besides its diverse repertoire of virulence factors, *K.pneumoniae* isolates are increasingly resistant to multiple antimicrobial agents including carbapenems, quinolones and aminoglycosides (5, 6,7). Most of the strains isolated in Bulgarian hospitals are multidrug resistant according to the National surveillance system BulStar (8). The typing of such strains is of great epidemiological importance for understanding their spread and preventing the dissemination of virulence and resistance determinants to other bacteria.

In this pilot study, we report the implementation of MLST technique for typing of Bulgarian *K.pneumoniae* strains producing various carbapenemases.

MATERIALS AND METHODS

Bacterial strains

A total of six *K.pneumoniae* strains producing various types of carbapenemases were investigated (Table 1). These strains were isolated from different Bulgarian hospitals and submitted to the National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance (NRL-CMAR). Carbapenemase encoding genes were determined by PCR and sequencing using previously published methods (9).

DNA extraction

DNA isolation was performed from *K.pneumoniae* culture grown on Mueller-Hinton agar by suspending one loopful to 150µl PrepMan™ Ultra reagent (Life technologies) and incubation at 100°C for 10 min. Af-

ter centrifugation of the samples at 13 300 rpm for 5 minutes, the supernatant was pipetted in a new tube. The DNA was diluted to approximately 25 ng/µl and stored at -20°C before further analyses.

PCR amplification of house-keeping genes for MLST

MLST is based on the determination of the nucleotide sequence of certain number of genes where stable, evolutionary meaningful mutations are manifested, the so called house-keeping genes. The current *K.pneumoniae* MLST scheme described by Diancourt et al. involves seven genes namely *rpoB, gapA, mdh, pgi, phoE, infB, and tonB* . PCR was performed on all isolates according to protocol 2 described at the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Amplicons obtained after the amplification reaction were verified on agarose gel electrophoresis, purified by PEG8000 precipitation, suspended in nuclease-free water and DNA adjusted to approximately 15 ng/µl. Sequencing reactions were performed with DTCS Quick Start Kit (Beckman Coulter, USA) using sequencing primers from Protocol 2 http://bigsdb.web.pasteur.fr/klebsiella/primers_used.html and following manufacturer's instructions. After removal of unincorporated dyes by Acetate-EDTA-Ethanol-glycogen, samples were suspended in 30µl SLS and sequenced on the GenomeLab GeXP Genetic Analysis System (Beckman Coulter) applying the LFR-a method. The trace analysis, manual editing (when necessary) and alignment were performed on Geneious v7.1.9 (Biomatters Ltd.) and sequence types (STs) were determined within *K.pneumoniae* MLST database. Phylogenetic analyses including determination of Clonal Complexes (CCs) and Clonal Groups (CGs) were established by software package goeBURST (10). A clonal complex is defined as a group of isolates that differ only at a single locus (Single Locus Variant, SLV) and is named after the putative ancestral ST. A Clonal Group is defined as the subset of STs in a certain CC containing the ancestral ST, one of its SLVs, and all of the next secondary SLVs in the branch (11).

RESULTS

In total five sequence types were discriminated among the six strains studied (Table 1). Each carbapenemase type appeared to be associated with different ST except for the KPC-2 producers linked to both ST15 and ST258.

ST11 and ST258 comprising NDM-1 and KPC-2 producers are evolutionary related and belonged to a single CC258 (Fig. 1). The other KPC-2-producer PR2761 belonged to CG15 and CC14 (Fig. 2A), while PR2874, a VIM-1-producer, was classified as ST147 within a separate CC147 (Fig. 2B). *K.pneumoniae* PR2899 producing OXA-48 carbapenemase was determined as ST530. However, PR2899 could not be associated to a clonal group. It is classified as the so-called singleton that is either evolutionarily separated from the other sequence types or currently a genetic relationship with other CG could not be established.

Table 1. MLST data for *K. pneumoniae* strains

Strain	Gene and Allele Number							ST	CG	CC	Carbapenemase type
	rpoB	*gapA*	*mdh*	*pgi*	*phoE*	*infB*	*tonB*				
PR2899	4	2	2	1	1	39	24	530	-	-	OXA-48
PR2682	1	3	1	1	1	3	4	11	11	258	NDM-1
PR2830	1	3	1	1	1	3	4	11	11	258	NDM-1
PR2761	1	1	1	1	1	1	1	15	15	14	KPC-2
PR2874	4	3	6	1	7	4	38	147	147	147	VIM-1
PR2671	1	3	1	1	1	3	79	258	258	258	KPC-2

Abbreviation: Sequence type, ST; Clonal group, CG; Clonal complex, CC

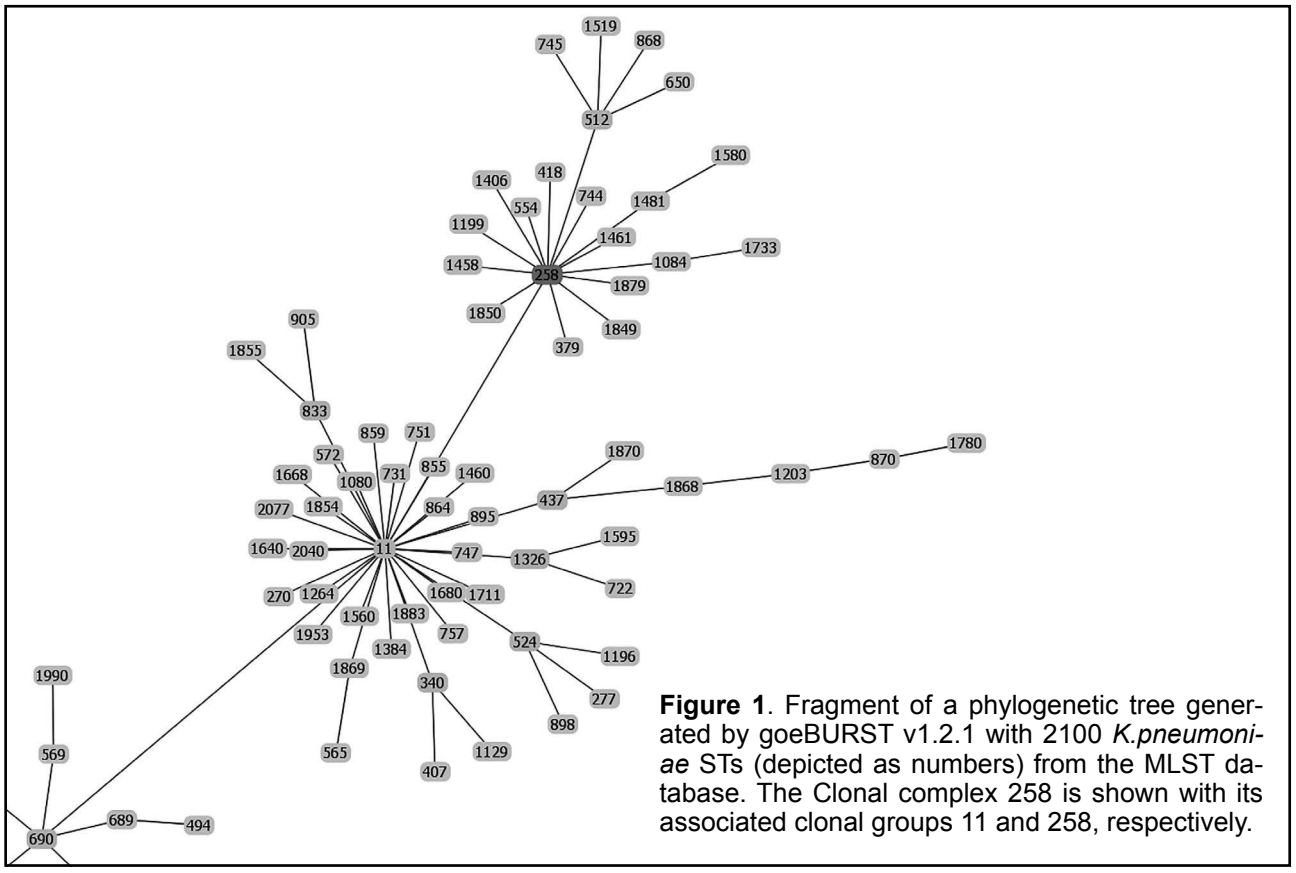

Figure 1. Fragment of a phylogenetic tree generated by goeBURST v1.2.1 with 2100 *K.pneumoniae* STs (depicted as numbers) from the MLST database. The Clonal complex 258 is shown with its associated clonal groups 11 and 258, respectively.

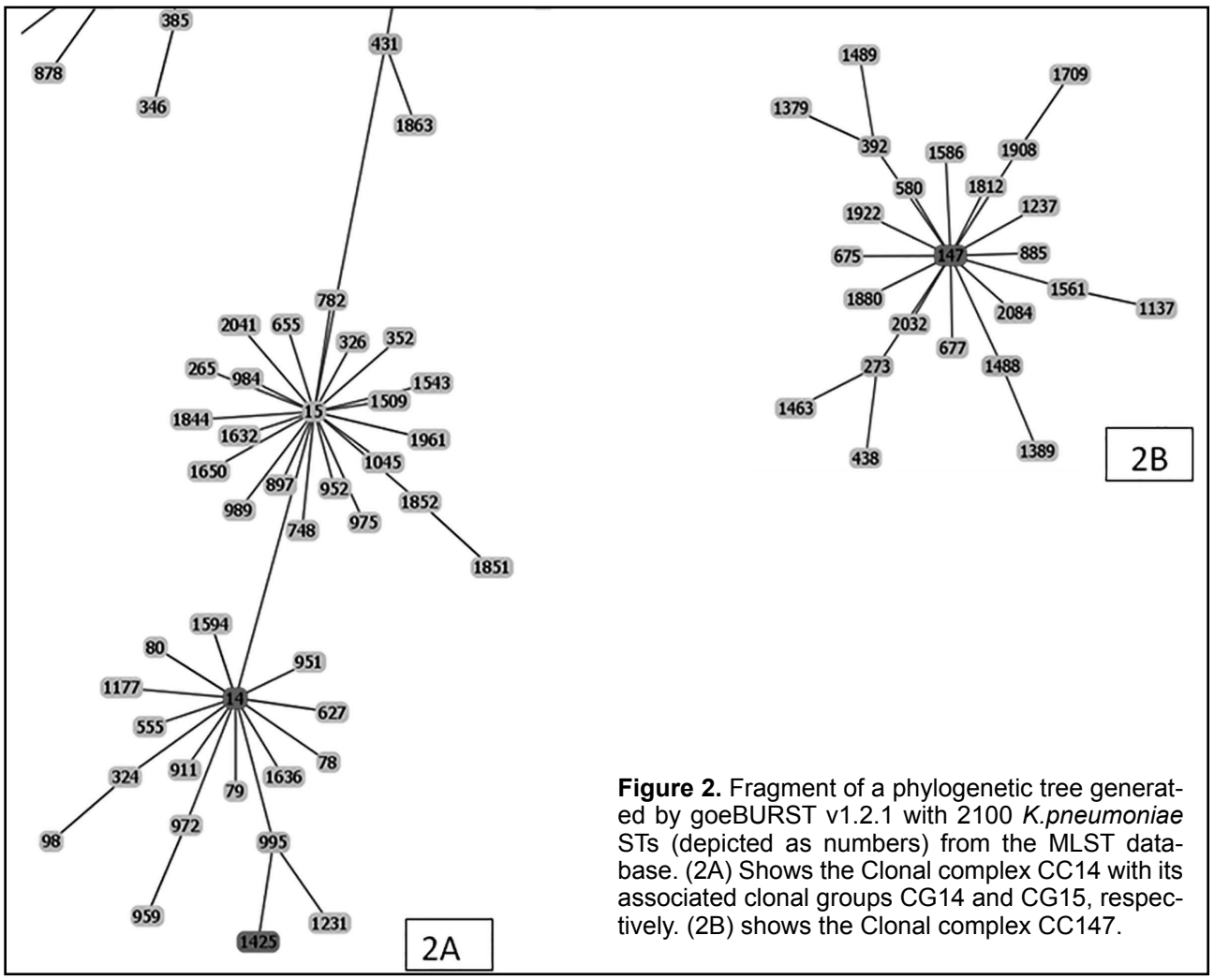

Figure 2. Fragment of a phylogenetic tree generated by goeBURST v1.2.1 with 2100 *K.pneumoniae* STs (depicted as numbers) from the MLST database. (2A) Shows the Clonal complex CC14 with its associated clonal groups CG14 and CG15, respectively. (2B) shows the Clonal complex CC147.

DISCUSSION

The MLST technique, based on determining the nucleotide sequence of house-keeping genes became a standard in the molecular typing of pathogenic bacteria. Mutations occur very rarely in these genes, and therefore they represent a good indicator of the ongoing changes in the genome of pathogenic strains (12). Currently, with over 2100 STs, the *K.pneumoniae* MLST database is one of the largest, most developed and representative ones including diverse isolates from all over the world.

Concerning the lab protocols, while the technique is usually straightforward, there are some critical factors that might impair the results, e.g. the quality of the extracted genomic DNA, the purification of the PCR sequencing reaction products, the separation method on the DNA sequencing machine etc. (13). Therefore, the objective of this study was the laboratory implementation of the MLST methodology by testing it on different carbapenemase producers (Table 1).

