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PROBLEMS
of Infectious and Parasitic Diseases

NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 41, NUMBER 1/2013

ISSN 0204-9155

1504 Sofia; 26, Yanko Sakazov Blvd.
Tel.: +359 2/ 846 83 07, Fax: +359 2/ 943 30 75
e-mail: infovita@ncipd.org

PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 41, NUMBER 1/2013

Preprinting and Printing by Maker Arts & Alliance print

CONTENTS

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2. Drafting the article or revising it critically for important intellectual content; and
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ACKNOWLEDGEMENTS

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

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MULTIDRUG RESISTANT TUBERCULOSIS IN BULGARIA – MICROBIOLOGICAL ASPECTS

S. Yordanova, E. Bachiyska, Y. Atanasova, Y. Todorova, A. Baikova, T. Kantardjiev

National Reference Laboratory of Tuberculosis,
National Center of Infectious and Parasitic Diseases
(NRL TB, NCIPD), Sofia, Bulgaria

ABSTRACT

The aim of the study was to examine the multidrug resistant tuberculosis (MDR-TB) in Bulgaria in 2012 using microbiological methods (phenotypic and genetic testing) and to describe its distribution in the country.

The clinical isolates of 67 patients were confirmed in NRL TB, NCIPD as MDR-TB in 2012. The conventional drug susceptibility testing (DST) was performed by BACTEC MGIT 960 System to first (FLDs) and second line drugs (SLDs) using the following critical concentrations: Streptomycin (STR) 1,0 μg/ml, Isoniazid (INH) 0,1 μg/ml, Rifampin (RMP) 1,0 μg/ml, Ethambutol (EMB) 5,0 μg/ml, Ofloxacin (OFL) 2,0 μg/ml, Amikacin (AMK) 1,0 μg/ml, Kanamycin (KAN) 5,0 μg/ml and Capreomycin (CAP) 2,5 μg/ml. The mutations in the relevant genes were detected by GenoType® MTBDR*plus* and GenoType®MTBDR*sl*.

Nearly half of the MDR-TB strains (47,76%) were resistant to all of the tested FLDs. The majority of the MDR-TB isolates were sensitive to SLDs – 83,58%, whereas 8,96% (n= 6) were defined as XDR-TB (Extensively drug resistant tuberculosis). The most common mutation in the *rpo*B gene was S531L (88, 06%). The C15T mutation in the regulatory region of the *inh*A gene was much more frequent (n=29) than the S315T1 one in *kat*G (n=18).

The highest prevalence of MDR-TB in Bulgaria was in the districts of Plovdiv, Montana and Vidin. There were five cases of MDR-TB in the penitential system found in prisoners. One of these patients was a woman.

Key words: *M.tuberculosis* complex, multidrug resistant tuberculosis, extensively drug resistant tuberculosis

INTRODUCTION

Although the mortality and incidence rates of tuberculosis (TB) are decreasing slowly worldwide, multidrug resistant tuberculosis remains a major concern [6]. According to WHO Bulgaria is among the 27 high MDR-TB burden countries with the estimated 2, 0 % (CI 1,1-3,2) multidrug resistance among new cases and 26 % (CI 19-33) among previously treated cases for 2012 [6].

The aim of this microbiological study was to examine MDR-TB strains in Bulgaria throughout 2012 using phenotypic and genetic testing and to describe its distribution in the country.

ADDRESS FOR CORRESPONDENCE:
Stanislava Yordanova
NRL TB, NCIPD, Sofia
44A Stoletov Blvd, Sofia 1233
Bulgaria
Phone: +359 894 389 093
E-mail: s.yordanova79@gmail.com

MATERIALS AND METHODS

This study includes all confirmed as MDR-TB strains in NRL TB, NCIPD in 2012. The patients were from all over the country. The strains were isolated from sputa – 98,51% (n = 66) and from pleural fluid – 1,49% (n = 1). According to their history of TB treatment 19,40% (n=13) of the patients were qualified as new cases and 80,60% (n=54) were previously treated cases. Every patient is represented by a single strain in the data processing.

Confirmation of *M.tuberculosis* complex was done by BD MGIT™ TBc Identification Test – a chromatographic test, which detects MPT64 in liquid cultures.

The conventional drug susceptibility testing (DST) was performed by BACTEC MGIT 960 System to FLDs and SLDs as follows: STR – 1,0 μg/ml; INH – 0,1 μg/ml; RMP – 1,0 μg/ml; EMB – 5,0 μg/ml; OFL – 2,0 μg/ml; AMK – 1,0 μg/ml; KAN – 5,0 μg/ml and CAP – 2,5 μg/ml.

The molecular tests (Line Probe Assays) GenoType® MTBDR*plus* and GenoType® MTBDR*sl* were also performed in order to detect the mutations in target genes: *rpo*B, *kat*G, *inh*A, *gyr*A and *rrs* [3].

RESULTS AND DISCUSSION

Most of the cases were between 25 and 64 years old – 88,06% (n= 59) . The age structure of the MDR-TB patients is shown in ***Figure 1***.

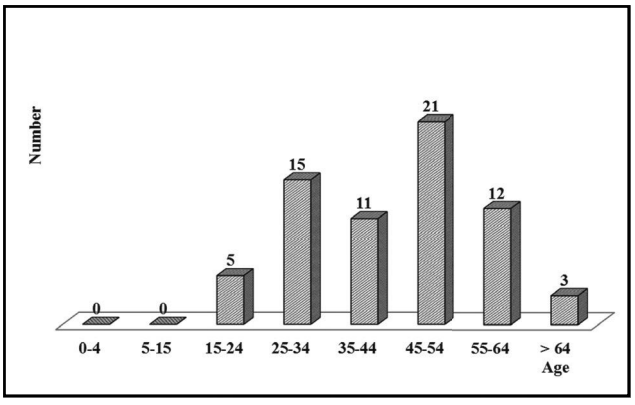

Figure 1: Age structure of MDR-TB patients confirmed in NRL TB in 2012

There were no children diagnosed with MDR-TB in 2012. Significant decrease of MDR-TB was observed in the age group over 65, compared to 2011 [1].

The Male/female ratio was 3,46:1 (77,61% (n=52) men and 22, 39% (n=15) women). Compared with the

results for 2011 (2,77:1), there is a slight difference in the gender distribution [1]. The sex ratio varied within the age groups.

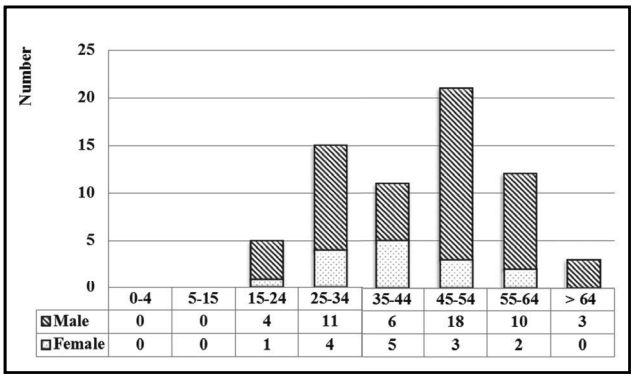

Figure 2: Gender distribution of the MDR-TB patients, confirmed in NRL TB, 2012 by age groups

Almost equal gender distribution of MDR-TB patients (1,2:1) was observed in the age group of 35 to 44 years old. The most significant difference in male/ female ratio was in the age group of 45 to 54 years old (6:1).

Distribution of MDR-TB in Bulgaria by districts is shown in ***Table 1***. The highest level of MDR-TB was established in the district of Plovdiv – n = 11, of which eight were previously treated cases. Next in rating by number of MDR-TB were the districts of Montana and Vidin, followed by Vratsa and Sofia city. Five of the MDR-TB patients were prisoners, one of whom was female [4].

The phenotypic performance of the clinical isolates according their FLDs resistance pattern is shown in ***Figure 3***.

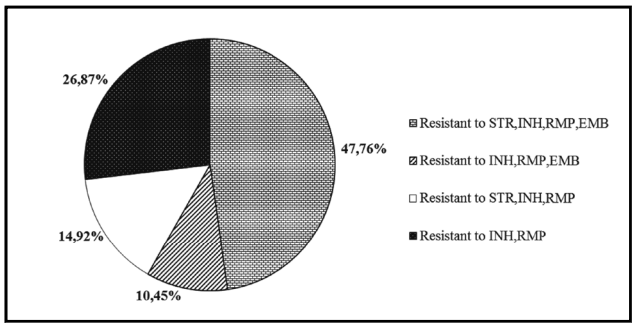

Figure 3: FLDs resistence pattern of MDR-TB clinical isolates in 2012

The largest number of the isolates were simultaneously resistant to all of the tested FLDs– 47,76% (n=32). Resistant to Isoniazid and Rifampin only showed 18 of the examined isolates (26, 87%).

The majority of the MDR-TB strains – 83,58% (n=56) were phenotypically susceptible to SLDs (OFL, AMK, KAN and CAP). Resistance to Ofloxacin was found in four clinical isolates (5,97%). Resistance to injectable SLDs (AMK, KAN and CAP simultaneously), without any resistance to Fluoroquinolones, was found in one strain. No resistance to single injectable drugs was detected. Six MDR-TB clinical isolates (8,96%) were defined as XDR-TB, two of them newly confirmed in 2012. Four of XDR-TB showed resistance to OFL, to all of the tested injectable drugs and Moxifloxacin but were susceptible to Ofloxacin. No case of XDR-TB resistant to OFL and to a single injectable drug was detected in Bulgaria in 2012. The results of phenotypical second line drugs testing can be seen in ***Figure 4***.

Table 1**:** Distribution of Bulgarian MDR-TB cases, confirmed in 2012 by NRL TB

District	Number MDR-TB	Number XDR-TB	District	Number MDR-TB	Number XDR-TB
Blagoevgrad	0	0	**Plovdiv**	11	4
Burgas	3	0	**Razgrad**	0	0
Varna	3	0	**Ruse**	1	0
Veliko Tarnovo	0	0	**Silistra**	0	0
Vidin	6	1	**Sliven**	1	0
Vratsa	5	0	**Smolyan**	0	0
Gabrovo	3	1	**Sofia city**	5	0
Dobrich	3	0	**Sofia -district**	2	0
Kardjali	0	0	**Stara Zagora**	2	0
Kjustendil	0	0	**Targovishte**	1	0
Lovech	3	0	**Haskovo**	1	0
Montana	7	0	**Shumen**	1	0
Pazardzhik	2	0	**Yambol**	0	0
Pernik	2	0	**Prisons**	5	0
Pleven	0	0			

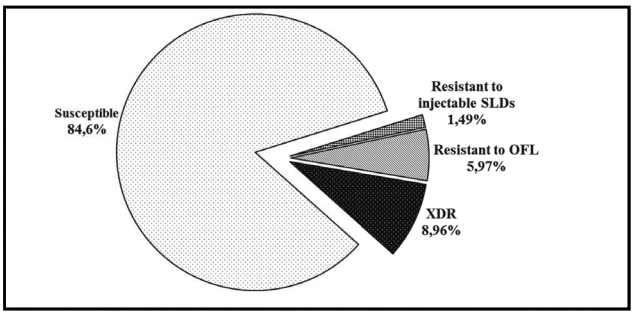

Figure 4: Phenotypical second line drugs susceptibility pattern of MDR-TB clinical isolates in 2012

The molecular tests GenoType ® MTBDR *plus* and GenoType® MTBDR*sl* can detect *M.tuberculosis* complex from acid fast bacilli positive sputa or culture and the most frequent mutations, associated with resistance to the main anti-TB drugs. The summarised results of the molecular testing are shown in Tables 2, 3 and 4.

The most frequent Rifampin resistance determining mutations in Bulgarian MDR-TB isolates for 2012 occurred in codon 531 of the *rpo*B gene – 91, 05% (n=61). S531L substitution was found in 88, 06% (n=59) of the MDR-TB isolates. In one of the 67 MDR-TB (1,49%) the test did not detect mutation in the 81 - bp Rifampicin Resistance Determining Region of the *rpo*B gene. In order to identify the cause of the confirmed phenotypical resistance to Rifampicin of the strain overall sequencing of the *rpo*B gene is required.

The mutations associated with Isoniazid resistance are located in several genes and regions. According to different authors, between 50% and 95% of INH - resistant strains had a mutation in the *kat*G gene, codon 315 and 20 to 35% - in the regulatory region of the *inh*A gene [9, 10, 11, 13]. Other genes associated with the INH resistance are *ahp*C, *oxy*R, whether combined or not with mutations in codon 315 of the *kat*G gene [11, 12, 13].

In 26,87% (n=18) of the Bulgarian MDR-TB clinical isolates the test did not detect mutation in the target genes. The phenotypic Isoniazid resistance in that strains suggests involvement of others genes or mechanisms of resistance.

C15T in the *inh*A gene was far more frequent than S315T1 mutation in the *kat*G gene (43,28% and 26,87%, respectively), while in 2011 there were no differences in their occurrence – 39,7% [1].