K.pneumoniae PR2899 classified as ST530 expressed the Ambler class D OXA-48 carbapenemase possessing weaker but significant carbapenemase activity (14). OXA-48 had first been identified from a clinical *K.pneumoniae* isolate recovered in Istanbul, Turkey, in 2001 (14). bla_{OXA-48} is usually encoded on a plasmid-mediated Tn*1999* transposon mostly related to *K. pneumoniae, E. coli* and *C. freundii*. ST530 is a relatively rare sequence type restricted to North Africa, Middle East and Turkey (9, 15).

PR2671, a multidrug resistant KPC-2 producer was associated with ST258 as reported in many other studies. This clone emerged as the dominant vehicle of KPC over the past decades and now its spread is considered pandemic with reports of various outbreaks involving mostly multidrug resistant *K.pneumoniae* (16). A recently reported colistin-resistant KPC-producing ST258 circulating in Greece, Spain and Hungary is particularly alarming and could represent a novel threat with no available options for treatment (17).

The NDM-1 producers in this study (PR2682 and PR2830) were determined as ST11 which is very close to ST258 differing only by single locus variant and belonged to one common clonal complex CC258. As with ST258, this sequence type was resistant to most antimicrobial agents with sensitivity only to gentamicin, colistin and tigecycline. Its proximity to ST258 is probably the main cause of nosocomial outbreaks in various parts of the world. Moreover, it seems that ST11 is the major *K.pneumoniae* clone carrying NDM-1 on the Balkans (18).

The second KPC-2 producer (PR2761) from our study is ST15 which is one of the first identified high-risk clones. It is not as widespread as ST11 and ST258 and does not cause nosocomial outbreaks with high frequency but exhibits significant resistance to β-lactam antibiotics. *K.pneumoniae* ST15 is often associated with diverse carbapenemases including VIM, KPC, NDM among others and is reported in Asia (China, India, Vietnam) Europe (Germany, Italy, Greece) and Africa (Madagascar, Côte d'Ivoire) (11).

The multidrug resistant VIM-1 producer (PR2874) was grouped as ST147. This sequence type forms a distinct clonal complex (CC147) without apparent evolutionarily linkage to the major sequence types causing nosocomial outbreaks. Nevertheless, ST147 plays an increasingly important role in the intercontinental spread of antimicrobial resistance as evidenced recently with a patient returning from India to Canada (19). Combination of VIM and ST147 was previously described in Greece where VIM-1 resided in an unusual IncR plasmid (20). This ST in combination with plasmid bla_{VIM} gene is resistant but its adaptive abilities are limited and for now is localised only in certain regions.

CONCLUSION

The study results revealed that NDM-1-producing *K.pneumoniae* strains belonged to sequence type ST11, VIM-1 producer was classified as ST147, KPC-2 producers linked to both ST15 and ST258, while OXA-48 producer was determined as ST530. The detection of exclusively high-risk international clones such as ST11, ST15, ST147 and ST258 is a worrisome finding that needs further investigations.

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METHOD FOR DETECTION OF GROWTH PROPERTIES OF NUTRIENT MEDIA AFTER BEING USED IN SEDIMENTATION TEST IN CLEAN ROOMS

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ABSTRACT
Requirements for the absence of microbial contamination in the air of premises from a certain class of purity, as well as in laminar flows, situated in different types of laboratories were confirmed by the sedimentation test. The specific conditions in these facilities, however, questioned whether the nutrient media used in the test, keep their growing qualities and are able to register the presence of microbes in the air. A comparative method was successfully developed to assess the growth properties of manufactured batch culture media as a percentage following their use in the sedimentation method. The preserved growth properties show the reliability of the media as an indicator of microbial contamination.
Key words: growth properties, sedimentation method, validation

INTRODUCTION

According to the regulations (directives) of the World Health Organisation for Good Production Practice, the programme for monitoring of the environment includes the sedimentation method.
The requirements for Good Laboratory Practice (1) in pharmaceutical microbiology laboratories (2), some sources of Quality assurance in Bacteriology and Immunology (3), Medical Standard in Virology (4), Medical Standard for Infectious diseases (5) and also the Requirements for Good Production Practice of veterinary products (6), insist on detecting (monitoring) cleanness of the air and surfaces in laminar flow cabinets. Good Manufacturing Practices requirements for environmental monitoring are especially important in the production of antibiotics, given the trend of increasing antibiotic resistance (7, 8, 9). These demands are essential to the work with infectious materials and microbiological cultures, as non-adherence may lead to compromised results of clinical tests and health hazards for laboratory personnel. The lab technicians must be trained in all aspects of work with cell cultures and potential pathogens (10). They must consider the local safety guidance as well as all local and national laws and regulations. The knowledge on how to handle complications, if such occur, is also needed. When working with cell (microbiological) cultures, Good Laboratory Practice (11) requires a standard that prevents contamination with bacteria, fungi, and mycoplasma or cross contamination with other cell cultures. Contamination can lead to a series of problems, many of which can be underestimated. Damages of the cell lines due to bacteria and fungi contamination are well known and easily detected. The consequences of mycoplasma contamination can vary and are harder to detect; for instance, slowing down of cell growth, induction of morphological changes, chromosome aberration, changing the cell metabolism and inability to recover after cryoconservation (12). Due to these exact reasons, the assurance and especially the demonstration of aseptic conditions is an important step in working with cell cultures. Confirmation of the preserved growth properties of the nutrient media involved in the sedimentation test gives reliability of aseptic conditions under which it was worked.
Technical specification ISO/TS 11133-2 : 2004 (13) requires dilutions of microorganisms to be used as a control of the quality of growth medium (substrate). Also the type of the inoculum (source material for inoculation) and the method of inoculation must be standardised. The requirement for inoculation with no more than 100 colony-forming units (CFU) when testing the sterility of bio-products is issued by the European Pharmacopoeia (14). The same rule for the microbiological inoculums used in the test is set by "WHO Good Practices for Microbiology Laboratories" (1) and requires 50 to 200% reproducibility of the inoculums.

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MATERIALS AND METHODS

Sedimentation Method
Petri dishes, 90 mm in diameter, with Soybean-Casein Digest Agar (SCDA) and Sabouraud Agar (SA) were used. SCDA was used to detect aerobic microbes in the air whereas SA was used to detect fungi and yeasts. The plates were opened and the lids placed next to them. According to the regulations (directives) of the World Health Organisation for Good Production Practice, the duration of the air cleanness control before starting work is five minutes and during work – throughout the whole time, but no more than four hours. The Petri plates with SCDA were incubated at 32.5 ± 2.5^{0}C for three days, and those with SA at 22.5 ± 2.5^{0}C for five days.

Sterility test of nutrient media used in the sedimentation method
Petri plates with nutrient media used in the sedimentation test were incubated under appropriate conditions for short-term or full sterility test. For short-term sterility test plates were introduced in appropriate for

bacteria and fungi conditions for 24 hours. For full sterility test SCDA plates were incubated at 32.5 ± 2.5^{0}C for 72 hours and those with SA were incubated at 22.5 ± 2.5^{0}C for five days period.

Growth properties test of the growth medium
Growth properties of the medium were tested with control strains following validated method according to ISO/TS 11133-2:2004 (5). Working culture colonies from the respective control strains were suspended in sterile buffered saline, except for *A. brasiliensis* where sterile injection water was used. Standardising was performed with ten-fold dilution in sterile buffered saline/sterile injection water. Inoculums were diluted up to 10^2 CFU following standard (validated) method for each microorganism.
0.1 ml of the diluted strain was spread over the whole agar surface with sterile spatula (swab). The inoculated growth media were cultivated at 32.5 ± 2.5^{0}C for bacteria and yeasts, and at 22.5 ± 2.5^{0}C for fungi.

RESULTS
Monitoring of microbiological contamination of the air in premises with a specific level of sterility or laminar flow cabinets is performed by the personnel working there. Validating the ability of the media, with growth substrate provided by "Bul Bio-NCIPD", to support growth in the sedimentation test was conducted for three consecutive days in a premise class "A" by a lab technician. The test required double amount of Petri plates for each control strain (ten SCDA plates and four SA plates) to be left opened for four hours during actual working process. The sedimentation spots were chosen considering places with the strongest laminar flow of circulating air (~ 0.42m/s) for the examined premise.
Petri plates of each growth substrate were placed in their respective incubation conditions for detecting growth following exposure in the sedimentation test. SCDA plates were incubated at 32.5 ± 2.5^{0}C and those with SA at 22.5 ± 2.5^{0}C.
Half of the plates from each kind, five SCDA and two SA, were tested with short-term sterility test for growth promotion. The rest of the plates were subjected to full sterility test to verify the absence of microbiological contamination of the air that could alter the results for growth properties of the tested substrates. Later on their ability to support growth was also tested. All SCDA plates detecting bacterial growth were incubated at 32.5 ± 2.5^{0}C for 24 to 72 hours and SA plates for fungi were incubated at 22.5 ± 2.5^{0}C for up to five days.
The results obtained from testing the ability of each medium to support growth were measured in colony-forming units (CFU) and were compared to the results from the microbiological control of the same batch tested following production and dispatch as regular lot. The percentage of reproducibility was determined using the cross rule (X- rule). The number of colonies obtained with the produced batch media in the test for ability to support growth was considered as 100% and the number of CFU measured after the sedimentation test was "x" %.
Results data represent three days` measurements of each SCDA medium (Table 1) and SA medium (Table 2) after short-term and full sterility test.

Table 1. Testing of the growth properties of SCDA plates, lot number 470613, included in the sedimentation test.

Up to 100 CFU of the microorganisms	% of reproducibility after 24 hours sterility test			% of reproducibility after the whole sterility test		
Staphylococcus aureus ATCC 25923	107	113	127	107	117	113
Pseudomonas aeruginosa ATCC 9027	108	134	121	145	55	82
Escherichia coli ATCC 25922	109	80	98	80	98	96
Bacillus subtilis ATCC 6633	154	17	31	25	43	82
Candida albicans ATCC 10231	122	533	411	500	100	111

Table 2 Testing of the growth properties of SA plates, lot number 730613, included in the sedimentation test.

Up to 100 CFU of the microorganisms	% of reproducibility after 24 hours sterility test			% of reproducibility after the whole sterility test		
Candida albicans ATCC 10231	200	1225	1063	1150	113	163
Aspergilus brasiliensis ATCC 16404	50	125	106	119	63	94

DISCUSSION

The validation shows no alteration in the growth properties of the tested SCDA and SA media after being used in the sedimentation test under the most unfavourable conditions, namely, the long four-hour exposure and positioning at spots with the strongest air flow in laminar flow cabinets. The growth potency of the substrate was preserved after additional incubation exposure of the plates to test the sterility after being subjected to the sedimentation method; particularly with SCDA plates the first test was conducted for 24 hours, the second test for 72 hours and five days in the case of SA plates. The thickness of the agar decreased from 4 mm to 0.5-1.0 mm for both substrates, but nevertheless, the established microbial growth was up to 100 CFU from the validated level of dilution of each tested control strain.

When estimating the percentage of reproducibility for media tested to support growth at the stage of production dispatching, after the sedimentation test and later on following the sterility test, results were different from those mentioned in "WHO Technical Report Series" (1). The standard norm of 50-200% was present only in two strains. Lower level of reproducibility was measured with *Bacillus subtilis* ATCC 6633, and level above the upper limit was measured with *Candida albicans* ATCC 10231.

CONCLUSION

When tested under extreme conditions with regard to humidity, the plates used in the sedimentation test maintained their growth properties while monitoring the environment in class "A" premises. The demonstrated stable qualities of SCDA and SA substrates, regardless of the agar drying out, guarantee reliability in monitoring the environment in premises class "A" where the presence of even one CFU can compromise the cleanness.

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LACK OF TYPE-SPECIFIC ANTIBODIES TO HERPES SIMPLEX VIRUS IN A PATIENT WITH GENITAL HERPES: A CASE REPORT

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ABSTRACT
We present a woman with clinically manifested HSV2 infection, as evidenced by PCR, while repeatedly seronegative for HSV2IgG and HSV2IgM by gG-based ELISA. Immunodeficiency affecting antiviral immunity was excluded. Our results underline the advantage of combined type-specific serology and viral DNA detection for accurate diagnosis and management of HSV infection.
Keywords: genital herpes, HSV2, type-specific serology

INTRODUCTION

Herpes simplex virus (HSV) type 2 (HSV2) is a common sexually transmitted infection and the most common cause of genital herpes although the role of HSV type 1 (HSV1) is increasing. The majority of HSV infections in adults are usually benign. However, these viruses could be responsible for serious diseases of ocular or central nervous system and neonatal HSV infection is associated with very high morbidity and mortality. Moreover, it was shown that HSV associated genital ulcerations increase the risk for HIV and HPV infection. At the same time, effective specific therapy for treatment of HSV infection is available. Therefore, a prompt and accurate diagnosis of HSV infection is essential for a favourable outcome.
Clinical diagnosis of genital herpes is usually based on visual examination and disease history, but it is not quite accurate because the lesions are often atypical and may be confused with other genital dermatoses. Therefore, laboratory confirmation is recommended by guidelines for the management of genital herpes. Current laboratory diagnosis of HSV infection relies on virus detection and HSV type-specific serology (1). HSV DNA detection by polymerase chain reaction (PCR) is the preferred diagnostic method for virus detection because of its high sensitivity and specificity and is one of the best methods for diagnosing genital herpes during active infection in patients with lesions.
Here we present a case of HSV2 associated genital herpes, confirmed by PCR, while repeatedly negative by HSV type-specific serology (TSS), emphasising the role of HSV DNA detection methods for diagnosis of genital herpes.

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CASE PRESENTATION

The patient was a 36-year-old woman who developed genital lesions for the first time. She was examined by her gynaecologist who suspected HSV infection. The patient was prescribed antiviral treatment with Valacyclovir, later replaced by Acyclovir but she had not been able to take them due to stomach pain. To confirm diagnosis serum sample was tested for HSV2 IgG, HSV2 IgM, HSV1 IgG and HSV1 IgM by enzyme-linked immunosorbent assay (ELISA). The results were negative. In the following 10 months tree new episodes with genital lesions were registered. HSV TSS testing was performed but the results remained negative. At the time of the last episode the patient was invited for examination in our Reference Laboratory for herpes virus infections in Sofia. The visual examination revealed typical for genital herpes lesions - painful vesicles and erosions with surrounding redness and swelling of the labia. The patient did not report any general symptoms. The blood sample was negative for HSV2 IgG, HSV2 IgM, HSV1 IgG and HSV1 IgM antibodies by ELISA (Euroimmun AG, Lübeck, Germany). Sterile Dacron swab was used to collect lesion specimen for PCR analysis. HSV-2 was detected by nested PCR assay (Fig. 1A) as described by Aurelius et al. (2). The result was confirmed by the RT PCR (Fig.1B) using commercial PCR kit AmpliSens® HSV-typing-FRT (AmpliSensÒ, Moscow, Russia). The sample was negative for HSV1.
To exclude a primary or secondary immune deficiency state, basic humoral and cellular immunity param-

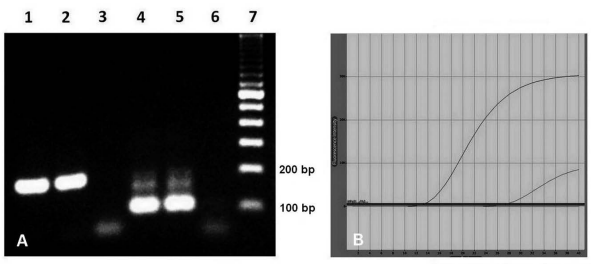

Figure legend

Figure1. PCR-based detection of HSV2 in genital lesion. A/ Results of nested PCR - 2% agarose gel showing 183 bp (first PCR) and 100 bp (second PCR) long PCR products. Line 1 (first PCR) and line 4 (second PCR) – lesion sample positive for HSV2; line 2 (first PCR) and line 5 (second PCR) – positive control; lines 3 and 6 – negative control. The molecular weight in base pairs (bp) is shown on the right (lane 7). B/ RT PCR of genital lesion sample using commercial PCR kit AmpliSens® HSV-typing-FRT(AmpliSens®, Moscow, Russia). The graph shows positivity for HSV2 (curve on the left) and internal control (curve on the right) of genital lesion sample. The sample is negative for HSV1.

eters were tested 2 months later, in the absence of clinically manifested infection. The results showed that the total IgM, IgG and IgA serum immunoglobulin concentrations were within the age-matched reference ranges. Peripheral blood flow cytometry revealed no quantitative cellular immune deficiency. Except for a non-significant lymphocytosis, the percentages and absolute counts of lymphocyte subsets were within the age-matched reference ranges. The presence of differentiated memory and effector CD4 and CD8 T cells excluded a major functional T lymphocyte defect. As previously shown, the quantitative expression of CD38 is a sensitive marker of continuous immune activation (3). In our case, there were no data of active viral replication according to the number of CD38 molecules expressed by CD4 and CD8 T cells. (Table1).

DISCUSSION

We present a case of a woman with typical clinical manifestations of genital herpes and HSV2-positive lesion sample by PCR while consistently seronegative for HSV1 IgG, HSV1 IgM, HSV2 IgG and HSV2 IgM by gG-based ELISA.

Table 1. Evaluation of the patient's basic cellular and humoral immunity parameters.

Parameter	Unit	Result	Reference range
IMMUNOPHENOTYPING[a]			
Lymphocytes	cells x 10^9/ l	**3.51**	1.0 - 2.8
Total T Ly CD3+	cells x10^9/ l	**3.36**	1.6 - 6.7
T Ly CD3+CD4+	cellsx10^9/ l	**1.36**	1.0 - 4.6
T Ly CD3+CD8+	cellsx10^9/ l	**0.91**	0.4 – 2.1
B Ly CD19+	cellsx10^9/ l	**0.63**	0.6 – 2.7
NK cells CD3-CD56 +	cellsx10^9/ l	**0.42**	0.2 – 1.2
Total T Ly CD3+	%	**67**	54 – 76
T Ly CD3+CD4+	%	**39**	31 – 54
Naïve CD4+CD45RA+CCR7-	%	**34**	30 - 61
Memory CD4+CD45RA-CCR7-	%	**65**	35 - 70
T Ly CD3+CD8+	%	**26**	12 – 28
Naïve CD8+CD45RA+CCR7+	%	**48**	28 – 59
Memory CD8+CD45RA-CCR7-	%	**34**	14 – 34
Effector CD8+CD45RA+CCR7-	%	**18**	5 – 54
Terminal CD8+CD28-CD57+	%	**12**	5 - 20
B Ly CD19+	%	**18**	15 – 39
NK cells CD3-CD56 +	%	**12**	3 – 17
Ratio CD4/CD8	-	**2.56**	1.3 – 3.9
Activated T CD4+CD38+	ABC[b]	**2073**	600 – 4500
Activated T CD8+CD38+	ABC	**1529**	510 - 2800
TOTAI SERUM IMMUNOGLOBULINS			
IgM	g/l	**4.5**	0.9 – 4.5
IgG	g/l	**13.4**	8.0 – 18.0
IgA	g/l	**2.4**	0.6 – 2.8

[a]Multicolor flow cytometry with TRUCount tubes (BD Biosciences) for direct absolute count determination and BD FACSCanto II analyzer were used.
[b]Antibody-binding capacity for CD38-PE was determined with QUANTIBRITE TM kit (BD Biosciences) for quantitation of phycoerythrin fluorescence by flow cytometry.

HSV TSS is recommended for management of genital herpes, especially for differentiating between primary and established infection. Although transient false-negative results have been reported with particular assays equivocal results are primarily interpreted as seroconversion phase (4). The median time to seroconversion after HSV infection varies depending on the detection method from three to 12 weeks. In addition, seroconversion may precede the first clinical manifestation of HSV infection (5). On the other hand, there is no evidence that immune response to HSV wanes over time or that seroreversion occurs, even with treatment (6). Therefore, it was legitimate to expect positive TSS results in our case, at least during the later samplings.

A wide range of primary or secondary immune deficiencies could be another cause for the absence of HSV-specific immunoglobulins. A number of subtle immune defects have been described, affecting specific signaling pathways and proteins, important for the control of individual pathogens. Thus, disorders of TLR3 and the UNC-93B (a highly conserved endoplasmic reticulum protein)-IFN-α/β pathway confer selective predisposition to herpes simplex virus (7) leading to severe clinical manifestations (encephalitis). However, the medical history of the patient, the physical examination data, and the tested cellular and humoral immunity parameters excluded a general or more specific deficiency affecting anti-viral immunity.

The currently used assays for type-specific detection of HSV antibodies are based on gG as this is the only glycoprotein that differentiates between HSV1 and HSV2. At the same time, several studies demonstrated that the reliability of gG-based HSV type specific seroassays is overestimated as they measure immune responses to a single viral protein or to individual epitopes. Mutations or deletions in gG2-gene of some HSV-2 strains have been identified, resulting in lack of gG-2 expression or production of truncated forms, and leading to reduced or absent immune response to gG-2, while the pathogenicity of these strains and clinical manifestations in humans were similar to wild HSV-2 infection (8, 9). Further, positive-to-negative shifts in gG-1- and gG-2-based type-specific seroassays, more prominent for HSV-2, were described (10). Genetic variability of the gG-1 gene has been reported for certain clinical isolates of HSV1, and in several cases a gG-1-reactive monoclonal antibody (MAb) was found to be unreactive when tested against mutated strains (11).

In our case, it is quite possible that the HSV2 strain was carrying mutation/s within the immunogenic region of the gG-2 gene, resulting in an absent or very weak antibody response to gG-2 and consequently a false-negative result by ELISA in spite of the typical clinical manifestation and positive PCR result. This case clearly demonstrates that a negative result for type-specific HSV antibodies does not eliminate the possibility of HSV infection. Therefore, the laboratory diagnosis of HSV infections should rely on both, direct virus detection (most commonly HSV DNA detection by PCR) and type-specific serology.

This possibility should be considered in management of patients with genital herpes, including pregnant women.

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TRYPANOSOMA INFECTION IN MEDITERRANEAN MOUSE (*MUS MACEDONICUS* PETROV & RUŽIĆ, 1983) IN BULGARIA

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ABSTRACT
Trypanosoma musculi is a non-pathogenic stercorarian trypanosome which is infective only to mice. The present study reveals a *Trypanosoma* infection in Mediterranean mouse (*Mus macedonicus* Petrov & Ružić, 1983) from the rice fields in Plovdiv region, Bulgaria. The average established prevalence of the parasite in *Mus macedonicus* was 17.1% with higher infection rate in male (21.1%) compared to female (12.5%) species. All trypomastigotes exhibited morphological features typical of the subgenus *Herpetosoma* (*Stercoraria* section) to which *T. lewisi*–like parasites belong. These features included: size of approximately 25 ± 5 μm, free flagellum, characteristic "C shape", visible undulating membrane, oval-shaped subterminal kinetoplast and a nucleus at the anterior end. The characteristic morphology and the presence of infection only in individuals of *Mus macedonicus* allow us to make an evidence-based assumption that the observed parasite is *Trypanosoma musculi*. Future characterisation should include molecular methods to confirm the registered species.
Key words: *Mus macedonicus, Trypanosoma musculi, parasitology, acridine orange*

INTRODUCTION

Virtually all vertebrate taxa can be infected by numerous species of trypanosomes. All tend to circulate between blood-sucking insects or leech vectors and vertebrate hosts whilst undergoing biochemical and morphological changes (1). The rodent trypanosomes are well adapted to their respective hosts and have a high degree of host peculiarity – *Trypanosoma lewisi* in rats, *Trypanosoma musculi* in mice, *Trypanosoma grosi* in some species of *Apodemus, Trypanosoma microti* in voles (2). Since these trypanosomes are extracellular parasites they are exposed to the humoral immune response of the host at all times. Although the *Herpetosoma* subgenus is not known to be pathogenic to its indigenous hosts, cases were occasionally reported in association with human diseases (3, 4). *Trypanosoma musculi* is a non-pathogenic stercorarian trypanosome which is infective only to wild mice living in the Mediterranean basin, West Africa, and Central America. It is host-specific for *Mus musculus* (5) and its life cycle and morphological features resemble those of other African trypanosomes. Upon ingesting trypanosome-infected fleas or flea faeces, mice become parasitaemic as indicated by peripheral blood smears. *Trypanosoma musculi* is closely related to, and morphologically indistinguishable from *T. lewisi*, a parasite which is restricted to rats. There have been few studies on this parasite, perhaps because it was assumed that all rodent stercorarian trypanosomes would behave like *T.lewisi*, the type-species which has been studied extensively (6).
Besides *Trypanosoma equiperdum* (7), which causes the disease "dourine" in horses, known also as equine syphilis, there are no other reports of trypanosome infections in rodents or other representatives of the wild fauna in Bulgaria.
The aim of the study is to present data on the distribution and morphological characteristic of *Trypanosoma musculi* in Mediterranean mice *Mus macedonicus* from Bulgaria.

MATERIALS AND METHODS

The study area covers a region of rice fields between the town of Saedinenie and the village of Tsalapitsa near Plovdiv (Fig. 1). The whole area is a complex of wetlands used for rice production and bounded by low-lying embankments and channels, as well as wet meadows located in close proximity thereto. The rice fields are located near a well-developed urban area and along the main highway in Southern Bulgaria.
A total of 35 specimens from 19 male and 16 female mice of *Mus musculus* were collected. The trapping was performed between March 2014 and May 2015. Sherman live traps were placed at dusk, left active overnight, and collected on the next morning. The mice were brought to the laboratory, where they were sexed and weighed. Blood samples were obtained from the caudal vena cava. A drop of blood was placed onto the slide and using a pusher slide a thin smear was prepared. Two peripheral blood smears from each animal were made on pre-cleaned microscope slides. Slides were air dried at room temperature for 24 h and fixed in absolute methanol for 10 min. The coded slides were stained with fluorochrome dye acridine orange (0.1% solution supplemented in Sörensen's phosphate buffer) for 1 min, rinsed and cover-glassed immediately before evaluation with fluorescence microscopy Leica DM 1000 equipped with appropriate for acridine orange I3 filter. Acridine orange method has a high diagnostic capacity to detect different parasites in blood smears because of its higher speed of reading and sensitivity when compared with common bright field microscopy using Giemsa staining and the opportunity to detect DNA/RNA through staining in different colour. The technique is recommended for a fast diagnosis especially in countries with endemic areas where diseases like malaria, sleeping sickness, Lyme disease, babesiosis, and spirochetemia occur. The acridine orange is appropriate dye for detecting cases of low-level parasitaemia (Fig. 2).