According to conventional DST to SLDs, eight isolates were resistant to Moxifloxacin and Ofloxacin and two were Moxifloxacin resistant, while being Ofloxacin susceptible. In the last ones GenoType ® MTBDR*sl* did not detect mutation in the *gyr*A gene. The lack of mutation in *gyr*A in Fluoroquinolone resistant strains is not rare, which gives grounds for searching other resistant mechanisms [2, 5, 7, 8, 14]. Despite their cross-resistance to Fluoroquinolones, there are reports for Moxifloxacin resistant but Ofloxacin sensitive *M.tuberculosis* complex strains [14].

Table 2: GenoType ® MTBDR *plus* results for Rifampin resistance of MDR-TB clinical isolates confirmed in 2012

n (%)	*rpo*B	Nucleotide/aminoacid substitution	MTBDR*plus* interpretation	BACTEC MGIT 960 System
1 (1,49%)	WT 1-8	0	RMP - S	RMP - R
1 (1,49%)	ΔWT2/3	*Q 510-517L/P, del514-516**	RMP - R	RMP - R
1 (1,49%)	ΔWT3/4	*D516Y,del515**	RMP - R	RMP - R
1 (1,49%)	ΔWT3/4, MUT1	D516V	RMP - R	RMP - R
1 (1,49%)	ΔWT7	*H526R/P/Q/N/L/S/C**	RMP - R	RMP - R
1 (1,49%)	ΔWT7, MUT2B	H526D	RMP - R	RMP - R
2 (2,99%)	ΔWT8	*S531P/Q/W; L533P**	RMP - R	RMP - R
59 (88, 06%)	ΔWT8, MUT3	S531L	RMP - R	RMP - R

* *To specify the nucleotide/aminoacid substitution in the corresponding codons, sequencing of the gene is required*
Δ – deletion; WT – wild type; MUT – mutation; RMP – Rifampin; R – resistant; S- susceptible

Table 3: GenoType ® MTBDR *plus* results for Isoniazid resistance of MDR-TB clinical isolates confirmed in 2012

n (%)	*kat*G	*inh*A	Nucleotide/ aminoacid substitution	MTBDR*plus* interpretation	BACTEC MGIT 960 System
18 (26, 87%)	ΔWT1, MUT1	WT	S315T1	INH - R	INH - R
29 (43, 28%)	WT	ΔWT1, MUT1	C15T	INH - R	INH - R
2 (2, 98%)	ΔWT1, MUT1	ΔWT1, MUT1	S315T1/C15T	INH - R	INH - R
18 (26, 87%)	WT	WT	0	INH - S	INH - R

Δ – deletion; WT – wild type; MUT – mutation; INH – Isoniazid; R – resistant; S- susceptible

Table 4: GenoType® MTBDR *sl* results for Fluoroquinolones susceptibility of Bulgarian MDR-TB clinical isolates in 2012

n (%)	*gyr*A	Nucleotide/aminoacid substitution	MTBDR*sl* Interpretation	BACTEC DST result
4 (5,97%)	ΔWT2, MUT1	A90V	FQ – R	OFL/MFX - R
1 (1,49%)	ΔWT2, MUT2	S91P	FQ – R	OFL/MFX - R
2 (2,99%)	ΔWT3, MUT3A	D94A	FQ – R	OFL/MFX - R
1 (1,49%)	ΔWT3, MUT3C	D94G	FQ – R	OFL/MFX - R
2 (2,99%)	WT1-3	0	FQ – S	OFL – S MFX - R
57 (85,07%)	WT1-3	0	FQ – S	OFL – S

Δ – deletion; WT – wild type; MUT – mutation; FQ – Fluoroquinolones; OFL – Ofloxacin; MXF – Moxifloxacin; R - resistant; S - susceptible

The most common *gyr*A mutation in the study was A90V (GCG → GTG) in 40 % of the cases (***Table 4***). According to conventional DST testing seven of the MDR-TB strains were resistant to Amikacin, Kanamycin and Capreomycin. In all of them the A1401G mutation in the *rrs* gene was detected, which determines high level resistance to Amikacin and Kanamycin. Comparable results were reported in the preceding one-year period [1].

CONCLUSIONS

The prevalence of MDR-TB was among the previously treated patients (80,60%). The most frequent drug susceptibility pattern to FLDs was resistance to all of tested ones (47,76%). Sensitive to SLDs were 83,58% of the MDR-TB isolates. Extensively drug resistance was found in 8,96% (n= 6) of the tested in 2012 MDR-TB strains. The C15T mutation in the regulatory region of the *inh*A gene was much more frequent (n=29) than the S315T1 one in *kat*G (n=18). The highest prevalence of MDR-TB in Bulgaria was in the districts of Plovdiv, Montana and Vidin, followed by Vratsa and Sofia city.

ACKOWLEDGEMENTS

This study was possible thanks to the consumables and reagents provided by The Global Fund to Fight AIDS, Tuberculosis and Malaria: BUL-607-G02-T, BUL-809-G03-T.

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ANTIMICROBIALS' RESISTANT SALMONELLA STRAINS TESTED FOR SUSCEPTIBILITY TO HYBRID MATERIAL WITH INCLUDED SILVER NANOPARTICLES

***Martin Iliev*[1]**

[1]Bul Bio-National Centre of Infectious and Parasitic Diseases

ABSTRACT:
Salmonella infections are of great public importance because of the wide geographical distribution, high infectiveness regardless of age and the fact that they are antropozoonoses. The most common causes of salmonellosis have been isolated from human serovars *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Salmonella enterica* serovar Infantis is isolated with high frequency in poultry farms. Because of reported increase in antibiotic resistance of isolated Salmonellas, monitoring of silver resistance in clinical Salmonella isolates was undertaken. The purpose is to establish the possibility of a therapeutic choice of hybrid materials comprising silver nanoparticles /AgNps/ in isolates that are resistant to more than two antibiotics. The test was conducted by the method of two-fold serial macro-dilutions according CLSI M26-A and certain MBC /Minimal Bactericidal Concentrations/ of the hybrid material PVA /polyvinyl alcohol/ /AgNps against the tested strains. Tests performed found synergistic effects in the combination of ceftazidime and PVA / AgNps. The strains with the highest levels of MBC (1.1 mg / L) would be subject of testing for a carriage of genes for silver resistance.

INTRODUCTION:
Salmonella Typhimurium causes 25% of the 1.4-million salmonellosis infections a year in the United States. Most persons infected with *Salmonella* sp. develop diarrhoea, fever, and abdominal cramps 12 - 72 hours after infection. The illness usually lasts 4 - 7 days, and most people recover without treatment. However, in some cases, the diarrhoea may be so severe that the patient needs to be hospitalized [1]. In other countries such as Bulgaria, Finland, United Kingdom, Sweden, Germany, Norway etc. salmonellosis infections caused by *S. Enteritidis* are registered every year [2]. In the European Union (EU), the serovars *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most frequent causes of gastroenteritis in humans. Food borne diseases caused by non-typhoid Salmonella are found to be a major public health problem in many parts of the world [3]. Fallah SH et al. alarmed that high level of resistance to antibacterial drugs commonly used for human and poultry can be a threat for the community health. The most common isolated stereotype of his study was *Salmonella enterica* serovar Infantis (79.5%). Although *Salmonella* Typhimurium or *Salmonella* Enteritidis were isolated from meat materials the prevalence of food contamination by *Salmonella* Infantis has been shown to be increasing. Their results indicate that among the *Salmonella* strains 88.7% had resistance to more than 3 antibiotics [4]. This literature search warrants looking for another way to combat the increasing antimicrobial resistance among the isolated *Salmonella* strains [5]. Such a choice may be the growing assortment of hybrid materials containing silver nanoparticles [6, 7, 8].

ADDRESS FOR CORRESPONDENCE:
Martin Iliev
Bul Bio-NCIPD
1504 Sofia, Bulgaria
26 Yanko Sakazov
e-mail: martin.iliev7@gmail.com

Aim:
The main goal of the research is testing the susceptibility of resistant to different antimicrobials *Salmonella* spp. to hybrid material with included silver nanoparticles in an established model [9].

Materials and methods:
The used hybrid material with silver nanoparticles, stabilized in polyvinyl-alcohol (PVA/AgNps), was synthesized through thermal reduction of silver nitrate in 100°C [10]. It was used in two samples with different silver concentration, determined by ICP -175.9 mg/L and 2032 mg/L. With them ten strains of *Salmonella* spp. were tested, using methods already established in the practice for testing of biological activity of such kind of hybrid materials, such as the modified Disk Diffusion Method [11], the method of macro-dilutions [12] and the method for testing presence of synergism [13].

Results and discussion:
The tested clinical Salmonella spp. strains were isolated mostly from people but one of them *(Salmonella Kentucky 1257)* originated from chicken.
At first, the susceptibility of the Salmonella spp. strains to antibiotics was determined (Table 1, page 10).
By testing with Disk Diffusion Method, in a set of discs impregnated with the sample (silver concentration 175.9 mg/L) lack of any zones of full suppression of the tested salmonellas was observed. The testing with discs impregnated with the second sample (silver concentration 2032 mg/L) showed zone of full inhibition only by two Salmonella spp. strains (Table2, Figure 1, page 10).
The lowest values of Minimal Bactericidal Concentration was observed in four of the tested strains, but only in one of them the result corresponded to the showed zone of full inhibition (Table 3, page 11).

Table 1: Results of testing antibiotics of clinical strains Salmonella spp., isolated from people. (The examinations were made by National Reference Laboratory of Enteric Pathogens, "National Centre of Infectious and Parasitic Diseases")By testing with Disk Diffusion Method, in a set of discs impregnated with the sample (silver concentration 175.9 mg/L) lack of any zones of full suppression of the tested salmonellas was observed. The testing with discs impregnated with the second sample (silver concentration 2032 mg/L) showed zone of full inhibition only by two Salmonella spp. strains (Table2, Figure 1).

Antibiotic	**Zone of inhibition in mm and interpretation according CLSI**																
Strain	**CTX**	**FOX**	**Cb**	**CAZ**	**CXM**	**CF**	**A**	**AMC**	**G**	**T**	**C**	**Cp**	**Nx**	**ST**	**AN**	**ESBL**	**CTXM / AmpC**
Salmonella Infantis 6	27S	21S	-	25	-	-	22S	24S	25S	6R	26S	26	6R	6R	24S	-	-
Salmonella Infantis 475	28S	20S	-	26S	-	-	19	23	24S	6R	20S	22	6R	6R	24S		
Salmonella Typhimurium 47	29S	24S	6R	25S	19I	18S	6R	16I	24S	6R	6R	29S	6R	6R	24S	-	-
Salmonella Paratyphi B 176	6R	24S	-	12R	-	-	6R	6R	6R	17	26S	31	20	6R	-	+	
Salmonella Infantis 210	30S	21S	24S	24S	18	18	22	24	27	6R	23	23	6R	6R	27	-	-
Salmonella Agona 568	-	11R	-	-	-	-	6R	8R	23S	6R	18	19	6R	6R	23S		AmpC
Salmonella Brandenburg 854	14R	26S	6R	22S	6R	6R	6R	21S	6R	26S	30S	36S	26S	6R		+	+
Salmonella Brandenburg 891	14R	26S	6R	24S	6R	6R	6R	21S	6R	26S	29S	35S	25S	6R	6R	+	+
Salmonella Kentucky 1257	19I	14R	26S	16I	17I	6R	6R	9R	27S	6R	31S	32S	22S	28S	24S		AmpC
Salmonella Corvallis 1888	16I	27S	6R	25S	6R	6R	6R	20S	11R	19S	27S	31S	22S	27S	25S	+	+

Legend: CTX- Cefotaxime, FOX –cefoxitin, Cb-carbenicillin, CAZ-ceftazidime, CXM-cefuroxime, CF-cephalothin, A-ampicillin, AMC-amoxicillin clavulanic acid, G-gentamicin, T-tetracicline, C-chloramphenicole, Cp-ciprofloxacin, Nx-nalidix acid, ST-sulfametoxasole/trimetoprime, AN-amicacin, ESBL- producing of Extended-Spectrum Beta-lactamase, CTXM / AmpC- producing of CTX-M enzymes and Amp C enzymes.