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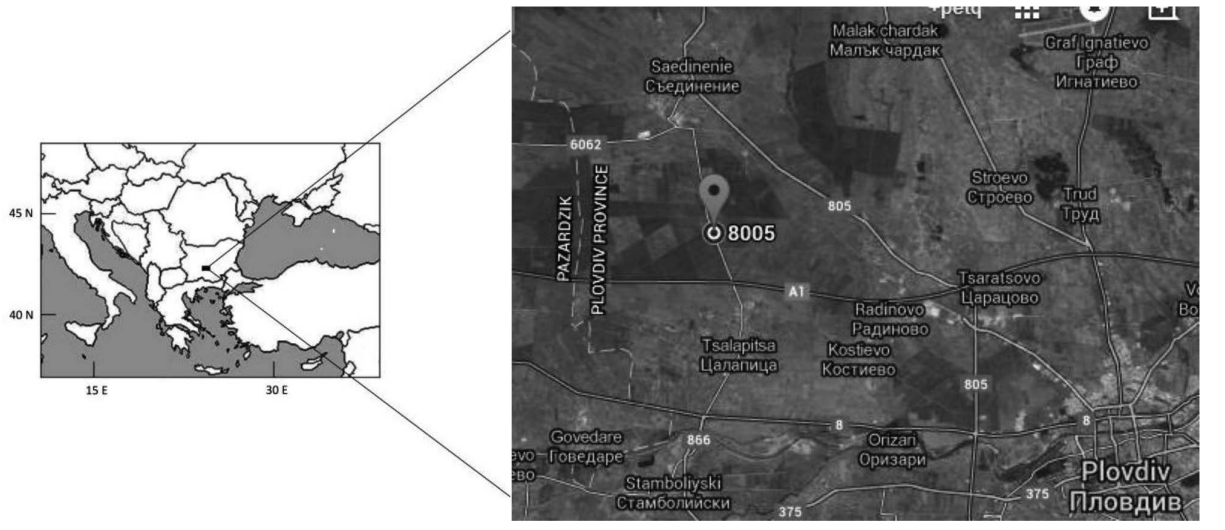

Fig. 1. Location of the study area with sites of *Trypanosoma* infection (coordinates: N 42°23'57.30", E 24°56'20.41").

RESULTS

The established prevalence in *Mus macedonicus* was 17.1% with 6 infected specimens out of 35 investigated. A lower infection rate was found in female (2 /16) as compared to male mice (4 /19) with 12.5% and 21.1%, respectively.

The registered species of *Trypanosoma* was a trypomastigote form, which lived in extracellular fluids and circulated freely among red blood cells (Fig. 2). The average size of the parasite was 25 ± 5 µm (n=187). The smallest parasite observed was 15 µm and the largest was 32 µm. The kinetoplast was oval-shaped, subterminal, located in the very sharp and thin posterior end. Nucleus was more in the anterior part of the body. In all observed forms the flagellum was free and the undulating membrane was not large but clearly visible. These characteristic features (Fig. 3) were compared with described morphological characteristics of different rodent's *Trypanosoma* species.

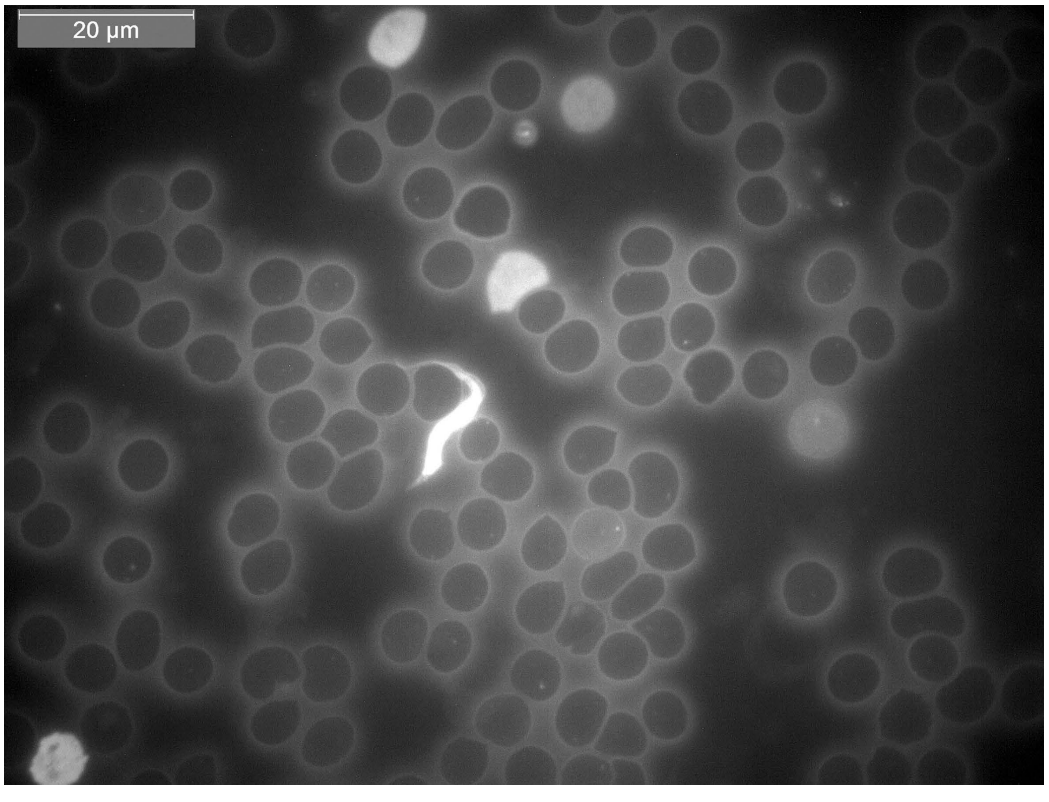

Fig. 2 Microphotograph of *Trypanosoma musculi* from *Mus macedonicus* in rice fields near Plovdiv. A thin blood smear showing trypanosomes stained with acridine orange. Parasites (orange) are easily recognisable alongside red blood cells (mature – dark green; young – red) (magnification 1000x).

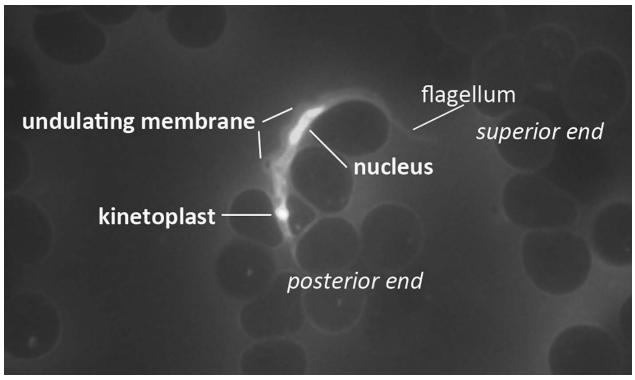

Fig. 3. Morphological characteristics of adult trypomastigote form.

DISCUSSION

The trypanosomes described here comply with the known morphological characteristics reported for the so-called *T. lewisi*-like group of subgenus *Herpetosoma* (*Stercoraria* section). Unlike *T. lewisi* which is specific to rats and cannot grow in other rodents (8), *T. musculi* (also known as *T. duttoni*) is specific to mice, resides in the blood and does not undergo intracellular stages (9). The "C shape" and free flagellum of the trypanosome are the most characteristic features of the *Herpetosoma* subgenus (4) and both are observed in the micrographs shown in Fig. 2 and Fig. 3. The characteristic morphology combined with restricted host specificity lead us to the assumption that the trypanosome described here is, in fact, *T. musculi*.

Our results demonstrate that male mice are two-fold more susceptible to infection than female mice with 21.1% versus 12.5%, respectively, which is in accordance with previous reports. According to Greenblatt and Rosenstreich (10), the decreased resistance of male mice to *T. rhodesiense* infection was a result of their inability to control parasite growth. The lack of difference between susceptible and resistant mice was further shown to be independent of an X-linked gene (10). The various strain-specific susceptibility rates to *T. brucei brucei* were used by Šíma *et al.* (11) to identify chromosomal loci conferring susceptibility to *T. brucei brucei* infection. Our data does indeed support a differential genetic regulation of *T. brucei* susceptibility between male and female mice. Sex-dependent susceptibility to other pathogens has also been observed (11). Therefore, the difference in susceptibility between male and female *Mus macedonicus* to *T. musculi* may also be due to differential genetic regulation in the two sexes.

On the basis of literature data, it can be assumed that the vectors transmitting *T. musculi* to its mammalian host *Mus macedonicus* are fleas that live in the fur of the host animal and feed on its blood. In addition to *T. lewisi*, at least nine other species of trypanosomes can infect rodents through fleas. Such species include *T. musculi* (synonym: *T. duttoni)* of house mice, *T. rabinowitschi* of hamsters, *T. neotomae* of wood rats, and T. *grosi* of the European wood mouse (*Apodemus sylvaticus).* Fleas are also common vectors of trypanosomes specific to certain birds, shrews, voles, and lagomorphs (12).

The pathogenesis of trypanosomes depends on the virulence of the agent, the degree of invasion, and the physiological condition of the animal species (13). Studies have shown that the bone marrow is severely stressed during infection with *T. musculi.* As a result, the entire lymphoreticular system is dedicated to the production of cells to combat the infection taking place in the peritoneal space (14). Following elimination of *T. musculi* from the circulating blood stream by the host's immune system, the mice become resistant to subsequent infections. However as the trypanosomes are able to persist in the vasa recta of the kidneys during the lifespan of the mice, it has been proposed that the kidneys represent an immunologically privileged site for *T. musculi* chronic infections (9, 15).

The two classical diseases associated with human trypanosomes are sleeping sickness and Chagas disease. Humans are innately protected against most *Trypanosoma* species (16) found in livestock and many other mammals. Recently, however, atypical human infections caused by other *Trypanosoma* species (or sub-species) have been reported, specifically due to *T. brucei brucei, T. vivax, T. congolense, T. evansi, T. lewisi,* and *T. lewisi*-like. These cases are reviewed in Truc *et al.* (4). Although it is classically believed that the rat-specific *T. lewisi* is non-pathogenic to humans, recent reports indicate that *T. lewisi* and other species like *T. evansi* could be potentially pathogenic in humans. For example, symptoms of a *T. lewisi*-like infection were reported in a Thai infant (3). It was therefore important to study the prevalence of *T. lewisi* infections in various rodents normally found in Thailand in order to identify possible sources of human infection (3). These results are therefore relevant to the trypanosome infection identified in *Mus macedonicus.* Particularly important is that the sites of sample collection were in the outskirts of Plovdiv and its industrial and agricultural areas. We therefore suggest that the degree of human impact may influence the transmission of *Trypanosoma* spp*.* and hence increase the risk of infection to humans.

This is the second report of *Trypanosoma musculi* identified in the blood of the Mediterranean mouse *Mus macedonicus* in Bulgaria. Among several species of mammals captured in the region near Plovdiv (*Mus macedonicus*, *Apodemus* spp*., Microtus arvalis*) *Trypanosoma* was detected in 23.5 % of *Mus macedonicus* (17). We can make the conclusion that *T. musculi* infection exists in the Mediterranean mice colony near Plovdiv. This finding confirms the important role of Mediterranean mice as a reservoir for trypanosome infection.

Further studies, such as molecular assays and additional investigations of potential vectors and reservoirs, are required to fully characterise the *Trypanosoma* species identified here.

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GENETIC STABILITY OF BCG VACCINE PRODUCTION IN BULGARIA

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ABSTRACT

A century ago, Albert Calmette and Camille Guérin began a daunting task, which is unmatched even today, that led to the most widely used vaccine in human history – BCG vaccine. There is little doubt that BCG will continue to play a role in TB control. It remains part of upcoming clinical trials as an integral component of new vaccinesor a primer to be boosted by new components. In the last years the importance of BCG characterisation and control has been strongly emphasised especially in the light of new knowledge based on the development of sophisticated genome analysis techniques.

The paper describes the results from the molecular typing performed on Bulgarian BCG strain Sofia SL222 and commercial BCG Lots focusing on their specific genetic characteristics in regard to monitoring the consistency of production and checking for genetic variations arising during the maintenance of the sub-strain for more than 30 years.

The genetic stability of Bulgarian BCG vaccine was confirmed by using two different and powerful tools of the post-genomic era – VNTR and DNA microarray hybridisation.

Key words: BCG vaccine, BCG sub-strains, Comparative genomics, Whole-genome microarray, VNTR

INTRODUCTION

Today, almost 95 years after the discovery of Calmette and Guérin, the BCG vaccine remains the most used vaccine in the world. Between 1921 and 2015more than three billion people have been vaccinated with various BacilleCalmette – Guérin (BCG) vaccines without significant side effects. Despite of its variable efficacy (between 0 and 80%) BCG has proved its role in the protection against severe forms of childhood tuberculosis and TB meningitis in neonatal age. The current global epidemiology of tuberculosis (1) and the emergence of drug-resistant strains (1, 2) have resulted in efforts to improve BCG or to develop an entirely new vaccine against tuberculosis. Due to the recent progress of molecular genetics differences have been demonstrated between *M.bovis* BCG and *M.tuberculosis*, between *M.bovis* BCG and virulent *M.bovis* as well as genetic distinction between different BCG strains. The understanding of genetic differences between the strains may provide insights into the determination of protective immunity. On the other hand, the necessity for improvement of quality control of BCG vaccines has been identified as a WHO priority (3). WHO has recognised the need to improve both characterisation of BCG vaccine and the assays used for its quality control, taking into account recent advances in genetics and molecular biology. The following tools of molecular biology can be applied in quality control of BCG vaccines: (i) Detection of DNA specific for virulent mycobacteria - possible use indetecting virulent mycobacteria in BCG vaccine; (ii) Determination the capacity of a given product to protect against virulentchallenge in animal models; (iii) Characterisation of BCG sub-strains; (iv) Possibility of monitoring the consistency of BCG vaccinesproduction; (v) Check forvariations arising during the production etc. (4).