Table 2: Testing resistant to antibiotics clinical strains *Salmonella sp.* with hybrid material PVA/AgNps (2032 mg/l) by DDM for determination of silver resistance

Disks impregnated with sample №	**№1-2032 mg/l**	**№2-1016 mg/l**	**№3-508 mg/l**	**№4-254 mg/l**
Strain	**Zone of inhibition in mm**			
Salmonella Infantis 6	0	0	0	0
Salmonella Infantis 475	0	0	0	0
Salmonella Typhimurium 47	0	0	0	0
Salmonella Paratyphi B 176	7mm	0	0	0
Salmonella Infantis 210	0	0	0	0
Salmonella Agona 568	12mm	10mm	9mm	7mm
Salmonella Brandenburg 854	0	0	0	0
Salmonella Brandenburg 891	0	0	0	0
Salmonella Kentucky 1257	0	0	0	0
Salmonella Corvallis 1888	0	0	0	0

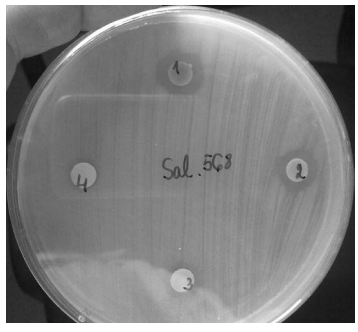

Figure 1: DDM of impregnated with hybrid material disks against Salmonella Agona 568

Table 3: Determination of the susceptibility of clinical strains Salmonella spp. by macro-dilution method MIC

Tube	**№1**	**№2**	**№3**	**№4**	**№5**	**№6**	**№7**	**№8**	**№9**	
Silver concentration (mg/l)	35,2	17,6	8,8	4,4	2,2	1,1	0,5	0,3	0,14	**MBC**
Final silver concentration with added standardized to 0,5MF bacterial suspension	17,6	8,8	4,4	2,2	1,1	0,5	0,3	0,14	0,07	
***Salmonella* Infantis 6**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	41 CFU	confluent growth	confluent growth	confluent growth	1,1
***Salmonella* Infantis 475**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	>300 CFU	confluent growth	confluent growth	confluent growth	1,1
***Salmonella* Typhimurium 47**	0 CFU	0 CFU	0 CFU	0 CFU	1 CFU	confluent growth	confluent growth	confluent growth	confluent growth	2,2
***Salmonella* Paratyphi B 176**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	>300 CFU	confluent growth	confluent growth	confluent growth	1,1
***Salmonella* Infantis 210**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	0,3
***Salmonella* Agona 568**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	>300 CFU	confluent growth	confluent growth	0,5
***Salmonella* Brandenburg 854**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	9 CFU	confluent growth	confluent growth	confluent growth	1,1
***Salmonella* Brandenburg 891**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	confluent growth	0,5
***Salmonella* Kentucky 1257**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	>100 CFU	confluent growth	confluent growth	confluent growth	1,1
***Salmonella* Corvallis 1888**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	confluent growth	0,5

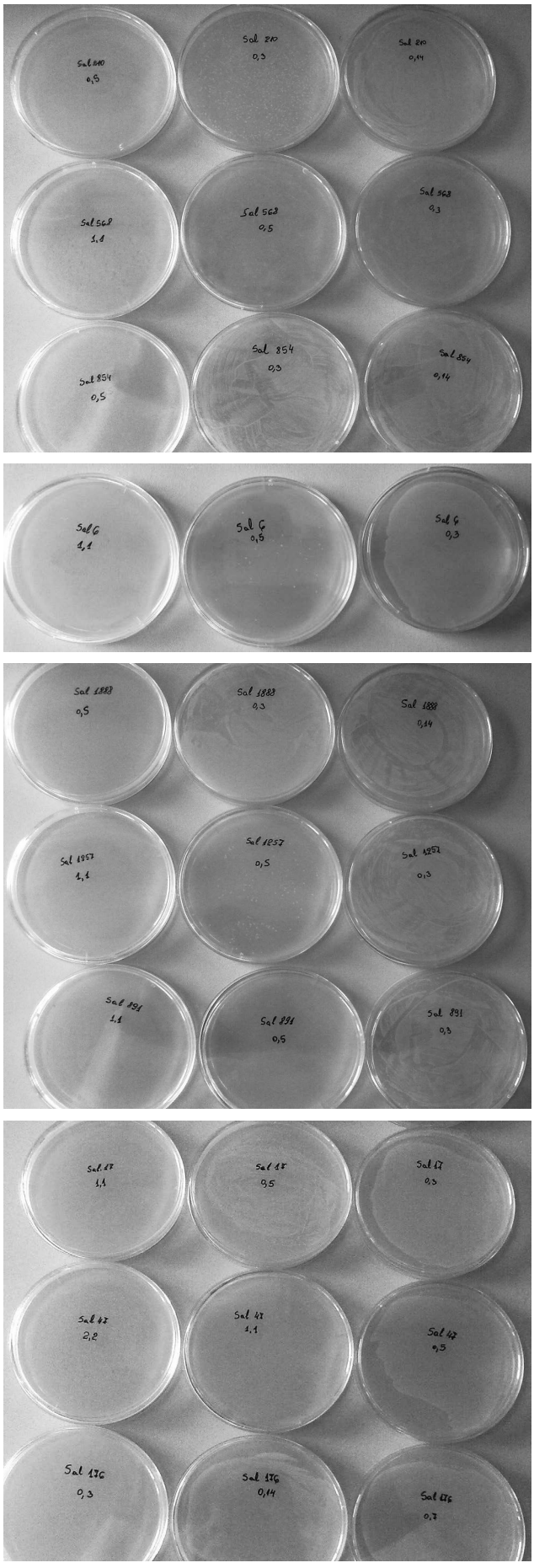

Figure 2: Macro-dilution method for determination of MBC of the tested salmonella strains to the hybrid material with 175.9 mg/L silver concentration.

The determined values of MBC were in the range from 0,3 to 2,2 mg/L silver. By testing the bactericidal properties of hybrid materials, the presence of any zone of inhibition is accepted as indicative for the existence of bactericidal activity. Therefore, the main method for determination of silver resistance remains the method of macro-dilutions (Figure 2).

The possibility for combination of an antibiotic with the hybrid material was studied for the purpose of establishing the existence of synergism on Salmonella Paratyphi B 176 (Table 4, Figure 3).

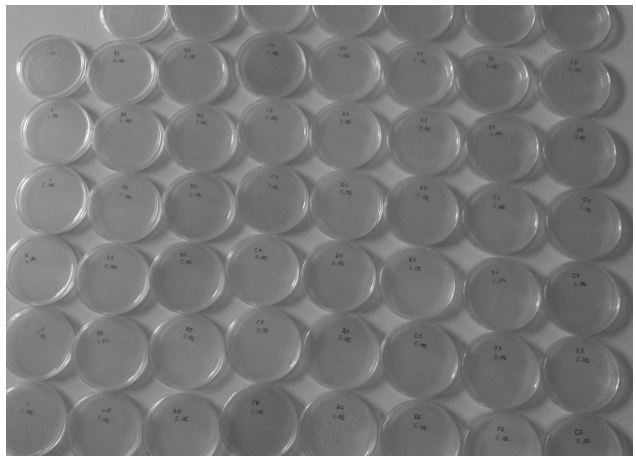

Figure 3: Testing for presence of synergism by combining of PVA/AgNps with ceftazidime on Salmonella Paratyphi B 176.

The calculation of Fractional bactericidal concentration (FBC) (EUCAST, 2000) on the combined action of ceftazidime and the hybrid material was:

$$\mathbf{FBC(Cz)} = \frac{\mathbf{MBC}\left(\mathbf{Cz\ in\ the\ presence\ of}\ \frac{\mathbf{PVA}}{\mathbf{AgNps}}\right)}{\mathbf{MBC\ (Cz\ alone)}}$$

$$FBC\ (Cz) = \frac{8}{4} = \mathbf{2}$$

$$FBC\left(\frac{PVA}{AgNps}\right) = \frac{MBC\left(\frac{PVA}{AgNps}\ in\ the\ presence\ of\ Cz\right)}{MBC\ \left(\frac{PVA}{AgNps}\ alone\right)}$$

$$FBC\ \left(\frac{PVA}{AgNps}\right) = \frac{0{,}5}{7{,}5} = \mathbf{0{,}07}$$

$$\sum \mathbf{FBC} = \mathbf{FBC\ (Cz)} + \mathbf{FBC}\left(\frac{\mathbf{PVA}}{\mathbf{AgNps}}\right) = \mathbf{2{,}07}$$

The result was higher than 2, and according to EUCAST [14], the combined effect is reported as antagonism.

Conclusion: By testing the bactericidal properties of hybrid materials, the presence of any zone of inhibition is accepted as indicative for the existence of bactericidal activity. Therefore, the main method for de-

Table 4: Determining of MBC by combining of PVA/AgNps with ceftazidime on Salmonella Paratyphi B 176.

		Ag 30	15	7,5	3,75	1,875	0,937	0,468
		0	0	0	0	0	0	0
Cz 32	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0
8	0	0	3	0	3	3	0	0
4	9	0	82	9	10	3	11	12
2	27	0	0	>100	22	25	64	43
1	72	0	0	0	>100	71	67	56
0,5	>100	0	0	1	0	>100	>100	87

termination of silver resistance remains the method of macro-dilutions. No synergistic effect was observed on Salmonella Paratyphi B 176 in combination with the hybrid material due to Ceftazidime strong bactericidal activity of the silver-containing liquid polymer. However, we can assume that there is a reason to test the strains with a MBC equal or more than 1.1 mg/L for the presence of a plasmid with silver resistance caring genes.

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Funding:
The present work is funded by the Project № DFNI-B 01/29 (10.12.2012).

ESBL PRODUCING STRAINS KLEBSIELLA AND THEIR SUSCEPTIBILITY TO HYBRID PVA/AgNps MATERIAL

***Martin Iliev*[1]**

[1]Bul Bio-National Centre of Infectious and Parasitic Diseas

ABSTRACT:
Klebsiella infections are of great public importance because of the wide geographical distribution. *Klebsiella* species are ubiquitous in nature. Drug resistant isolates remain an important hospital-acquired bacterial pathogen, add significantly to hospital stays, and are especially problematic in high impact medical areas such as intensive care units. The purpose of this paper is to establish the possibility of a therapeutic choice of hybrid materials comprising silver nanoparticles /AgNps/ in isolates that are resistant to more than two antibiotics. The test was conducted by the method of two-fold serial macro-dilutions according to CLSI M26A and certain MBC /Minimal Bactericidal Concentrations/ of the hybrid material PVA/polyvinyl alcohol//AgNps against the tested strains. Synergistic effect was observed on tested *Klebsiella* strain in combination with the hybrid material due to Ceftazidime strong bactericidal activity of the silver-containing liquid polymer. However, we can assume that there is a reason to test the strains with a MBC equal or more than 1.1 mg/L for the presence of a plasmid with silver resistance carrying genes.

INTRODUCTION

The resistance of clinically significant bacteria in Bulgaria was estimated to be at a medium level when compared with many other surveillance sources worldwide [1]. Traditionally one of the most isolated microorganism from the bacterial pathogens in Bulgaria is *Klebsiella* spp. According to summarized data from BulSTAR, 2010 in Bulgaria *Klebsiella* spp. were isolated in 13% of the positive samples from sputum, 11,5% - from blood cultures, 10,1% - from urines, 6,8% - from surgical site infection, 6,5% - from wound secretions and 0,7% - from throat secretions. Enterobacteriaceae producing expanded-spectrum β-lactamases (ESBL) are major problem worldwide, causing outbreaks as well as sporadic infections. The ESBL *Klebsiella* pneumonia resistant isolates were determined in Bulgaria in 2010 to be 50,7% from blood cultures, 37,8% from surgical site infection, 12% - from wound secretions and 1,8% from sputum [2]. Interesting research works are directed to study the genes responsible for resistance of *Klebsiella* spp. [3, 4]. A way to combat the increasing antimicrobial resistance among the isolated pathogen bacteria may be the growing assortment of hybrid materials containing silver nanoparticles [5, 6, 7] and their application for treatment [8]."

ADDRESS FOR CORRESPONDENCE:
Martin Iliev
Bul Bio-NCIPD
1504 Sofia, Bulgaria
26 Yanko Sakazov
e-mail: martin.iliev7@gmail.com

AIM:
Testing the susceptibility of. *Klebsiella* spp. with predetermined antimicrobials resistance to hybrid material with included silver nanoparticles in an established model [9]

Materials and methods:
The used hybrid material with silver nanoparticles, stabilized in polyvinyl-alcohol (PVA/AgNps), was synthesized through thermal reduction of silver nitrate in 100°C [10]. It was used in two samples with different silver concentration, determined by ICP - 175.9 mg/L and 2032 mg/L. With them ten strains of *Klebsiella* spp. were tested, using methods already established in the practice for testing of biological activity of such kind of hybrid materials, such as the modified Disk Diffusion Method [11], the method of macro-dilutions [12] and the method for testing presence of synergism [13].