Bulgarian BCG sub-strain BCG Sofia SL222 is one of the most commonly used strains for immunisation against tuberculosis. More than 50 million doses per year are distributed through UNICEF and PAHO in almost 150 different countries all over the world. The Bulgarian BCG Laboratory was established in 1949 when its founder Dr Rodopska obtained the BCG strain from Institute Pasteur. Because of high prevalence of local lymphadenitis few years later the strain was replaced by BCG Russia. The freeze-drying was introduced in 1963. Nine years later, in 1972, National Regulatory Authorities approved Lot 222 as a Master Seed Lot and this act represents the beginning of the Bulgarian BCG sub-strain BCG Sofia SL 222.

BCG Sofia SL 222 was fully investigated in relation to its biological and immunological properties. The substrain possesses low level of residual virulence and moderate immunogenicity. Also, *Mycobacterium bovis* BCG Sofia reveals genetic characteristics which pertain only to the closest to the original Calmette and Guerin strains. The main characteristics of the Bulgarian BCG sub-strain are presence of RD2 Region which is typical for all early BCG strains; presence of DU2-type duplication (from Rv3299 to Rv3316) and 1.6-kb deletion that affects the Rv3697c and Rv3698 which is related to membrane protein in the cell wall structure.

Here we report our results from molecular typing performed on two commercial BCG Lots comparing them with results obtained for Master Seed Lot Sofia SL222 which was produced 30 years earlier. Determination of the genetic events in Master Seed Lot and in BCG commercial lots allowsus to check forvariations arising during the production, to monitor the consistency of production and to prove the stability of the product using a set of genotyping methods, including VNTR typing and whole-genome DNA microarray.

MATERIALS AND METHODS

1. DNA isolation and cells.

The following sources of DNA were used: a) BCG Sofia SL222; b) two commercial batches BCG vaccine (BB-NCIPD Sofia) Lot No906 and Lot No909.

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DNA was extracted using the method described by van Soolingen (5). The final concentration of DNA (0.525 µg/µl) was estimated using a spectrophotometer (Unicam, Helios γ).

2. VNTR:

VNTR – PCR primers (Table 1) were designed to anneal upstream and downstream of each tandem repeat locus. Each well of the 96 wells plate contained-QiagenHotStarTaq™ MasterMix Kit - 10 µl,VNTR –F primer (PE Biosystems)- 0.5 µl,VNTR –R primer (PE Biosystems)- 0.5 µl,H_2O – 7 µl,cells – 2 µl. PCR was performed in a total volume of 20 µl under the following conditions: 15 min at 94°C; [0.5 min at 94°C; 1 min at 68°C; 2 min at 72°C] x 30 cycles; 10 min at 72°C; ∞ at 4°C. Isolates were amplified using primers for ETR-A through ETR – F.

Table 1.VNTR-primers` sequences used in this study.

VNTR	Fluorescent Label dye	Primer sequence
ETR A - F*	FAM	AAATCGGTCCCATCACCTTCTTAT
ETR A - R**		CGAAGCCTGGGGTGCCCGCGATTT
ETR B - F	HEX	GCGAACACCAGGACAGCATCATGGGTT
ETR B – R		GGCATGCCGGTGATCGAGTGGCTATA
ETR C - F	HEX	GTGAGTCGCTGCAGAACCTGCAG
ETR C – R		GGCGTCTTGACCTCCACGAGTG
ETR D - F	NED	CAGGTCACAACGAGAGGAAGAGC
ETR D – R		GCGGATCGGCCAGCGACTCCTC
ETR E - F	FAM	CTTCGGCGTCGAAGAGAGCCTC
ETR E – R		CGGAACGCTGGTCACCACCTAAG
ETR F - F	NED	GGTGATGGTCCGGCCGGTCAC
ETR F - R		GTGCTCGACAACGCCATGCC

*F- Forward primer; **R- Reverse primer

Dilution, pooling and filtering of PCR products were performed after finishing the PCR cycle. Gel mix with the following composition was prepared:Urea (Sigma) - 9.0 g,Long Ranger ®gel solution 50% - 2.5 ml,dH_2O - 13.5 ml.After Gel mix filtering (Whatman Filter) 125 µl Ammonium Persulphate (10%) and 17.5 µl TEMED were added. Using a syringe the gel was carefully poured between the glass plates. The gel thickness was 0.2 mm. By inserting the flat edge of a 96-well comb into the gel 96 wells with 0.4 mm well area were made. After the gel polymerisation the samples were loaded using a 96-well porous comb. Each sample required: 1.0 µl VNTR-PCR product,0.3 µl Gene Scan 2500 ROX (internal line standard),0.7 µl Formamide/Blue Dextran Loading Buffer. On each separate “tooth” of the 96-well comb 0.6 µl of the sample was loaded. The samples were analysed by Automated Fragment Analysis, using ABI PRISM 377® DNA Sequencer (Applied Biosystems) with the following parameters: Running time - 3.15 h,Laser power - 40.0 mW,Gel temperature - 49°C,Electrophoresis power - 200 W; Electrophoresis voltage - 60 V. The size of the PCR fragments was estimated using GeneScan and Genotyper software package.

3. DNA microarray analysis

DNA from cells grown onto Löwenstein-Jensen medium was extracted using the method described by van Soolingen et al. (5).

Whole genomic DNA from *M.tuberculosis* H37 Rv,BCG Sofia (Master Seed Lot SL222), BCG Lot No906, and BCG Lot No909 were used as templates for direct incorporation of fluorescent nucleotide analogues (Cy_3 and Cy_5dCTP) by randomly primed polymerisation reaction. The *M.tuberculosis* H37 Rv sample contained:DNA – 3 µl,random primers (Life Technol.) – 1 µl,water – 37.5 µl. The samples of Bulgarian BCG vaccine contained:genomic DNA – 3.9 µl,random primers (Life Technol.) – 1 µl,water – 36.5 µl (per each sample).

After heating at 95°C for 5 min the following were added to each sample:10xREact 2 buffer (Life Technol.)– 5 µl,dNTPs [5mM dA/G/TTP; 2mM dCTP] (Life Technol.) – 1 µl,Klenow (Life Technol.) – 1 µl. 1.5µl of Cy_5dCTP (Amersham Pharmacia Biotech) was added to BCG Sofia samples and 1.5µl of Cy_3dCTP (Amersham Pharmacia Biotech) was added to *M.tuberculosis* H37Rv.Samples were incubated at 37 °C in a dark place for 90 min.

The microarray slides (BMG at St George’s Hospital Medical School, London, UK), covered with poly-L-lysine and spotted, were incubated in pre-hybridisation solution at 65°C for 20 min. The pre-hybridisation solution contained:20xSSC – 8.75 ml,20% SDS – 250 µl,BSA (100 mg/ml) – 5 ml,H_2O – to 50 ml.

The purification of Cy3/Cy5 labelled DNA was performed using QiagenMinElute PCR Purification kit (Qiagen). The hybridisation of the strains being compared was performed to total volume of 16.0 µl:Cy3/Cy5 labelled DNA sample – 10.5 µl,filtered 20xSSC – 3.2 µl,filtered 2% SDS – 2.3 µl. The hybridisation mixture was denatured at 95°C for 2 min, cooled slightly and applied onto the slide at the edge of the arrayed area,and after that covered with a 22mm^2 cover slip. The microarray hybridisation cassette (Telechem International) was sealed and submerged in a water bath in dark for 16-20 h. After hybridisation the slides were washed for 2 min in:20xSSC – 20 ml (1xSSC),20% SDS – 1 ml (0.05% SDS),H_2O – to 400 ml; followed by 2 min washing in:20x SSC – 2.4 ml,H_2O – to 800 ml.After centrifugation (for drying purpose) the slides were scanned using a dual-la-

ser Affymetrix 428 Array scanner at λ=532 nm and λ=635 nm.The Affymetrix 428 Array scanner is a digital confocal laser scanning epifluorescent microscope for viewing fluorescent-dye tagged samples on slides. The instrument converts laser-induced fluorescence of the dye-tagged material into gray-scaled binary data or false-coloured date.

Fluorescent spot intensities were quantified using ImaGene 4.0 software (BioDiscovery Inc.). For each spot background fluorescence was subtracted from the average spot fluorescence to produce a channel-specific value.

The data were further analysed using GeneSpring 5.0 software (Silicon Genetics). This software integrates microarray experimental results with the genomic positions of the hybridisation targets and displays corresponding Open Reading Frames (ORFs).

The geometric mean of the normalised red/green (Cy5/Cy3) ratio was calculated for each sample using data from three experiments.

To confirm the putative deletions, PCR primers were designed to approach the predicted edges of each deletion so that the amplified region would span the missing genomic locus for those strains in which it is deleted.

RESULTS

1. VNTR

VNTR typing is a rapid and reproducible method for strain differentiation and evolutionary studies on bacteria (6, 7, 8). Despite of its basic application as a genotyping technique for *M.tuberculosis* molecular epidemiology, we used this method to compare allelic profiles of BCG Sofia SL222 and two commercial BCG lots. When the results for all six VNTR loci ware combined, the allele profiles of tested BCG vaccines were identified. Each allele corresponds to a different number of repeat units as determined by PCR. Multiple samples from each source yielded PCR product of identical size at all loci. The VNTR profile of Bulgarian BCG vaccine was determined as 5-5-5-2-3-3.1. All tested BCG samples revealed matching allele profiles.

The number of tandem repeats in each locus in the tested BCG vaccines is presented on Table 2.

Table 2. Allele profiles of BCG Sofia SL 222 and Lots No906 and 909 analysed by VNTR typing, compared with *M.tuberculosis* H37Rv

Sample	Number of tandem repeats in each locus						Allele profiles
	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	ETR-F	
BCG Sofia SL222	5	5	5	2	3	3.1	5-5-5-2-3-3.1
BCG LotNo906	5	5	5	2	3	3.1	5-5-5-2-3-3.1
BCG LotNo909	5	5	5	2	3	3.1	5-5-5-2-3-3.1
M.tuberculosis H37Rv	***3***	***3***	***4***	***3***	***3***	***3.2***	***3-3-4-3-3-3.2***

VNTR are located both within coding regions (ETR-A) and intergenic regions (ETR-B to F). Consequently, these DNA repeats, which are located within ORFs, encode amino acid repeats and variations in repeat numbers lead to size variations in the proteins. When the VNTR loci are located in the intergenic regions they represent bi-directional transcription terminator and may contain regulatory elements (6).

VNTR D is known to contain a 24 bp deletion in one of the tandem repeats. The naming convention reflects the presence of this deletion by an asterisk(*). Product designated as D5* would have 5 tandem repeats each of them with size 77 bp and the asterisk shows that 24 bp deletion has occurred in only one of the tandem repeats.

Analysis of VNTR – F is more complex. This locus contains a 55 bp tandem repeat and a 79 bp tandem repeat. The different number of 55 bp and 79 bp tandem repeats is manifested by different size of the product. For example a product of 469 bp would have 3 copies of 79 bp and 2 copies of 55 bp and would be designated as F3.2.

In our study we combined the analysis of six of these loci (ETR-A to ETR-F) in order to identify the strain and to confirm its consistency during the production cycle. Comparing the allele profiles of BCG Sofia SL 222 with those of two commercial lots we proved the stability of Bulgarian BCG for a period longer than 30 years.

3. DNA MICROARRAY

The whole genome microarrays were constructed by spotting onto poly-L-lysine coated slides of PCR products derived from portions of each of the 3924 predicted ORFs of *M.tuberculosis* H37Rv. Application of BCG genomic DNA to this slide demonstrated those regions of *M.tuberculosis* genome that have been deleted from *M.bovis* BCG (9).

The original results from microarray analysis showed that 116 genes of the genome of BCG Sofia are deleted from a total of 3924 genes of *M. tuberculosis* H37Rv, spotted onto the slide, which presents 2,95% of ORFs in H37Rv genome. After subtracting those, which are included in RD regions and IS6110, the genes Rv2887, Rv2353c, Rv3698 and Rv3203 were additionally analysed by confirmatory PCR analysis. Data on these genes, obtained from DNA microarray analysis are presented in Table 3.

Table 3. Genes deleted from BCG Sofia, Lot No906, and Lot No909 compared to *M.tuberculosis* H37Rv.