RESULTS AND DISCUSSION:
The tested clinical *Klebsiella* spp. strains were isolated from humans.
At first, the susceptibility of the *Klebsiella* spp. strains to antibiotics was determined (Table 1).
Testing a set of discs impregnated with the sample (silver concentration 175.9 mg/L) with the Disk Diffusion Method showed no zones of full suppression of the tested *Klebsiella*s. The testing with discs impregnated with the second sample (silver concentration 2032 mg/L) showed zone of full inhibition on discs, impregnated with the highest silver concentration in eight of the tested strains. Only in two of them full inhibition zone was presented in all available disks, impregnated with dilutions of this sample (Table2, Figure 1).
The lowest values of Minimal Bactericidal Concentration was observed in four of the tested strains, but only in two of them the result corresponded to the showed zone of full inhibition (Table 3).
The determined values of MBC were in the range from 0,3 to 1,1 mg/L silver. By testing the bactericidal properties of hybrid materials, the presence of any zone of inhibition is accepted as indicative for the existence of bactericidal activity. Therefore, the main method for determination of silver resistance remains the method of macro-dilutions (Figure 2).
The possibility for combination of an antibiotic with the hybrid material was studied for the purpose of establishing the existence of synergism by combining of PVA/AgNps with ceftazidime on *Klebsiella* 2494 (Table 4, Figure 3).
The calculation of Fractional bactericidal concentration (FBC) (EUCAST, 2000) on the combined action of ceftazidime and the hybrid material was:

$$FBC(Cz) = \frac{MBC(Cz \text{ in the presence of } {}^{PVA}/_{AgNps})}{MBC\ (Cz\ alone)}$$

FBC(Cz)=0,25/2=0,125

$$FBC({}^{PVA}/_{AgNps}) = \frac{MBC(PVA/AgNps \text{ in the presence of } Cz)}{MBC\ ({}^{PVA}/_{AgNps}\ \text{alone})}$$

FBC(PVA/AgNps)=0,234/0,937=0,25

ΣFBC= FBC (Cz)+FBC (PVA/AgNps)= 0,375

Table 1: Results of testing antibiotics of clinical strains *Klebsiella* spp., isolated from humans. (The examinations were made by National Reference Laboratory of "Control and Monitoring of Antibiotic Resistance", "National Centre of Infectious and Parasitic Diseases")

Antibiotic	Zone of inhibition in mm and interpretation according CLSI																		
CLINICAL STRAINS	CAZ	AMC	CTX	FOX	FEP	IPM	MEM	CF	TZP	ATM	CXM	Pip	GM	NET	NA	SXT	AN	Cip	INTERPRETATION
Klebsiella 2438	R	I	R	S	R	S	S	R	S	R	R	R	R	R	R	R	R	S	ESBL
Klebsiella 2455	R	R	R	S	R	S	S	R	S	R	R	R	R	S	R	R	S	R	ESBL
Klebsiella 2456	R	R	R	S	R	S	I	R	R	R	R	R	R	R	R	R	I	R	ESBL
Klebsiella 2470	R	R	R	R	R	S	R	R	R	R	R	R	R	I	R	R	S	R	ESBL, Hodge (-), CRE (+)
Klebsiella 2485	R	I	R	S	R	S	S	R	I	R	R	R	S	S	R	R	S	R	ESBL
Klebsiella 2489	R	I	R	R	R	S	I	R	I	R	R	R	R	R	R	R	R	R	Hodge (-)
Klebsiella 2493	R	I	R	S	R	S	S	R	S	R	R	R	R	R	I	R	R	I	ESBL
Klebsiella 2494	R	I	R	I	R	S	S	R	I	R	R	R	R	R	I	R	R	I	ESBL
Klebsiella 2503	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	Hodge (-)
Klebsiella 2506	R	R	R	S	R	S	S	R	I	R	R	R	R	S	R	R	S	R	ESBL

Legend: CAZ - Ceftazidime, AMC - Amoxicillin Clavulanic acid, CTX - Cefotaxime, FOX - Cefoxitin, FEP - Cefepime, IPM - Imipenem, MEM - Meropenem, CF-Cephalothin, TZP - Tazobactam/Piperacillin, ATM - Aztreonam, CXM - Cefuroxime, Pip - Piperacillin, GM - Gentamicin, NET - Netilmicin, NA – Nalidix acid, SXT –Sulfamethoxazole/Trimethoprim, AN - Amikacin, Cip - Ciprofloxacin, ESBL- producing of Extended-Spectrum Beta-lactamase

Table 2: Testing resistant to antibiotics clinical strains *Klebsiella* spp. with hybrid material PVA/AgNps (2032 mg/l) by DDM for determination of silver resistance

Disks impregnated with sample №	№1-2032 mg/l	№2-1016 mg/l	№3-508 mg/l	№4-254 mg/l
Strain	**Zone of inhibition in mm**			
Klebsiella 2438	0	0	0	0
Klebsiella 2455	7mm	6mm	6mm	6mm
Klebsiella 2456	0	0	0	0
Klebsiella 2470	7mm	0	0	0
Klebsiella 2485	7mm	6mm	6mm	6mm
Klebsiella 2489	8mm	0	0	0
Klebsiella 2493	6mm	0	0	0
Klebsiella 2496	8mm	0	0	0
Klebsiella 2503	7mm	0	0	0
Klebsiella 2506	7mm	0	0	0

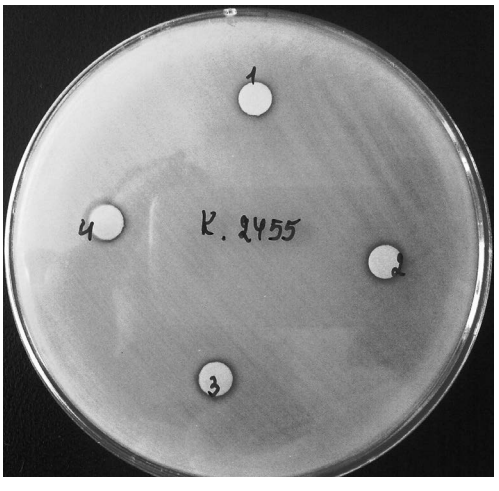


Figure 1: DDM of impregnated with hybrid material disks (№1-2032 mg/l, №2-1016 mg/l, №3-508 mg/l, №4-254 mg/l) against Klebsiella 2455

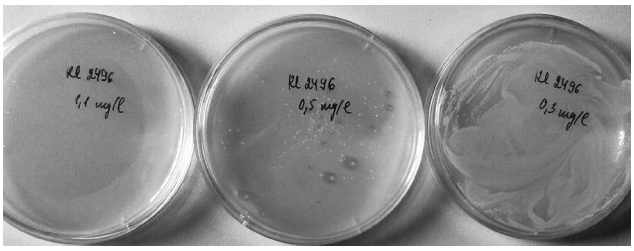
Table 3: Determination of the susceptibility of clinical strains *Klebsiella pneumoniae* by macro-dilution method MIC

Tube №	№1	№2	№3	№4	№5	№6	№7	№8	№9	MBC
Silver concentration (mg/l)	35,2	17,6	8,8	4,4	2,2	1,1	0,5	0,3	0,14	
Silver concentration (mg/l) with standard 0,5MF bacterial suspension	17,6	8,8	4,4	2,2	1,1	0,5	0,3	0,14	0,07	
***Klebsiella* 2438 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	0,3
***Klebsiella* 2455 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	0,3
***Klebsiella* 2456 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	0,3
***Klebsiella* 2470 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	>300 CFU	confluent growth	confluent growth	0,5
***Klebsiella* 2485 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	0,3
***Klebsiella* 2489 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	confluent growth	0,5
***Klebsiella* 2493 petri dishes**	0 CFU	0 CFU	0 CFU	0 KOE	0 CFU	0 CFU	confluent growth	confluent growth	confluent growth	0,5
***Klebsiella* 2496 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	>300 CFU	confluent growth	confluent growth	confluent growth	1,1
***Klebsiella* 2503 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	25 CFU	confluent growth	confluent growth	0,5
***Klebsiella* 2506 petri dishes**	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	0 CFU	confluent growth	confluent growth	confluent growth	0,5

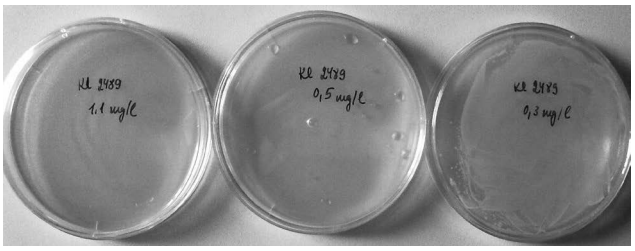
Table 4: Determining of MBC by combining of PVA/AgNps with ceftazidime on *Klebsiella* 2494.

Ag / **Cz**	30 0	15 0	7,5 0	3,75 0	1,875 0	**0,937 0**	0,468 1
32 0	15 / 16 0	7,5 / 16 0	3,75 / 16 0	1,875 / 16 0	0,937 / 16 0	0,468 / 16 0	**0,234** / 16 **0**
16 0	15 / 8 0	7,5 / 8 0	3,75 / 8 0	1,875 / 8 0	0,937 / 8 0	0,468 / 8 0	0,234 / 8 0
8 0	15 / 4 0	7,5 / 4 0	3,75 / 4 0	1,875 / 4 0	0,937 / 4 0	0,468 / 4 0	0,234 / 4 0
4 0	15 / 2 0	7,5 / 2 0	3,75 / 2 0	1,875 / 2 1	0,937 / 2 2	0,468 / 2 0	0,234 / 2 0
2 0	15 / 1 0	7,5 / 1 0	3,75 / 1 0	1,875 / 1 5	0,937 / 1 11	0,468 / 1 4	0,234 / 1 5
1 2	15 / 0,5 0	7,5 / 0,5 0	3,75 / 0,5 0	1,875 / 0,5 53	0,937 / 0,5 4	0,468 / 0,5 4	0,234 / 0,5 22
0,5 4	15 / **0,25 0**	7,5 / 0,25 0	3,75 / 0,25 0	1,875 / 0,25 0	0,937 / 0,25 29	0,468 / 0,25 9	0,234 / 0,25 32

Figure 2: Macro-dilution method for determination of MBC of the tested *Klebsiella* strains (2a-2j) to the hybrid material with 175.9 mg/L silver concentration.



2a



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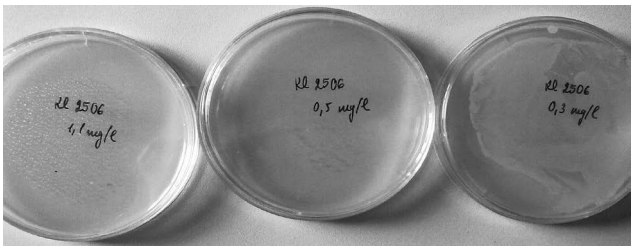

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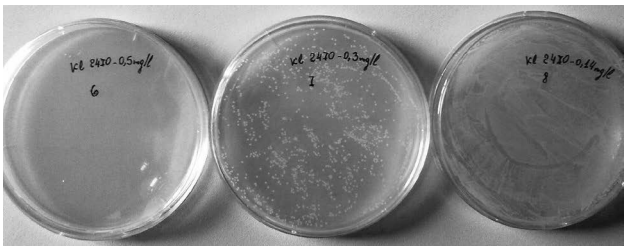

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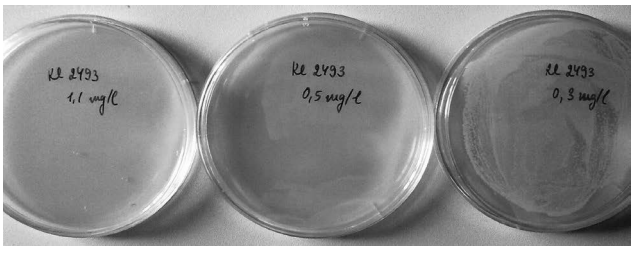

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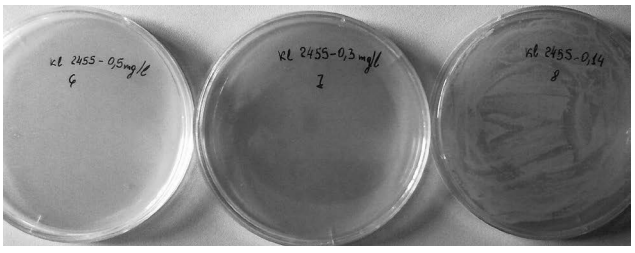

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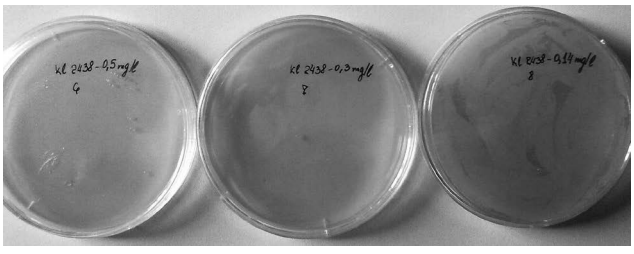

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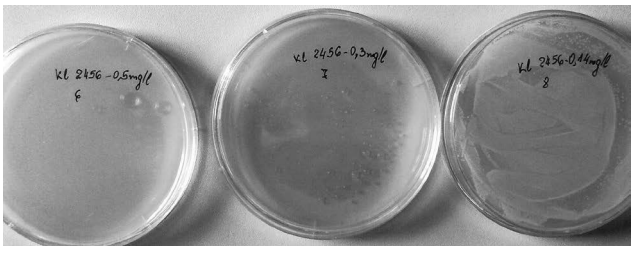

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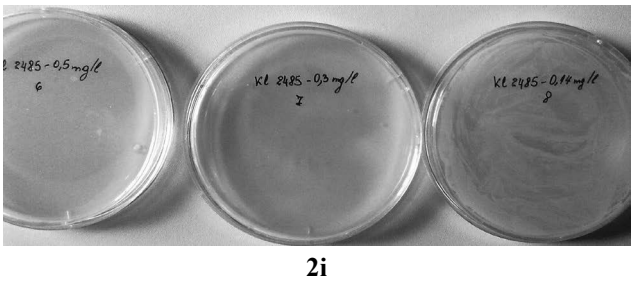

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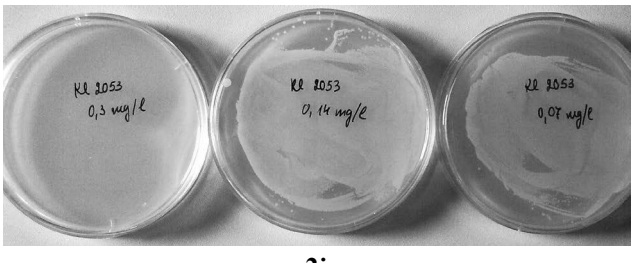

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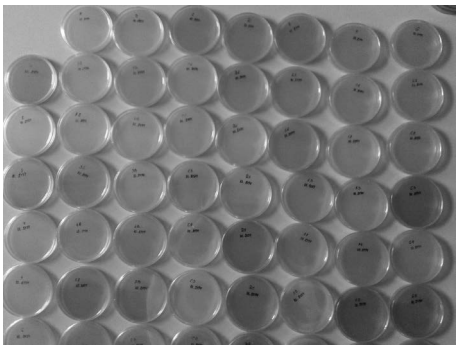

Figure 3: Testing for presence of synergism by combining of PVA/AgNps with ceftazidime on Klebsiella 2494.