Gene designation	P-value	Expected size	Product
Rv2887	0.083	392 bp	Transcriptional regulator
Rv2353c (PPE 39)	0.026	198 bp	PPE-family protein
Rv3698	0.026	833 bp	Hypothetical protein
Rv3203	0.048	547 bp	Probable lipase

As the PCR was weak with Rv3698, the analysis was repeated with Rv3697c with the following primer sequence:
5'- GGG TAT CCT GCC ATA CAA TC – 3' [RD.Russia_f]
5'- CTT CGT TTG GCT TGA CTT CC – 3' [RD.Russia_r]

Data from PCR analysis with the above primers showed that the gene Rv3697c was deleted in the BCG Sofia genome (Master seed Lot SL222 and both BCG Lot No906 and 909), as well as the gene Rv2353c.
As expected, the deletion RD1 occurred in all BCG strains andwas also missing in BCG Sofia. RD2 region, which was deleted from all BCG strains obtained from Institute Pasteur later than 1931, is present in the genome of BCG Sofia. The region known as RD Russia, which is deleted from BCG Russia only, and which truncates part of both Rv3697c and Rv3698, was deleted in BCG Sofia as wellData on the regions deleted from the BCG Sofia genome are presented in Table 4.

Table 4. Deleted or truncated genes in the variable regions in BCG Sofia, Lot No906, and Lot No909.

Variable Region	ORF, gene	Salient functions
RD1	Rv3871-Rv3879c	PE, PPE, ESAT-6 system
RD3	Rv1573-Rv1588c	phiRv1 prophage
RD4	Rv1505c-Rv1516c	Possible polysaccharide system
RD5	Rv2346c-Rv2353c	ESAT-6, PE, PPE family members, phospholipases C
RD6	Rv3424- Rv3428c	PPE proteins, IS 1532
RD8	EphA-lpqG	Epoxide hydrolase, monooxygenase, lipoprotein, ESAT-6, PE, PPE protein family
RD9	CobL-Rv2075c	Precorrinmethylase, oxidoreductase, exported protein
RD10	Rv0221-Rv0223	Enoyl CoA hydratase, aldehyde dehydrogenase
RD11	Rv2645-Rv2695c	phiRv2 prophage
RD12	*sseC*-Rv3121	Thiosulfate sulfurtransferase, molybdopterin converting factor, methyltransferase, cytochrome P450
RD13	Rv1255c-Rv1257c	Transcriptional regulator, cytochrome P450, dehydrogenase

The genome of Bulgarian BCG revealed DU2 duplication, from Rv3299c to Rv3316. The same duplication was found in BCG Russia in previous analysis.
The described genetic properties are the same in Master seed Lot SL222 and both BCG Lot No906 and 909, which is a proof for absence of variations arising during the production and for consistency and genetic stability of the product for a period longer than 30 years.

DISCUSSION

It is well known that the differentiation between the BCG sub-strains could considerably improve the control and standardisation of these products. BCG sub-strains can be distinguished from each other by certain phenotypic and biochemical criteria (10), but the best means of differentiation could be achieved by investigation at the genomic level.

During the last two decades plenty of methods have been used in order to establish genetic specificity of BCG, to reveal the reason for *M.bovis* attenuation and to trace the phylogeny of *Mycobacterium tuberculosis* complex (11, 12, 13). Although the strains of *M.tuberculosis*complex vary in their host range, virulence and other phenotypes, they have highly conserved DNA sequences. The variability observed in tandem repeat loci contrasts with this sequence conservation observed in other parts of the genome. Polymorphism in TR loci may be a significant genetic mechanism underlying phenotypic variations among *M.tuberculosis* strains (14). VNTR are located both within coding regions (ETR-A) and intergenic regions (ETR-B to F). Consequently, these DNA repeats, which are located within ORFs, encode amino acid repeats and variations in repeat numbers lead to size variations in the proteins. When the VNTR loci are located in the intergenic regions they represent bi-directional transcription terminator and may contain regulatory elements (6). Supply et.al (15) have identified 41 such loci in *M.tuberculosis* genome and termed them mycobacterial interspersed repetitive units (MIRUs). Twelve loci were demonstrated to vary in tandem repeat numbers. These loci have formed the basis of PCR-based typing method, which is reproducible, sensitive and specific for *M.tuberculosis* complex. In our study we combined the analysis of six of these loci (ETR-A to ETR-F) in order to identify the strain and to confirm its consistency during the production cycle. Comparing the allele profiles of BCG Sofia SL 222 with those of BCG Lot No906 and Lot No909 we proved the stability of the Bulgarian BCG for a period longer than 30 years.

The genetic core of differences between BCG strains was presumed in early 1980's by Osborn (16). Due to differing methods of passage and storage BCG vaccines, although originating from one strain of *Mycobacterium bovis,* have produced many sub-strains showing phenotypic and genetic heterogeneity. These distinct characteristics have been used for the classification of different BCG sub-strains currently in use. The early BCG sub-strains, produced prior to 1926, differ from late sub-strains because the former secrete the MPB70 antigen, produce methoxymycolates, and contain two copies of the IS 6110 insertion sequence. A lot of methods with variable discriminatory properties have been used in order to classify the different BCG sub-strains. Using genomic subtraction Mahairas and his colleagues identified three genomic regions of difference (RD1, RD2, RD3) between *M.bovis* BCG and virulent *M.bovis* and *M.tuberculosis* strains (17). It was proven that the deletion of RD1 occurred in all BCG strains tested and at the same time RD1 was present in all other strains from *M.tuberculosis*complex. Most probably the culture conditions between 1908 and 1921 allowed the selection of a stable mutation in the parent *M.bovis* strain which is expressed by the deletion of RD1 and which is manifested by the loss of virulence (18, 19).

As a result of sequencing the TB genome it became possible to perform the comparative studies of BCG vaccines by whole-genome DNA microarray. By using this method 18 regions (RD1 to RD18) were found to have been deleted from BCG strains in relation to the H37Rv strain of *M.tuberculosis*. Some of these deletions vary between different BCG sub-strains. The deletion of RD2 occurred at Institute Pasteur between 1927 and 1931. Further deletion of RD14 occurred between 1938 and 1961 and is specific for BCG Pasteur. Other deletions that occurred away from Institute Pasteur are the loss of RD8 in Montreal between 1937 and 1948 and RD 16 in Uruguay or Brazil after 1925. This indicates that ongoing evolution of BCG strains was not confined only to Institute Pasteur. When the described deletions are superimposed on historical reports,the invaluable use of genomic analysis in clarification the in-vitro evolution of *M.bovis* BCG is demonstrated(9).

In our study we used one of the most powerful enabling technologies of the post-genomic era - microarray-based hybridisation. From this analysis it became clear that the genome of *M.bovis* BCG Sofia had undergone the same genetic events as its predecessor BCG Russia without revealing any genetic variations during the sub-cultivation in Bulgaria. The presence of RD2 region in its genome pertains BCG Sofia to the group of BCG strains, closest to the Calmette and Guérin original strain. Phenotyping differences between BCG strains, which differ in the presence or absence of RD2, are not readily apparent. The strains carrying the complete RD2 region (e.g. Japan, Russia) have been widely used as tuberculosis vaccines, as have the strains lacking RD2. However, there is no evidence that BCG efficacy could be associated with presence or absence of this region.

The region termed as BCG Russia, which covers 1603 bp, affecting both Rv3697c and Rv3698, and which was deleted from BCG Russia only, was found to be deleted in BCG Sofia also. The function of the deleted genes is still unknown but the encoded product is membrane protein probably related to the cell wall structure.

The duplication DU2, from Rv3299c to Rv3316, was revealed in the genome of Bulgarian BCG. This is the same duplication found in BCG Russia in previous observations. Tandem duplications are generally caused by unequal crossing-over between homologous sequences or by recombination of short DNA homologies. Dynamic duplications are observed in some bacterial species, suggesting that these events represent an important mechanism for generating genomic plasticity, e.g. increasing of gene dosage, generating novel functions, source of redundant DNA for divergence etc. (20). Although it is difficult to speculate about the exact role of these duplicates in BCG genome, their existence demonstrates once again the in vitro evolution of BCG daughter strains.

Although DNA-microarray technique cannot detect smaller deletions, point mutations, small genetic rearrangements, PGRS genes etc., it has the potential to provide important insights into BCG strains evolution and strain variations (21, 22, 23). Data obtained from microarray-based comparative hybridisation give us

the opportunity to find the exact place in the BCG genealogical tree, where BCG Sofia "fits" and also to monitor the consistency of the production and prove the genetic stability of the Bulgarian BCG vaccine.

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GENOTOXICITY BIOMONITORING OF ANTHROPOGENIC POLLUTION IN RICE FIELDS USING THE MICRONUCLEUS TEST IN STRIPED FIELD MOUSE (*APODEMUS AGRARIUS* PALLAS, 1771)

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ABSTRACT
Zoomonitoring of small mammal populations, exposed to potential mutagens, can provide an early detection system for the initiation of cell dysregulation. The striped field mouse (*Apodemus agrarius* Pallas, 1771) is an appropriate zoomonitor species suitable for genotoxicological research, especially due to its wide distribution, r-type reproductive strategy, relatively small home range, high trophic chain position and metabolic rate. The present study was carried out in differently polluted areas. The striped field mice were collected in the rice fields located near Plovdiv (Southern Bulgaria) and in background region of Strandzha Nature Park (Southeastern Bulgaria). The rice fields are exposed to different anthropogenic pollutants like heavy metals and polycyclic aromatic hydrocarbons, due to the nearby located highway and the use of various fertilizers in agricultural practices. The results showed that anthropogenic pollution in rice fields induces DNA and chromosomal lesions in striped field mouse's cells, which was well demonstrated by the micronucleus assay. The mean frequency of micronuclei in the individuals from the rice fields was significantly higher compared to the mean frequency of the individuals from the background region of Strandzha Nature Park. This proves the existence of geno- and cytotoxic effect in the region of the paddies. The micronucleus assay showed no gender differences. The statistically significant differences in mean frequencies of micronuclei in striped field mice both from the impact and from the background area demonstrated the good genomic sensitivity of the species against anthropogenic pollution. The obtained results confirm the importance of *Apodemus agrarius* as a zoomonitor species for biomonitoring studies in the species` characteristic habitats – wetlands.
Key words: biomonitoring, micronucleus test, *Apodemus agrarius*, rice fields

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INTRODUCTION

Rice fields, as wetlands, have high conservation value. Their heterogeneous landscape determines great biodiversity. Agricultural activities associated with rice production have a significant impact on the flora and fauna in rice fields. The main habitats are formed as a result of agricultural operations and their existence largely depends on the use of the land, associated primarily with rice production. The whole process is accompanied by prophylaxis against specific fungal and other diseases in rice, as well as fighting against various pests, both of which require multiple treatments of rice fields with fungicides, pesticides, insecticides, and rodenticides. The needs of farmers may have different degrees of adverse impact on habitats such as increased total mutagenic potential of the environment. These changes may affect the diversity of small communities of mammals living in those agricultural landscapes (1).

Zoomonitoring of small mammal populations, exposed to potential mutagens, can provide an early detection system for the initiation of cell dysregulation. Small mammals possess the features of good zooindicator species because they react promptly to environmental impacts as the intensity of changes occurring in there organisms correlates with the intensity of external stress factors. The striped field mouse (*Apodemus agrarius)* is an appropriate zoomonitor species suitable for genotoxicological research, especially due to its wide distribution, r-type reproductive strategy, relatively small home range, high trophic chain position and metabolic rate. Mice of the *Apodemus* genus, which the striped field mouse belongs to, are among the most widely used rodents for evaluation of the anthropogenic impact on the environment. Most biomonitoring studies have been conducted on the wood mouse (*Apodemus sylvaticus* Linnaeus, 1758) and the yellow-necked mouse (*Apodemus flavicollis* Melichor, 1834), which were found to have excellent qualities as bio-indicator species (2–11). Like other species of the *Apodemus* genus, the striped field mouse also exhibits the features of species suitable for biomonitoring studies: it is widely spread and has high population density (12). However, compared to the wood mouse and yellow-necked mouse, the striped field mouse is subject to biomonitoring studies much more rarely. Velickovic (13) determined the effects of environmental pollution on the amount of aberrant bone marrow cells and the fluctuating asymmetry associated with them. Mitkovska et al. (14) used the micronucleus frequency in the striped field mouse for ecological risk assessment in the Strandzha Nature Park (Strandzha NP).

Various biomarkers are used to assess the degree of pollution, such as the micronucleus frequency (2, 6, 15, 16), chromosomal aberrations (4, 8, 10, 13), abnormalities in spermatocytes (6) or haematological parameters (8, 17, 18).

The micronucleus test (19) was developed as a technique to determine the cytogenetic damage induced by chemical mutagens and ionising radiation in the somatic cells of human and animal populations (20, 21). The test is a widely used cytogenetic method for detection of natural mutagens and has been successfully applied in wild rodents since 1978 (22–27). Of all *in vivo* methods used to asses genotoxicity, the one most commonly used is the *in vivo* erythrocytes micronucleus test in rodents (28–33). The detection of micronuclei in a population of dividing cells is an alternative method, replacing the more labour intensive chromosomal aberrations method.

The aim of this study is to assess the genotoxic effect of the environment in rice field areas through the application of an *in vivo* micronucleus test in the striped field mouse population.

MATERIALS AND METHODS

Study areas

The paddies study area is part of the European Ecological Network NATURA 2000 Protected area "Orizishta Tsalapitsa" (*Tsalapitsa rice fields*), code: BG 0002086 (Fig. 1). The exact coordinates of the investigated area are N 42.235730, E 24.562041. The site is a wetland with strong anthropogenic impact due to increased agricultural operations in the area and major roads passing nearby. The area of Strandzha Nature Park serves as a control in this study due to its insignificant anthropogenic impact, remoteness from major industry factories, lack of heavy traffic, and minor share of agricultural areas treated with pesticides.

Sample collection

A total of 31 individuals (21 from Tsalapitsa rice fields and 10 from Strandzha NP) of *A.agrarius* (Pallas, 1771) were studied (Table 1). The species is suitable for the established objective due to its strong biotopic association with wetland habitats and its distribution in both the impact area and the background area.

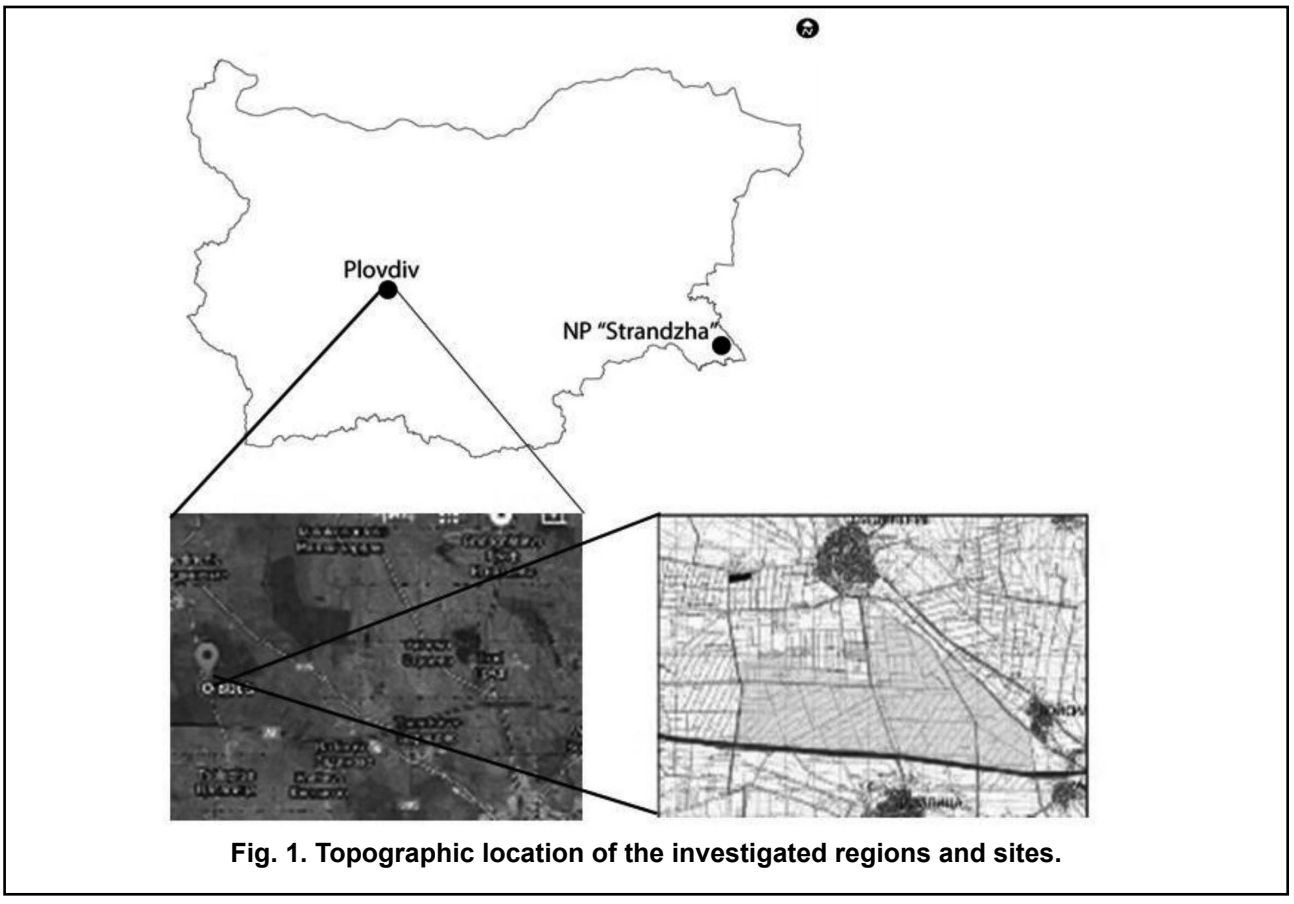

Fig. 1. Topographic location of the investigated regions and sites.

Table 1. Number of studied individuals within the investigated areas.

Species	Tsalapitsa rice fields		Strandzha Nature Park		Total	
Apodemus agrarius	*male*	*female*	*male*	*female*	*male*	*female*
	7	14	6	4	13	18

Methods

On-site studies in the investigated area were carried out during the period of March 2014 – May 2015. Animals were caught by setting live-capture traps (Sherman type) using the trap lines method.

In order to achieve the established objective an *in vivio* micronucleus test was performed on the striped field mouse population *in situ*. According to Hayashi et al. (34) whole blood smears were made on clean glass slides, dried at room temperature, fixed with methanol for 10 min, and stained with Acridine Orange (AO) supravital stain at the time of analysis. Two smears were prepared from each animal. The frequency of micronuclei (MN) was manually scored using a LED fluorescent microscopy (Leica DM 1000) equipped with an appropriate filter and digital photo camera. The cytoplasm of the polychromatic erythrocytes (PCEs) emits red fluorescence, while the micronuclei and the cells nuclei fluoresce yellow-green or yellow. This study used the criteria of Fenech et al. (35) for classification of the intracellular structures. The average micronucleus frequency, which represents the number of micronuclei per 2000 erythrocytes, both PCEs and normochromatic erythrocytes (NCEs), were calculated for each animal tested.

The data were analysed statistically by using the statistical software package Prism, version 4.02 (GraphPad Software, San Diego, CA, USA). First, the data were checked for normality by means of the D'Agostino and Pearson omnibus normality test. Since the micronucleus data did not pass the normality test ($p<0.0001$), non-parametric tests were applied to analyse the results. The Kruskal–Wallis test was used for comparing more than two samples, followed by Dunn's multiple comparison post-test.

RESULTS AND DISCUSSION

The *in vivo* micronucleus test was carried out with fluorescent dye Acridine Orange. Its advantage over the routine dyes (Giemsa, Papenhaym, etc.) is the resulting metachromasia, i.e. the ability to intercalate into double-stranded DNA giving green fluorescence (530 nm) or to bind electrostatically to the phosphate groups of single-stranded nucleic acids (RNA or DNA) fluorescing in red (640 nm). This has been successfully used for the registration of micronuclei in polychromatic and normochromatic erythrocytes. AO-stained micronuclei and nuclei of leukocytes fluoresce intense yellow-green, making them extremely easy to visualise under the microscope (Fig. 2, 3). This proves the applicability and reliability of the micronucleus test in screening for clastogenic and aneugenic activity of potential genotoxic agents in the environment. AO is suitable for distinguishing of different age generations in erythrocytes. The ribosomal RNA still present in the PCEs is removed during the ripening process (within 48 hours) and is absent in the NCEs. Therefore the PCEs are dyed in red. This characteristic of AO was used in the study for reporting the MN both in the PCEs and in the NCEs (Fig. 2).

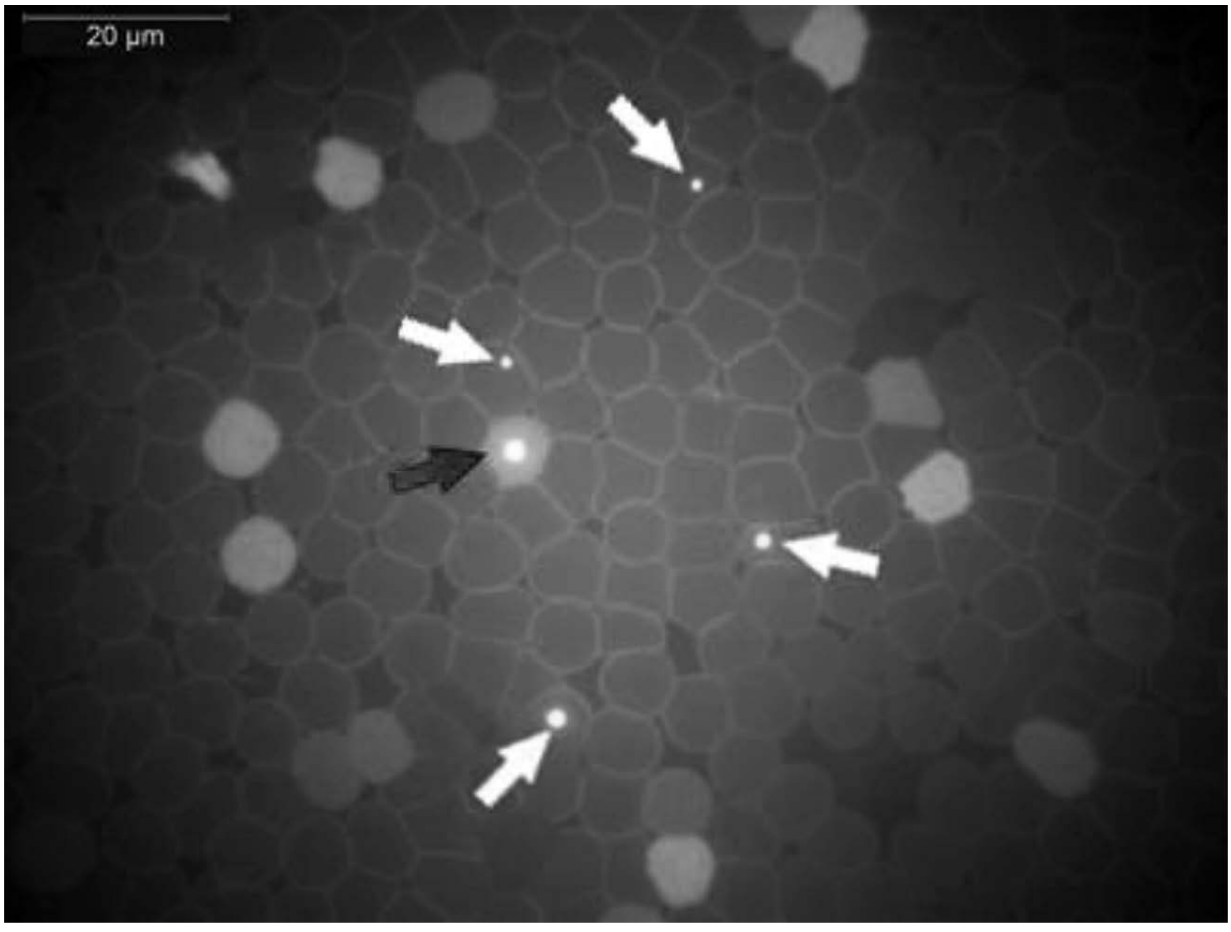

Fig. 2. Micronuclei in NCEs (white arrows) and PCE (red arrow) from peripheral blood of *Apodemus agrarius* from Tsalapitsa rice field with high MN frequency (x1000, *in imersion*).

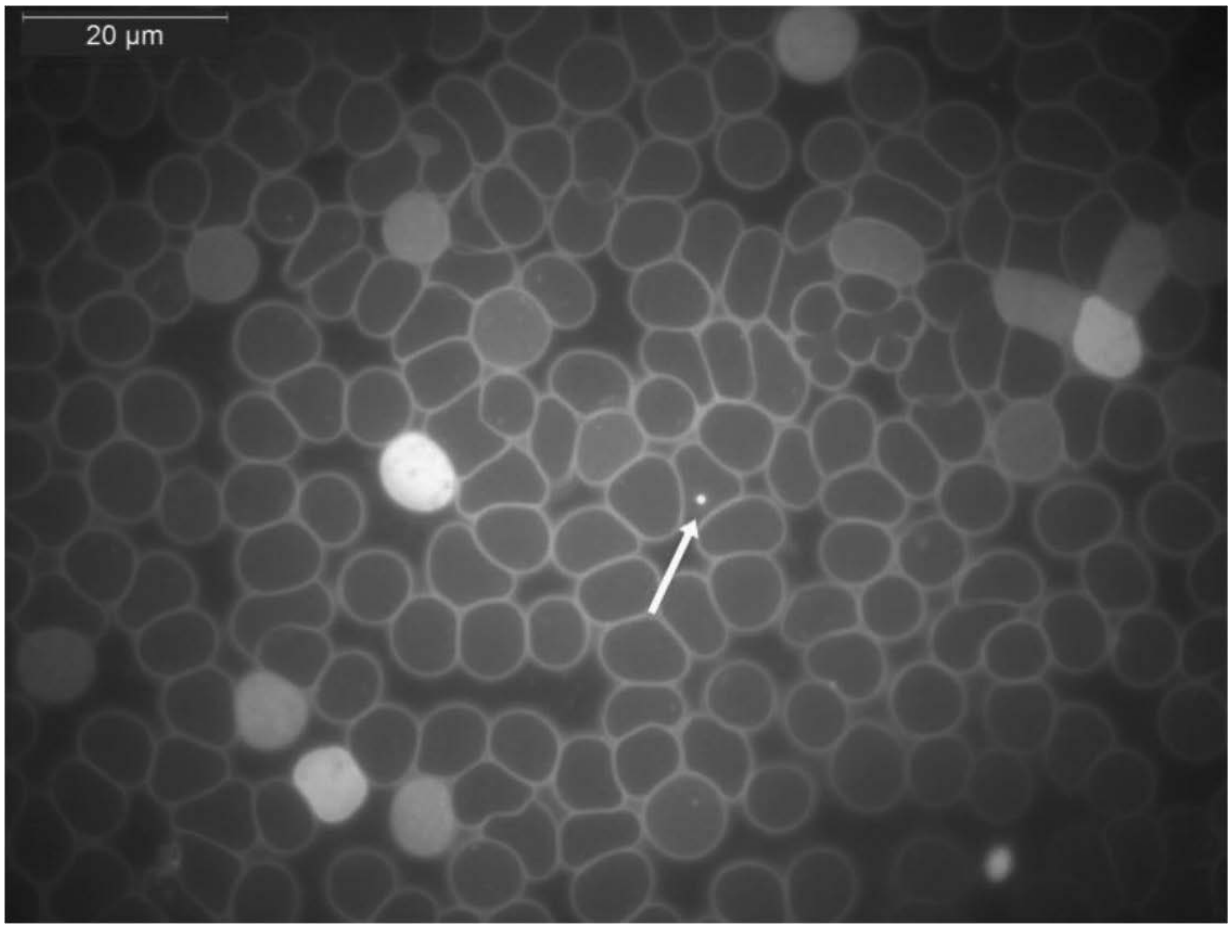

Fig. 3. Micronucleus in NCE (white arrows) from peripheral blood of *Apodemus agrarius* from Strandzha NP (x1000, *in imersion*).