The result was lower than 2, and according to EUCAST [14], the combined effect is reported as sinergism.

Conclusion: By testing the bactericidal properties of hybrid materials, the presence of any zone of inhibition is accepted as indicative for the existence of bactericidal activity. Therefore, the main method for determination of silver resistance remains the method of macro-dilutions. Synergistic effect was observed on *Klebsiella* 2494 in combination with the hybrid material due to Ceftazidime strong bactericidal activity of the silver-containing liquid polymer. However, we can assume that there is a reason to test the strains with a MBC equal or more than 1.1 mg/L for the presence of a plasmid with silver resistance caring genes.

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Funding:

The present work is funded by the Project № DFNI-B 01/29 (10.12.2012).

MOLECULAR EPIDEMIOLOGY SURVEILLANCE OF PURE HIV-1 SUBTYPES IN BULGARIA

Ivailo Alexiev Ivanov[1], Danail Beshkov[1], Ivaylo Elenkov[2], Mariana Stoicheva[3], Daniela Nikolova[4]

[1.] National Reference Laboratory of HIV, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.
[2.] Hospital for Infectious and Parasitic Diseases, Sofia, Bulgaria.
[3.] Medical University, Plovdiv, Bulgaria.
[4.] Medical University, Clinic of Infectious Diseases, Varna, Bulgaria.

ABSTRACT
Limited information is available to describe pure HIV-1 subtypes distributed in Bulgaria. Thus, the objective of the present study was to perform a comprehensive molecular epidemiological survey of HIV-1 in Bulgaria with a focus on pure HIV-1 subtypes to better characterize the HIV-1 epidemic. In this regard we analyzed 202 polymerase (*pol*) sequences from pure HIV-1 subtypes isolated from Bulgarians diagnosed until 2009. Epidemiologic and demographic information as well as phylogenetic analysis was used to infer HIV-1 evolutionary histories of studied populations. Our analysis classified 120 (59.4%) of the 202 Bulgarian HIV-1 samples to five different major pure subtypes. 104 (51.5%) were subtype B, 7 (3.5%) were subtype C, 5 (2.5%) were subtype A1, 2 (1.0%) were subtype F1, and two (1.0%) were subtype H. In addition, the phylogenetic analysis of subtype B sequences showed that this subtype has been introduced into Bulgaria on multiple occasions followed by local expansion. Our analysis revealed the presence of a substantial increase in the spread of HIV-1 subtype B among MSM which are among the most vulnerable groups of the population and highlights the importance of sustaining and expanding surveillance to prevent and control HIV-1 infection in Bulgaria.
Key words: HIV-1, subtype, molecular epidemiology, Bulgaria

INTRODUCTION
Human immunodeficiency virus type 1 (HIV-1) is characterized by enormous genetic heterogeneity and is divided into four major phylogenetic groups: M (major), N (new), O (outlier), and the recently identified group P [1]Universite de Rouen, France. jean-christophe.plantier@univ-rouen.fr</Address><ZZ_JournalStdAbbrev><f name="System">Nat.Med.</f></ZZ_JournalStdAbbrev><ZZ_WorkformID>1</ZZ_WorkformID></MDL></Cite></Refman>. Nine subtypes of HIV-1 group M (A–D, F–H, J and K) and few sub-subtypes, e.g. F1, F2, and A1 – A4 are currently recognized. In addition, a great variety of circulating recombinant forms (CRFs) and unique recombinant forms (URFs) have been identified, thus adding to the growing genetic complexity of HIV-1 [2]. Subtype B is predominant in the Americas, Western Europe, and Australia [3;4], subtype A prevails in the former Soviet Union (FSU) countries and Africa. Subtype C is the most abundant genetic form worldwide followed by subtypes A and B [5;6].
The Balkan region has been affected by a wide variety of HIV-1 genotypes with specific subtypes predominating in different countries. Subtype B is the primary subtype here, but to a lesser extent in Croatia, Greece, Montenegro, Slovenia, Serbia and Turkey. In contrast, the main subtype in Albania is A1 while in Romania subtype F1 is dominant with 95% and various subtypes and CRFs have also been found in Cyprus [7-17]. In addition, a study in Greece identified outbreaks within specific risk groups after the introduction of the A1 genotype [18]. Thus, the genetic diversity of HIV-1 in the Balkan region is much more complex compared to that reported in Western Europe.
Current epidemiological data indicates that the impact of the HIV-1 epidemic in Bulgaria is still limited [19-21]. From the beginning of 1986 up to the end of 2009, which is the period of the current study, 1100 HIV/AIDS cases were registered and of these 798 (72.6%) persons were male and 302 (27.5%) were female. Most infections were attributed to heterosexual transmission (747 (67.9%)), followed by intravenous drug use (IDU) (224 (20.4%)), men who have sex with men (MSM) (104 (9.5%)), blood transfusion (17 (1.6%)), and mother-to-child transmission (8 (0.7%)). Early in the epidemic most infections were acquired through heterosexual contact, but more recently the HIV-1 infection has become more common in IDUs (43.3%), followed by MSM (15.2%). The annual number of HIV-1 diagnoses increased from 15 - 20 in the early 1990s to 171 in 2009.
Given the recent changes in the HIV-1 epidemic in Bulgaria, defining the diversity of HIV genotypes in different vulnerable populations is of epidemiological importance [22-25]. Thus, the objective of the present study was to perform a comprehensive molecular epidemiological survey with a focus on pure HIV-1 subtypes to better characterize the introduction and transmission of these viruses of the HIV-1 epidemic in Bulgaria.

Materials and methods
Study population and specimen preparation
We collected blood samples from 202 (18.36%) patients out of the 1,100 persons diagnosed with an HIV-1 infection in Bulgaria from the onset of the epidemic until 2009. Blood samples were collected from different vulnerable populations including MSM, IDUs, newborns, and blood product recipients at the National HIV Reference Laboratory and hospitals in Sofia, Plovdiv and Varna. All collected blood samples were linked to the demographic and clinical data via an anonymous numerical coding system in accordance with the ethical standards of Bulgaria. Plasma was obtained and processed as previously described [22].

ADDRESS FOR CORRESPONDENCE:
Ivailo Alexiev
National Reference Laboratory of HIV
National Center of Infectious and Parasitic Diseases
44a Gen. N. Stoletov Blvd
1233 Sofia, Bulgaria
E-mail: Ivoalexiev@yahoo.com

Polymerase (*pol*) gene RT-PCR and sequence analysis

Viral RNA was extracted from one milliliter of plasma using the QIAamp® Ultra Sens™ Virus Kit. Generation of the protease (PR) and reverse transcriptase (RT) sequences of the HIV-1 *pol* gene was performed using either the Viroseq HIV-1 Genotyping Test (Abbott) and/or TruGene DNA Sequencing System (Siemens Healthcare) RT-PCR kits at the National HIV Reference Laboratory in Sofia, Bulgaria. Automated DNA sequencing was done using an Applied Biosystems model 310 sequencer or an OpenGene DNA sequencing system (Visible Genetics, Siemens) following the manufacturer's protocol.

All HIV-1 *pol* sequences were first analyzed using the Internet-based subtyping tools: REGA HIV-1&2 Automated (Version 2) (http://jose.med.kuleuven.be/ genotypetool/html/subtypinghiv.html) [26]R.</Authors_Primary><Authors_Primary>Vandamme,A.M.</Authors_Primary><Date_Primary>2005/10/1</Date_Primary><Keywords>Algorithms</Keywords><Keywords>analysis</Keywords><Keywords>Artificial Intelligence</Keywords><Keywords>Base Sequence</Keywords><Keywords>Chromosome Mapping</Keywords><Keywords>Dna,Viral</Keywords><Keywords>genetics</Keywords><Keywords>Genotype</Keywords><Keywords>Hiv-1</Keywords><Keywords>methods</Keywords><Keywords>Molecular Sequence Data</Keywords><Keywords>Pattern Recognition,Automated</Keywords><Keywords>Phylogeny</Keywords><Keywords>Sequence Alignment</Keywords><Keywords>Sequence Analysis,DNA</Keywords><Keywords>Software</Keywords><Reprint>Not in File</Reprint><Start_Page>3797</Start_Page><End_Page>3800</End_Page><Periodical>Bioinformatics.</Periodical><Volume>21</Volume><Issue>19</Issue><Misc_3>bti607 [pii];10.1093/bioinformatics/bti607 [doi]</Misc_3><Address>Evolution Group at the Zoology Department, University of Oxford, UK. tulio.deoliveira@zoology.oxford.ac.uk</Address><ZZ_JournalStdAbbrev><f name="System">Bioinformatics.</f></ZZ_JournalStdAbbrev><ZZ_WorkformID>1</ZZ_WorkformID></MDL></Cite></Refman> and the COntext-based Modeling for Expeditious Typing, version 0.5 (COMET HIV-1/2) - http://comet.retrovirology.lu/ programs to obtain preliminary HIV-1 subtype classification. Detection of recombination was evaluated using the programs RDP, 3Seq, GENECON, MaxChi, and Chimaera, implemented in RDP v3 [27].

An individual BLAST search for each of the 202 sequences was performed and the most similar GenBank sequences for each subtype and CRF were downloaded from the HIV Los Alamos sequence database for further sequence analysis (http://www.hiv.lanl.gov/). Nucleotide alignments were prepared using ClustalW v1.6 in BioEdit software packages followed by manual editing [28].

The best fitting nucleotide substitution model was inferred in MEGA5 to be the general time reversible model (GTR) with discrete gamma and invariant among-site rate variation [29]. Phylogenetic relationships were inferred using the ML method in MEGA5 with 1,000 nonparametric bootstrap replicates.

Potential epidemiological clusters were defined using a stringent set of criteria and included those sequences grouping together in phylogenetic analysis with posterior probabilities >0.97, >96% ML bootstrap support, and sharing >90% nucleotide identity per total sampling period between related sequences. The latter estimate is based on the 10^{-3} substitution rate for HIV-1 *pol* se-

Table 1. Distribution of age, gender, route of infection, place of presumed infection, and year of diagnosis in Bulgaria.

	Study Population	Registered individuals with HIV-1 in Bulgaria till 2009
	Number (%)	**Number (%)**
Total	202 (100)	1100 (100)
Age (years)		
<20	14 (6.9)	73 (6.6)
20-44	163 (80.7)	887 (80.6)
≥45	25 (12.4)	140 (12.7)
Gender		
Male	141 (69.8)	798 (72.6)
Female	61 (30.2)	302 (27.5)
Route of infection		
Heterosexual	140 (69.3)	747 (67.9)
MSM	34 (16.8)	104 (9.5)
Hemotransfusion	5 (2.5)	17 (1.6)
IDUs	19 (9.4)	224 (20.4)
Vertical	4 (2.0)	8 (0.7)
Presumed country of infection		
Bulgaria	175 (86.6)	981 (89.2)
Abroad	27 (13.4)	119 (10.8)
Diagnosis period		
1986-1995	22 (10.9)	146 (13.3)
1996-1999	25 (12.4)	110 (10)
2000-2005	78 (38.6)	324 (29.5)
2006-2009	77 (38.1)	520 (47.3)

quences generating a divergence rate of 1-2% per year between founder and transmitted viruses [31]. Antiretroviral resistance-associated mutations were detected using the Stanford genotypic resistance algorithm (http://sierra2.stanford.edu) and stripped from partial alignments containing potential transmission clusters to minimize clustering artifacts and selective evolutionary pressure of the treatment.

Statistical analysis

To assess the epidemiological associations with each of the pure HIV-1 subtype groups, multivariable logistic regression models were constructed, controlling the following demographic and risk mode variables: age at diagnosis, gender, mode of transmission, site of infection and diagnosis period. Odds ratios and 95% confidence limits were calculated. Single variable logistic regression models were also performed and results are reported for findings that differed from those of the multivariable models. In addition, linear trends (degrees of freedom = 1) in subtype prevalence and in proportion to infections by a risk transmission category were assessed using the Cochran-Armitage trend test. Differences in subtype diversity by population subgroups were assessed using a Fisher-Freeman-Halton test for nominal independence.

Results

Diversity of pure HIV-1 subtypes circulating in Bulgaria

Phylogenetic analysis classified 120 (59.4%) of the 202 Bulgarian HIV-1 samples into the five different major subtypes (Figure 1-4 and Table 2). 104 (51.5%) were subtype B, 7 (3.5%) - subtype C, 5 (2.5%) were subtype A1, 2 (1.0%) - subtype F1, and two (1.0%) were subtype H (Table 2). In addition, 82 (40.6%) sequences were classified in at least ten different CRFs or URFs (data not shown).

Identification of clusters among pure HIV-1 subtypes circulating in Bulgaria

Using stringent phylogenetic and nucleotide identity criteria as evidence of possible epidemiologically linked HIV-1 sequences, we identified clusters of highly associated sequences within subtype B and A1 clades (Figures 1 and 2). The identified different but highly related sequence sets were composed mostly of subtype B infections and one group of five subtype A1 sequences (326, 673, 687, 551 and 862). Male participants 326, 673, and 687 were all infected with HIV subtype A1 that shared >96.5% nucleotide identity over the 5 to 6 years of sampling and clustered together with high statistical support (Figure 2). Sequences 326 and 673 were 100% identical while those from persons 326 and 687 were 96.5% identical.

Two groups from Sofia (756, 1026 and 1031) and (986 and 1047) clustered significantly together in the subtype B clade (Figure 1). The intrapair HIV-1 *pol* nucleotide identity for both pairs was > 99.8%. Contact information was not available for the remaining two sequences (553 and 564) to further investigate their potential epidemiological links. These three sets of B sequences still clustered together after phylogenetic analysis of over 4,000 global subtype B sequences in the GenBank database (Figure 1), further supporting their molecular and possible epidemiological linkage.

Association of major HIV-1 subtypes with demographic and risk groups in Bulgaria

Subtype B is the main HIV-1 subtype found in our study and was found among approximately half (104/202, 51.5%) of the patient samples (Table 2 and Figure 1). Nine patients (8.7%) reported acquiring HIV-1 outside of Bulgaria; one each in England, Greece, Italy, South Africa, Romania, Spain, two in Germany and one patient did not report a specific country where he was infected. In a multivariable model, controlling for age, gender, risk mode, country of infection, and calendar period of diagnosis, relative to other subtypes, subtype B was independently higher in MSM (odds ratio (OR) = 16.0, p-value<0.001) and significantly lower in IDUs (OR=0.2, p-value=0.02) compared to heterosexual risk, and significantly lower in females (OR=0.3, p-value=0.002) compared to males and persons ≥ 45 years of age at diagnosis (OR=0.3, p-value 0.05) compared to persons 20-44 years of age (Table 3). There was a significant spike in the relative prevalence of subtype B during the period between 2000 and 2005 in our study population (OR=3.5, p-value=0.03). Phylogenetic analysis of over 4,000 global subtype B sequences showed that this subtype has been introduced in Bulgaria on multiple occasions followed by local expansion demonstrated by clustering of some Bulgarian sequences in this phylogenetic tree (Figure 1).

HIV-1 subtype C was seen in seven (3.5%) persons (Table 2 and Figure 3) and five (2.5%) patients were infected with subtype A1. Two persons (1.0%) were infected with subtype F1 (Table 2 and Figure 4) and their sequences clustered together with strong statistical support. Also, two persons (1.0%) were determined to be infected with subtype H (Table 2 and Figure 3). Collectively, infections with non-B subtypes (C, F1, A1, and H) (n=16, 7.9%) were more likely to occur among persons >45 years old (OR=9.6, p-value <0.001) compared to the year group 20 to 44 years (Table 3).

Trends in the HIV-1 subtype prevalence and total infections in high risk groups

To better understand the temporal trends of the epidemiology of the HIV-1 infection in Bulgaria and for the purpose of targeting prevention resources, we analyzed trends in the prevalence of HIV-1 subtypes, and trends in the major risk groups across four time periods (1986-1995, 1996-1999, 2000-2005, and 2006-2009) without controlling for explanatory variables. Subtype B did not significantly increase over time using multivariable or single variable analyses (data not shown) despite a significant spike in prevalence from 2000-2005 (OR=3.5, p-value=0.03). This is likely explained by overrepresentation of MSM in the study population and a simultaneous decrease in infected heterosexuals during that period.

The proportion of younger infected persons, aged 20 or younger <20, were significantly declining (trend test p<0.001). Patients infected through heterosexual contact and vertical transmission were declining, while those infected through MSM or IDU were increasing (p<0.001). During the early years of the epidemic, HIV-1 infection was greatest among Bulgarian citizens returning from abroad. However, there was an increasing trend in the proportion of persons infected in Bulgaria (p=0.02), particularly during 2006-2009 when 92.2% acquired infection within the country.

Figure 1. Global phylogenetic relationships of Bulgarian HIV-1 subtype B polymerase sequences. The 753-bp alignment consisted of >4,000 reference sequences sampled globally and 104 subtype B sequences from Bulgaria. Phylogenetic tree was inferred by the maximum likelihood method as implemented in the FastTree program (http://www.microbesonline.org/fasttree/) using a general time reversible (GTR) model with 20 gamma categories. The reliability of the clusters was assessed with the Shimodaira-Hasegawa test implemented in the FastTree program. Red diamonds indicate Bulgarian sequences.

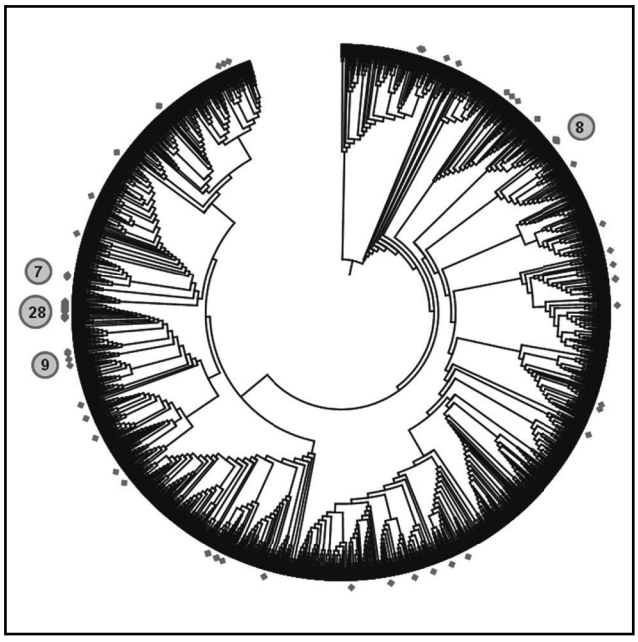

Figure 2. Inferred phylogenetic relationships of Bulgarian HIV-1 subtype A1. Tree was inferred using maximum likelihood analysis in MEGA5. Support for each node was determined using 1,000 bootstrap replications with only values >70 shown. Scale bar indicates the number of nucleotide substitutions per site. Antiretroviral resistance-associated mutations were stripped from the alignments. The 690-bp alignment consisted of 5 HIV-1 A1 strains from Bulgaria and 34 Group M reference sequences from the Los Alamos HIV database. The tree was rooted by using an HIV-1 subtype J strain as the outgroup. Bulgarian sequences are shown using bold branches and taxon names ending with the suffix (BG).

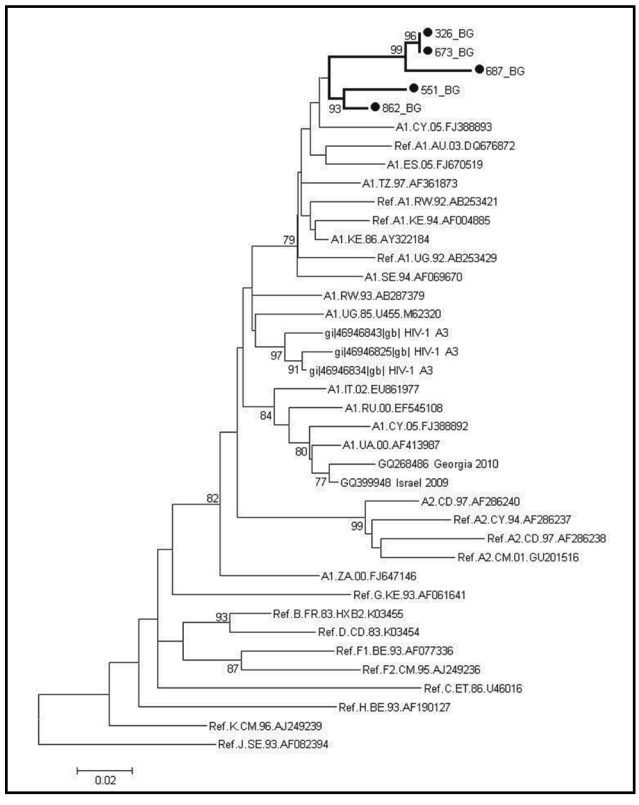

Figure 3. Inferred phylogenetic relationships of Bulgarian HIV-1 subtypes C and H. Tree was inferred using maximum likelihood analysis in MEGA5. Support for each node was determined using 1,000 bootstrap replications with only values >70 shown. Scale bar indicates the number of nucleotide substitutions per site. Antiretroviral resistance-associated mutations were stripped from the alignments. The 690-bp alignment consisted of 9 HIV-1 C and H strains from Bulgaria and 29 Group M reference sequences from the Los Alamos HIV database. The tree was rooted by using an HIV-1 subtype J strain as the outgroup. Bulgarian sequences are shown using bold branches and taxon names ending with the suffix (BG).

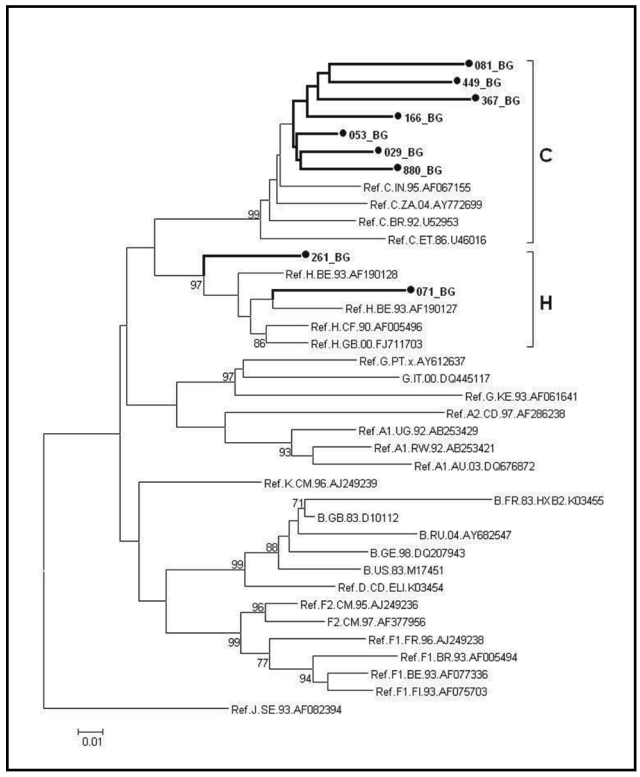

Figure 4. Inferred phylogenetic relationships of Bulgarian HIV-1 subtype F1. Tree was inferred using maximum likelihood analysis in MEGA5. Support for each node was determined using 1,000 bootstrap replications with only values >70 shown. Scale bar indicates the number of nucleotide substitutions per site. Antiretroviral resistance-associated mutations were stripped from the alignments. The 690-bp alignment consisted of 2 HIV-1 strains from Bulgaria and 27 Group M reference sequences from the Los Alamos HIV database. The tree was rooted by using an HIV-1 subtype J strain as the outgroup. Bulgarian sequences are shown using bold branches and taxon names ending with the suffix (BG).

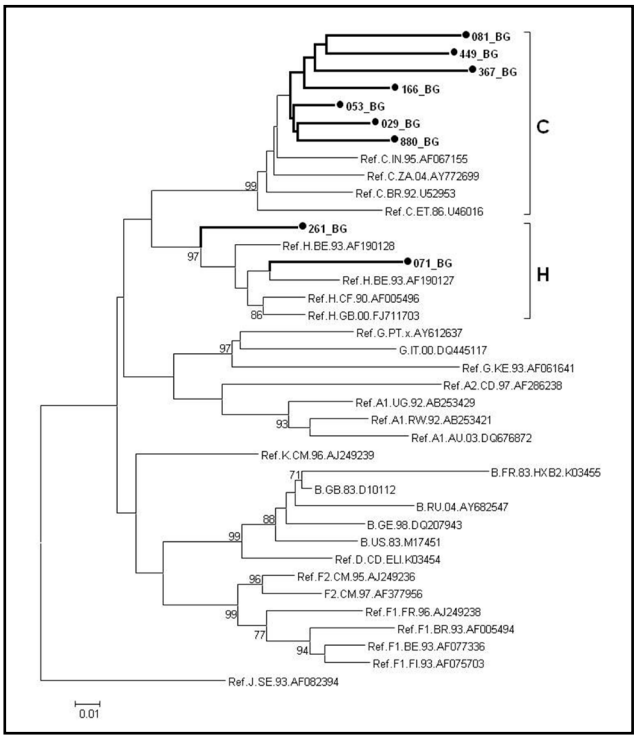

Table 2. Pure HIV-1 subtype diversity found in samples from 120 Bulgarian residents.