Gender dependence of average micronucleus frequency in peripheral erythrocytes

The resulting average micronucleus frequency (Table 2) of the studied striped field mice from both the impact area and the background area showed no statistically significant dependence on sex (K=2.948; P=0.3998) when applying the non-parametric Kruskal-Wallis statistic test and post-test according to Dunn's multiple comparison test. Female *A. agrararius*, both from the rice fields and the area of Strandzha NP **(**Fig. 1), had statistically insignificant (P>0.05) higher mean micronucleus frequency compared to that of male individuals.

Table 2. Micronucleus frequency in peripheral erythrocytes (PCEs and NCEs) of studied striped field mice within both the impact area and background area. N – sample size; SD – standard deviation.

Parameters	Tsalapitsa Rice Fields		Strandzha NP	
	male	*female*	*male*	*female*
N	7	14	6	4
Mean	0,04965	0,0062	0,0066	0,0050
SD	0,0644	0,0038	0,0030	0,0018
Minimum	0,0021	0,0005	0,0015	0,0024
Maximum	0,1646	0,0165	0,0104	0,0069

According to the obtained results, there is no gender dependence in the formation of micronuclei in the species *A. agrarius*. Such lack of gender dependence is established also by Heuser et al. (36) in laboratory tests with white mice (*Swiss albino line*) in peripheral erythrocytes. Kahl et al. (37) also found a complete absence of gender dependence in a study of the role of Vitamin C in the reduction of the micronucleus formed after nicotine treatment. The average micronucleus frequency in male and female individuals in biomonitoring studies of rodents also showed no gender differences as in the research work of da Silva et al. (26) and Cabarcas-Montalvo et al. (27). Mitkovska et al. (14) also established no statistically significant differences in the mean number of micronuclei in males and females of the Bulgarian populations of *A. agrarius* and other zoomonitor species of the genus *Apodemus, Mus* and *Microtus*.

Gender dependence of the average micronucleus frequency in polychromatic erythrocytes (PCEs)

When comparing the mean micronucleus frequency in polychromatic erythrocytes of studied individuals of both sexes in the impact area and background area the same trend is established. The studied locations revealed no statistically significant differences in the mean micronucleus frequency in polychromatic erythrocytes between male and female individuals (N=2.621; P=0.4539). In both impact area and background area, the males had statistically insignificant higher averages of micronuclei compared to females (Fig. 4).

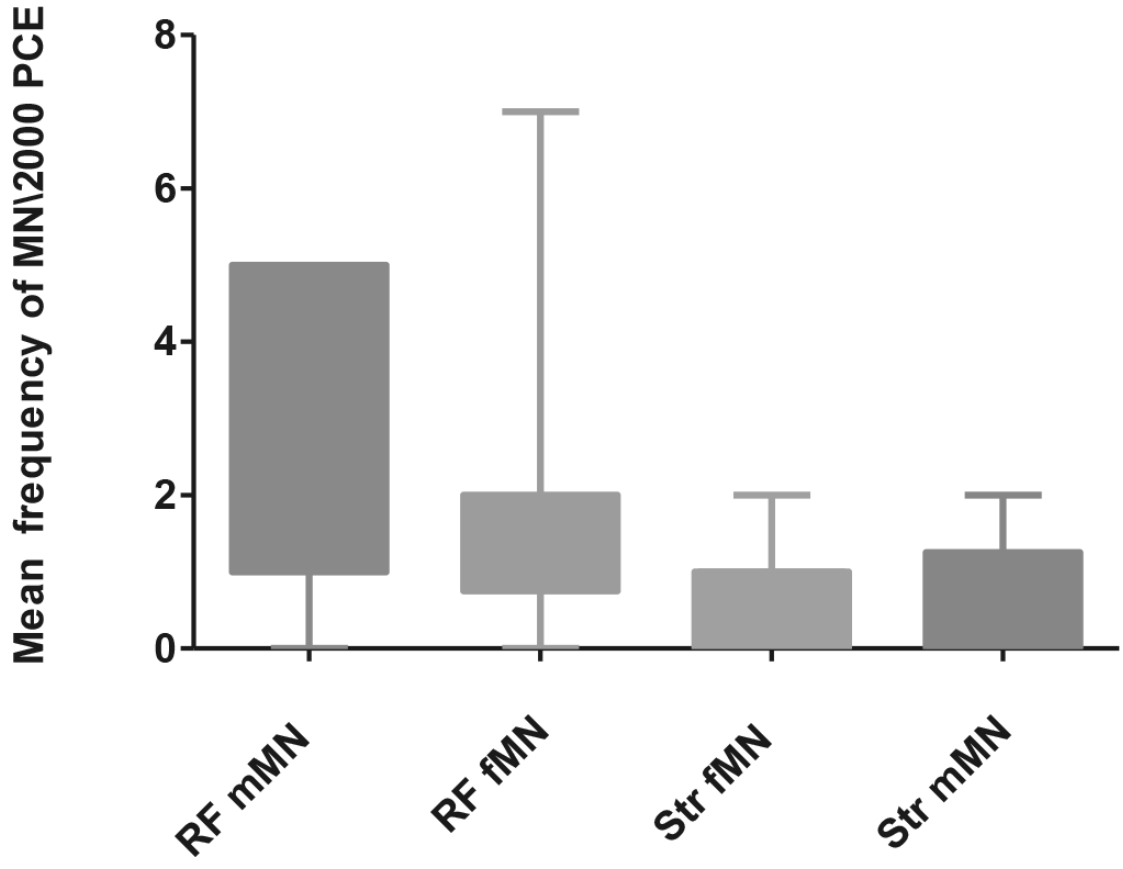

Fig. 4. Mean micronucleus frequency (MN) in polychromatic erythrocytes (PCEs) in both striped field mice sexes from the impact area and background area. RF – rice fields; Str – Strandzha NP; m – male; f – female.

Although these differences are insignificant, they probably illustrate the higher and faster reactivity of male individuals to directly acting environmental cytotoxic agents. This could be associated with the fact that they occupy a larger home range, which makes them more exposed to genotoxic and cytotoxic stress agents of different nature. These insignificant differences may be the result of random processes or circumstances.

Comparative analysis of micronucleus frequency in normochromatic erythrocytes (NCEs) and polychromatic erythrocytes (PCEs) in the studied species from the impact area and background area

The results obtained for the mean micronucleus frequency in normochromatic erythrocytes and polychromatic erythrocytes are shown in Fig. 5. For Tsalapitsa rice fields the obtained mean values of MN fall within the range of 0.0043 to 0.0147 for NCEs and 0.0005 to 0.0333 for PCEs and are similar to those obtained in other biomonitoring studies of small rodents (26, 27, 38).

The values obtained in the background area take an intermediate position when compared with published data from studies of highly polluted areas which have suffered environmental accidents (26) or on coal mining areas (27). The applied nonparametric Kruskal-Wallis test showed statistically significant differences (K=13.08, P=0.0045) between the obtained

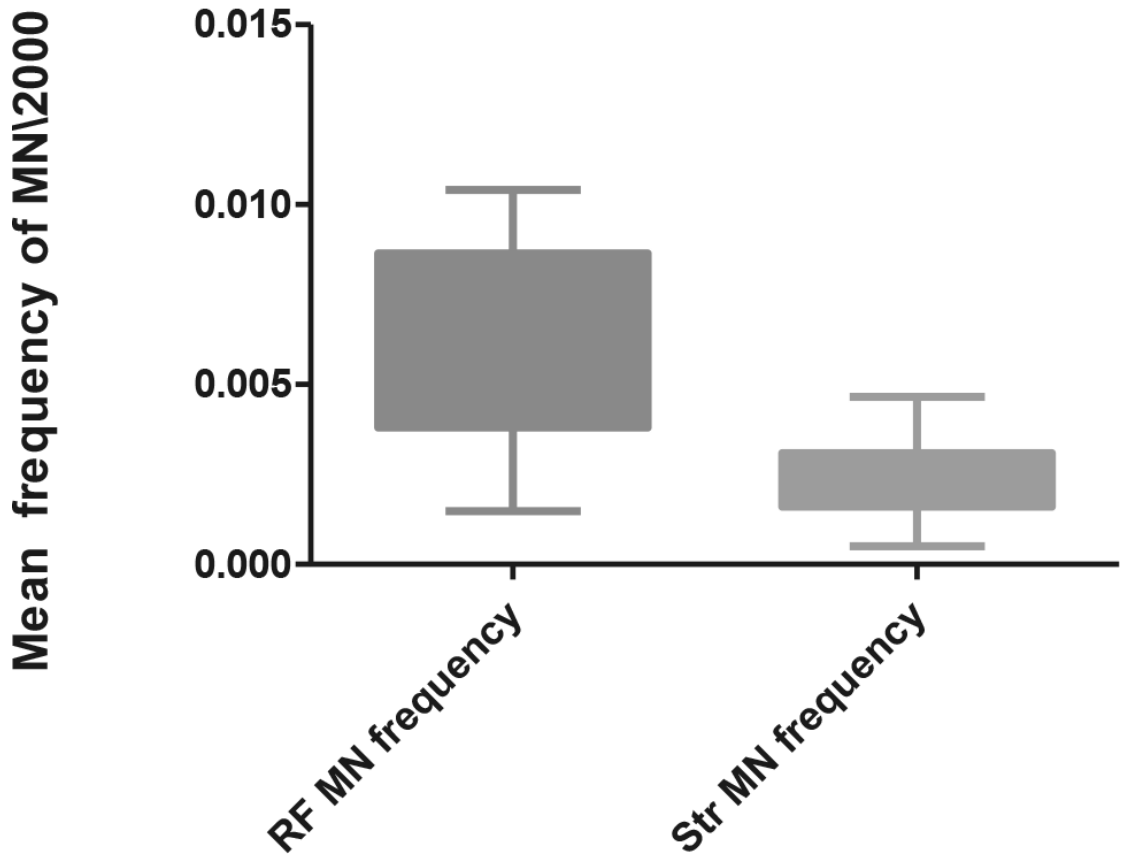

Fig. 5. Mean micronucleus frequency (MN) in peripheral erythrocytes (Ery) in striped field mice from the impact area and background area. RF – rice fields; Str – Strandzha NP.

mean micronucleus frequencies in individuals studied in the impact area and background area. Dunn's multiple comparison post-test showed intergroup differences only among the females from both areas (difference in average values – 16.43; $P<0.05$).

The results obtained by Mitkovska et al. (14) for the species of the *Apodemus* genus are evidence of the stronger impact of heavy metal pollution on micronucleus frequency compared to pollution in agricultural areas. It is a consequence based on the difference in the nature of both pollution types. For the area of Strandzha NP the mean micronucleus frequency values in PCEs and NCEs were similar to those obtained in the current study. This once again confirms the status of the region as an uncontaminated territory with extremely low anthropogenic impact. High mean micronucleus frequency in peripheral erythrocytes and significant differences in the number of micronuclei in polychromatic and normochromatic erythrocytes of striped field mice from the impact area compared to those in the background area prove the existence of genotoxic and cytotoxic impact in the region of Tsalapitsa rice fields. The high values of micronucleus frequency in the region of Tsalapitsa rice fields are evidence of permanent DNA damage that cannot be repaired. The total mutagenic potential of the environment in the area of Strandzha NP can be defined as weak and insignificant due to the resulting low values of the micronucleus frequency indicator in the studied zoomonitor species. High mean MN-frequency in polychromatic erythrocytes was found in striped field mice from the background area, which may be used as a biomarker for quick assessment of the effects of genotoxic agents that occur within several days. The genomic response of the striped field mouse to anthropogenic pollution, confirmed by statistically significant differences in the micronucleus frequency within the impact area and background area, proves the zoomonitor importance of the studied species for biomonitoring studies in wetland habitats and areas of conservation importance.

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