Subtype	Number	Prevalence (95% confidence limits)	Travel Outside of Bulgaria (number of persons)[1]
B	104	51.5 (44.4, 58.6)	England (1), Greece (1), Italy (1), South Africa (1), Romania (1), Spain (1), Germany (2), unspecified (1)
C	7	3.5 (1.4, 7.0)	unspecified (1)
A1	5	2.5 (0.8, 5.7)	Greece (1)
F1	2	1.0 (0.1, 3.5)	(0)
H	2	1.0 (0.1, 3.5)	(0)

Odds ratios (OR) are the estimated odds of a subtype occurring in the study population for a given risk factor relative to the referent group (Ref) as compared to occurrence of all other subtypes. *p*, is the p-value for differences in subtype prevalence compared to the referent group; CL, confidence limit.

Discussion

In the present study we describe molecular epidemiological assessment of 120 (59.4%) pure subtypes out of the 202 *pol* studied sequences, representing about 20% of infections identified to date. With consistence to our previous results, our data suggests that the HIV/AIDS incidence in the country remains low overall. Our study indicates that the HIV-1 epidemic in Bulgaria possess a significant diversity of more than 15 different HIV-1 subtypes, CRFs and URFs. The data suggest that there is a local networks within certain risk groups with specific HIV-1 genotypes and unequal subtype distribution among different risk groups [24]. Our results show that subtype B has been introduced into Bulgaria on multiple occasions, with local expansion observed among certain populations.

The distribution of HIV-1 subtypes in our study varied by age, sex, geography, and risk exposure. Persons infected via heterosexual or IDU transmission had the greatest variety of subtypes, compared to MSM (p<0.0001). Furthermore, we also observed that with the exception of two persons, all MSM were infected with subtype B. Our results are consistent with several studies from different European countries which found a predominance of subtype B in MSM [15;30-32]. There is also recent independent evidence that subtype B in Bulgaria may be phylogenetically related to subtype B strains identified in Russia, Greece and some other neighboring Balkan countries [30]. In addition, our phylogenetic and epidemiological analyses showed an absence or reduced prevalence of certain viral strains in specific populations. For example,

Table 3. Risk factors associated with pure HIV-1 subtypes in Bulgaria, results from multivariable logistic regression model.

	B (n=104)		C, A1, F1, H (n=16)	
	OR (95% CL)	*p*	OR (95% CL)	*p*
Age (years)				
≤20	1.3 (0.4, 4.6)	0.63	2.0 (0.3, 12.1)	0.44
20-44	Ref		Ref	
≥45	0.3 (0.1, 1.0)	0.05	9.6 (2.6, 35.0)	<0.001
Gender				
Male	Ref		Ref	
Female	0.3 (0.1, 0.6)	0.002	0.8 (0.2, 2.9)	0.71
Route of infection				
Heterosexual, Other	Ref		Ref	
MSM	16.0 (3.3, 77.4)	<0.001	0.7 (0.1, 3.9)	0.69
IDU	0.2 (0.1, 0.7)	0.02	Ref	
Presumed country of infection				
Bulgaria	2.4 (0.8, 6.9)	0.10	2.5 (0.4, 14.5)	0.30
Abroad	Ref		Ref	
Diagnosis period				
1986-1995	Ref		Ref	
1996-1999	2.0 (0.5, 7.5)	0.31	0.3 (0.1, 2.2)	0.24
2000-2005	3.5 (1.1, 11.2)	0.03	0.2 (0.1, 0.9)	0.03
2006-2009	2.3 (0.7, 8.1)	0.19	0.2 (0.1, 1.1)	0.06

neither MSM, nor individuals infected through blood transfusions were infected with HIV-1 CRF01_AE, which was the second most common genotype in Bulgaria (data not shown) [20]. These findings suggest a pattern of independent and limited transmission of some viral clades within certain population groups without viral exchange between them.

Analysis of longitudinal epidemiological and demographic data with HIV-1 genotype can provide important information regarding infection dynamics during different stages of the epidemic in Bulgaria. Using this strategy by year of HIV/AIDS diagnosis, we found a dissimilar rate of introduction and spread of different HIV-1 clades over time during the history of the epidemic in Bulgaria. Some clades persist, such as subtype B, while others like subtype C have declined. We also found that there was a significant increase in the number of infections in MSM with a concomitant decline in heterosexual and vertical infections.

We also found that during the early years of the epidemic, HIV-1 was most likely found in Bulgarians returning from abroad and who then transmitted HIV-1 via heterosexual contact. However, in recent years the significant increase in transmission of certain HIV-1 subtypes among IDUs and MSM is most likely due to the introduction and dissemination of certain subtypes within these groups.

Our results also suggest that the infection dynamics and HIV-1 genetic diversity of the HIV-1 epidemic in Bulgaria are fluctuating. An underestimation of MSM transmission in the early years of the epidemic due to stigma associated with reporting of homosexual contacts could also explain the observed increase in MSM infections.

The cross-sectional design of our study, which included convenience specimens, collected as patients were diagnosed or came into clinics for medical care, may not truly represent the other 80% of reported cases in Bulgaria. The effect of this possible sampling error in our analyses is unknown but may influence genotype distribution over time and in different populations. Also, our phylogenetic results are based on a single genomic region. Any potential association of infection with a geographic location is based on self-reporting and may be affected by recall or other biases and thus may require analysis of larger HIV-1 datasets from the purported country where the transmission was reported to confirm the origin.

In conclusion, we found that subtype B is the dominant HIV-1 genetic form in Bulgaria. In addition, most major subtypes were also introduced in the country. Our data also revealed the dynamic nature of the Bulgarian epidemic with unequal distribution of different HIV-1 genotypes among high risk populations. These findings emphasize the need for sustained and focused molecular epidemiological surveillance to identify transmission links that can be targeted by prevention strategies to control the HIV-1 epidemic in Bulgaria.

Acknowledgments

Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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MOLECULAR EPIDEMIOLOGY SURVEILLANCE OF HIV-1 CRFS AND URFS IN BULGARIA

Ivailo Alexiev Ivanov[1], Danail Beshkov[1], Ivaylo Elenkov[2], Mariana Stoicheva[3], Daniela Nikolova[4]

[1.] National Reference Laboratory of HIV, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.
[2.] Hospital for Infectious and Parasitic Diseases, Sofia, Bulgaria.
[3.] Medical University, Plovdiv, Bulgaria.
[4.] Medical University, Clinic of Infectious Diseases, Varna, Bulgaria.

ABSTRACT
Little is known about the introduction and dissemination of circulating recombinant forms of HIV-1 in Bulgaria. To fill the gap, we analyzed 202 polymerase (*pol*) sequences from Bulgarians diagnosed through 2009. The phylogenetic analysis showed that 120 (59.5%) persons were infected with one of five different HIV-1 subtypes (A1, B, C, F1 and H) and 82 (40.6%) individuals were infected with (CRFs) or unique recombinant forms URFs: 63 (31.2%) persons had one of six different CRFs: 01_AE, 02_AG, 04_cpx, 05_DF, 14_BG, and 36_cpx. We also identified for the first time an infection with two different clusters of unique A-like and F-like genetic forms in 12 persons (5.9%) and seven additional unique recombinant forms (3.5%), including a novel J/C recombinant. CRF01_AE was the most common recombinant form and was higher in women and IDUs relative to other risk groups combined. Our results show that the HIV-1 infection in Bulgaria reflects the shifting distribution of genotypes coincident with the changing epidemiology of the HIV-1 epidemic among different risk groups. The data of our study supports increased public health interventions targeting IDUs and highlights the importance of sustained and expanded surveillance to prevent and control the HIV-1 infection in Bulgaria.
Key words: HIV-1, subtype, molecular epidemiology, Bulgaria

INTRODUCTION

HIV-1 group M, which predominates in the global HIV/AIDS epidemic, can be further subdivided into subtypes (A–D, F–H, J, K), sub-subtypes (A1 to A4, F1 and F2), circulating recombinant forms (CRF01 to CRF51) and numerous unique recombinant forms (URFs) [1;2]Universite de Rouen, France. jean-christophe.plantier@univ-rouen.fr</Address><ZZ_JournalStdAbbrev><f name="System">Nat.Med.</f></ZZ_JournalStdAbbrev><ZZ_WorkformID>1</ZZ_WorkformID></MDL></Cite></Refman>. Subtype B is predominant in the Americas, Western Europe, and Australia, [3] while (the) Balkan region is much more complex compared to that reported in Western Europe [4-15], subtype B is dominating in Greece, Montenegro, Croatia, Slovenia, Serbia and Turkey. In contrast, the principal subtype in Albania is A1, 95% of HIV-1 in Romania is subtype F1, and various subtypes and CRFs have been found in Cyprus.
Our epidemiological data from the national registry indicates that the impact of the HIV-1 epidemic in Bulgaria is still limited [16]. From the beginning of 1986 up to the end of 2009, the period of our study, 1,100 HIV/AIDS cases were registered. Of these, 798 (72.6%) persons were male and 302 (27.5%) were female (including 327 persons with AIDS). 747 (67.9%) of infections were attributed to heterosexual transmission, 224 (20.4%) to intravenous drug use (IDU), 104 (9.5%) were men who have sex with men (MSM), 17 (1.6%) were blood product recipients, and 8 (0.7%) were newborns infected via mother-to-child transmission. In the early years of the epidemic in Bulgaria most infections were acquired through heterosexual contact, while more recently the HIV-1 infection has become more common in IDUs (43.3%), followed by MSM (15.2%).
Given the current HIV-1 epidemic in Bulgaria with regard to rising proportions of infected IDUs and MSMs, and the specific geographic location of Bulgaria at the intersection of Western Europe, Eastern Europe and the Middle East, defining the diversity of HIV genotypes in Bulgaria is of substantial importance [17-22]. In this regard, the objective of our study was to perform a comprehensive molecular epidemiological surveillance with a focus on distributed CRFs and URFs of the HIV-1 epidemic in Bulgaria.

Materials and methods

Ethics statement

All patients provided written informed consent to participate in this study. Our study received approval by the Ethical Committee at the National Centre of Infectious and Parasitic Diseases, Sofia Bulgaria. NCIPD IRB IORG0003505.

Study population and specimen preparation

In this study we analyzed 202 (18.36%) blood samples from 1,100 patients diagnosed with an HIV-1 infection in Bulgaria from 1986 until 2009. The patient's samples were collected from different vulnerable groups, including MSM, IDUs, newborns, and blood product recipients at the National HIV Reference Laboratory and in hospitals in Sofia, Plovdiv and Varna. Blood samples were linked to the demographic and clinical data through an anonymous numerical coding system in accordance with the ethical standards of Bulgaria. Plasma was obtained and processed as previously described [17].

Sample processing and sequence analysis

Viral RNA was isolated from one milliliter of plasma using the QIAamp® Ultra Sens™ Virus Kit 50. Generation of the protease (PR) and reverse transcriptase (RT) sequences of the HIV-1 *pol* gene was performed using either the Viroseq HIV-1 Genotyping Test (Abbott) and/or TruGene DNA Sequencing System (Siemens Healthcare) RT-PCR kits at the National HIV Reference Laboratory in Sofia, Bulgaria. Automated

ADDRESS FOR CORRESPONDENCE:
Ivailo Alexiev
National Reference Laboratory of HIV
National Center of Infectious and Parasitic Diseases
44a Gen. N. Stoletov Blvd
1233 Sofia, Bulgaria
E-mail: Ivoalexiev@yahoo.com

DNA sequencing was done using an Applied Biosystems model 310 sequencer or an OpenGene DNA sequencing system (Visible Genetics, Siemens) following the manufacturer's protocol.

All 82 HIV-1 *pol* sequences were analyzed using the internet-based REGA HIV-1&2 Automated Subtyping Tool (Version 2.0) (http://jose.med.kuleuven.be/ genotypetool/html/subtypinghiv.html) [23]R.</Authors_Primary><Authors_Primary>Vandamme,A.M.</Authors_Primary><Date_Primary>2005/10/1</Date_Primary><Keywords>Algorithms</Keywords><Keywords>analysis</Keywords><Keywords>Artificial Intelligence</Keywords><Keywords>Base Sequence</Keywords><Keywords>Chromosome Mapping</Keywords><Keywords>Dna,Viral</Keywords><Keywords>genetics</Keywords><Keywords>Genotype</Keywords><Keywords>Hiv-1</Keywords><Keywords>methods</Keywords><Keywords>Molecular Sequence Data</Keywords><Keywords>Pattern Recognition,Automated</Keywords><Keywords>Phylogeny</Keywords><Keywords>Sequence Alignment</Keywords><Keywords>Sequence Analysis,DNA</Keywords><Keywords>Software</Keywords><Reprint>Not in File</Reprint><Start_Page>3797</Start_Page><End_Page>3800</End_Page><Periodical>Bioinformatics.</Periodical><Volume>21</Volume><Issue>19</Issue><Misc_3>bti607 [pii];10.1093/bioinformatics/bti607 [doi]</Misc_3><Address>Evolution Group at the Zoology Department, University of Oxford, UK. tulio.deoliveira@zoology.oxford.ac.uk</Address><ZZ_JournalStdAbbrev><f name="System">Bioinformatics.</f></ZZ_JournalStdAbbrev><ZZ_WorkformID>1</ZZ_WorkformID></MDL></Cite></Refman> and COntext-based Modeling for Expeditious Typing, version 0.5 (COMET HIV-1/2) - http://comet.retrovirology.lu/ programs to obtain preliminary HIV-1 subtype classification. Possible genetic composition of URF was inferred using the BLAST sliding window algorithm implemented at NCBI (www.ncbi.nlm.nih.gov/projects/genotyping) and used to guide selection of reference sequences in bootscanning analysis. Manual bootscan analysis was done with the program SimPlot to confirm selected subtypes using the F84 nucleotide substitution model and a sliding window of 200-bp, a 40-bp step, with the transition/transversion ratio determined empirically [24]. Nucleotide distances were calculated with MEGA5 using TN93+G+I as a substitution model, respectively. Detection of recombination was also evaluated using the programs RDP, 3Seq, GENECON, MaxChi, and Chimaera, implemented in RDP v3 [25]. Antiretroviral resistance-associated mutations were detected using the Stanford genotypic resistance algorithm (http://sierra2.stanford.edu) and stripped from partial alignments containing potential transmission clusters to minimize clustering artifacts.

An individual BLAST search was performed for each of the sequences and the most similar GenBank sequences for each subtype and CRF were downloaded from the HIV Los Alamos sequence database for further sequence analysis (http://www.hiv.lanl.gov/). Nucleotide alignments were prepared using Clustal W v1.6 in BioEdit software followed by manual editing [26;27]. The complete data set alignments for phylogenetic analysis consisting of 82 Bulgarian sequences and similar sequences from the BLAST search and HIV-1 subtype reference sequences from the Los Alamos HIV database. To investigate the molecular epidemiology of HIV-1 in Bulgaria, we also analyzed subsets of Bulgarian and reference sequences by using selected genotype clusters inferred by maximum likelihood (ML) Bayesian analysis.

Phylogenetic relationships were inferred using Bayesian analysis with BEAST v1.6.2 [28] program. For the Bayesian analyses, a relaxed molecular clock model was used and each run consisted of two independent 800 x 10^6 Markov chain Monte Carlo (MCMC) generations with sampling every 800,000th generation and a constant coalescent tree prior. Convergence of the MCMC was assessed by calculating the effective sampling size (ESS) of the runs using the program Tracer v1.5 (http://beast.bio.ed.ac.uk/Tracer). All parameter estimates showed significant ESSs >1,200. The tree with the maximum product of the posterior clade probabilities (maximum clade credibility tree) was chosen from the posterior distribution of 8,001 sampled trees after burning in the first 1,000 sampled trees with the program TreeAnnotator version 1.6.2 [29].

Potential epidemiological clusters were defined using a stringent set of criteria and included those sequences grouping together in phylogenetic analysis with posterior probabilities >0.97, >96% ML bootstrap support, and sharing >90% nucleotide identity per total sampling period between related sequences. The latter estimate is based on the 10^{-3} substitution rate for HIV-1 *pol* sequences generating a divergence rate of 1-2% per year between founder and transmitted viruses [29].

Statistical analysis

The epidemiological associations was assessed with each of the major HIV-1 subtype groups, multivariable logistic regression models were constructed, controlling for the following demographic and risk mode variables: age at diagnosis, gender, mode of transmission, site of infection, and diagnosis period. Each CRF: 01_AE, 02_AG, rare CRFs, and URFs was modeled relative to all other subtype groups collectively. Odds ratios and 95% confidence limits were calculated. Single variable logistic regression models were also performed and results are reported for findings that differed from those of the multivariable models. In addition, linear trends (degree of freedom = 1) in subtype prevalence and in proportion of infections by risk transmission category were assessed using the Cochran-Armitage trend test. Differences in subtype diversity by population subgroups were assessed using a Fisher-Freeman-Halton test for nominal independence.

Results

Diversity of HIV-1 CRFs and URFs in Bulgaria

Phylogenetic analysis using ML and Bayesian MCC methods of *pol* sequences classified 82 (40.6%) sequences in at least ten different CRFs or URFs: 40 (19.8%) CRF01_AE, 15 (7.4%) CRF02_AG, 4 (2%) CRF05_DF, 2 (1.0%) CRF14_BG, one (0.5%) CRF04_cpx, one (0.5%) CRF36_cpx, and 19 (9.4%) sequences were found to be URFs (Figure 1, Table 2). In addition 120 (59.5%) of the 202 Bulgarian HIV-1

samples were classified into five different major subtypes (data not shown).

Identification of HIV-1 CRFs and URFs clusters within Bulgaria.

For cluster identification we used phylogenetic and nucleotide identity criteria as evidence of possible epidemiologically linked HIV-1 sequences. We identified one group of six and three pairs of highly associated sequences within CRF02_AG (Figure 1). A monophyletic lineage with high posterior probability containing six sequences was obtained from specimens collected over about a year from male IDUs who all lived in Plovdiv and the neighboring city of Haskovo. All six sequences shared >97.4% nucleotide identity. Three other clusters were found with subtype CRF02_AG and shared nucleotide identity of 99.4%, 98% and 95% respectively (Figure 1).

Sequence analysis showed that the F-like cluster shared 90.7 – 97.1% nucleotide identity which is greater than that within F1 (94 – 96.5%), F2 (95.8 – 97.1%), and CRF05_DF (94.9 – 96.6%). Likewise, the F-like Bulgarian sequences were equidistant from the F1, F2, and CRF05_DF subtypes sharing 75 - 79% nucleotide identity which is greater than the range of sequence identity between F1, F2, and CRF-05_DF (89 – 96%). Additional phylogenetic analysis showed that these seven sequences clustered with strong support with an F-variant from the former Soviet Union (FSU) (data not shown) [30].

Similarly, five persons that formed a distinct lineage between A1 and CRF01_AE in the phylogenetic analysis (Figure 1) showed that their genetic structure was similar and is composed of mostly A1 and unidentified sequences (data not shown). The nucleotide identities within this group were similar to those seen within A1 (94.2 – 95.3%), A2 (93.8 – 95.3%), A3 (93.8 – 98.6%), A4 (94.1 – 94.9%), and CRF01_AE (95.3 – 97.5%). In addition, this group of five Bulgarian sequences and the A1, A2, A3, A4, CRF01_AE reference sequences were nearly equidistant from each other sharing about 90 – 95%, which is similar to that between subtypes B and D in this region. The distinct relationship of these five A-like sequences to other subtype A sequences was also supported by phylogenetic analysis as were the A1, A2, A3, and A4 sub-subtypes (data not shown). The five A-like sequences clustered with other *pol* sequences from HIV-1 infected persons from Africa, FSU, and persons from Cyprus who reported infection in FSU that have also been described as A variants or A sub-subtypes and were shown to originate from Africa [31].

Association of circulating and complex recombinant HIV-1 forms with demographic and risk groups in Bulgaria

Phylogenetic and subtype analysis classified 63/202 (31.2%) HIV-1 sequences from Bulgaria as CRFs (Table 2 and Figure 1). CRF01_AE is the most common HIV-1 recombinant form in Bulgaria with 40 (19.8%) of the 202 sequences in our study. Based upon multivariable regression, compared to all other subtypes, CRF01_AE prevalence is significantly higher among females (OR=2.5, p=0.02) and IDUs (OR=4.0, p=0.02) relative to other risk groups combined (Table 3).

We found HIV-1 CRF02_AG to be the second most frequent CRF group in Bulgaria with 15/202 persons (7.4%) infected with this subtype (Table 2, Figure 1). In a single-variable regression analysis, compared to other subtypes, CRF02_AG infection was significantly higher during later diagnosis periods. However, this finding was explained by the coincidental increase in IDU infection over time. In the multivariate analysis, CRF02_AG prevalence was higher among IDU (OR=8.4, p=0.003) and females (OR=4.6, p=0.02) (Table 3) independent of other potential risk factors.

Four extremely rare HIV-1 genotypes were detected in Bulgaria among 8 patients (4.0%), including CRF04_cpx, CRF05_DF, CRF14_BG and CRF36_cpx, (Table 2, Figure 1). Our sequence analyses identified four (1.98%) persons infected with HIV-1 CRF05_DF, two of whom reported having travelled abroad.

Based upon multivariable analysis, some particular URFs were significantly less likely to occur among persons infected while in Bulgaria (OR=0.2, p=0.0074) (Table 3).

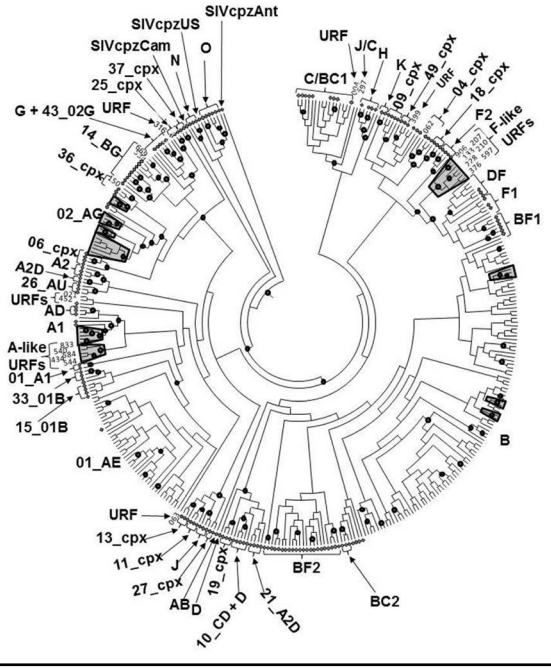

Figure 1. Inferred genetic relationships of 202 HIV-1 polymerase (*pol*) sequences from persons in Bulgaria. Bayesian inference performed using the BEAST software package. 170 HIV-1 reference *pol* sequences were used for genetic comparison. Reference sequences are indicated with a grey diamond. Final alignment length is 804 nucleotides. Posterior probabilities greater than 0.97 at nodes are shown with black dots. Clades shaded with grey trapezoids indicate possible epidemiologically-linked sequences supported with posterior probabilities >0.97. Genotype classification of the Bulgarian sequences is shown with respect to the reference sequences. Major subtypes, CRFs and URFs lineages in Bulgaria are shown.

Table 1. Distribution of age, gender, route of infection, place of presumed infection, and year of diagnosis in Bulgaria.

	Study Population	Registered individuals with HIV-1 in Bulgaria till 2009
	Number (%)	**Number (%)**
Total	202 (100)	1100 (100)
Age (years)		
<20	14 (6.9)	73 (6.6)
20-44	163 (80.7)	887 (80.6)
≥45	25 (12.4)	140 (12.7)
Gender		
Male	141 (69.8)	798 (72.6)
Female	61 (30.2)	302 (27.5)
Route of infection		
Heterosexual	140 (69.3)	747 (67.9)
MSM	34 (16.8)	104 (9.5)
Hemotransfusion	5 (2.5)	17 (1.6)
IDUs	19 (9.4)	224 (20.4)
Vertical	4 (2.0)	8 (0.7)
Presumed country of infection		
Bulgaria	175 (86.6)	981 (89.2)
Abroad	27 (13.4)	119 (10.8)
Diagnosis period		
1986-1995	22 (10.9)	146 (13.3)
1996-1999	25 (12.4)	110 (10)
2000-2005	78 (38.6)	324 (29.5)
2006-2009	77 (38.1)	520 (47.3)

Table 2. Diversity of HIV-1 CRFs and URFs in 120 samples from Bulgarian residents.

Subtype	**Number**	**Prevalence (95% confidence limits)**	**Travel Outside of Bulgaria (number of persons)[1]**
01_AE	40	19.8 (14.5, 26.0)	Nicaragua (1)
02_AG	15	7.4 (4.2, 12.0)	Ghana (1), Cyprus (1), unspecified (1)
04_cpx	1	0.5 (0.0, 2.7)	(0)
05_DF	4	2.0 (0.5, 5.0)	Democratic Republic of Congo (1), unspecified (1)
14_BG	2	1.0 (0.1, 3.5)	Spain (1)
36_cpx	1	0.5 (0.0, 2.7)	Libya (1)
URF	19	9.4 (5.8, 14.3)	Greece (1), Russia (1), South Africa (1), Nigeria (2), unspecified (3)

Table 3. Risk factors associated with HIV-1 CRFs and URFs, results from multivariable logistic regression model.

	01_AE (n=40)		02_AG (n=15)		04_cpx, 05_DF, 14_BG, 36_cpx, (n=8)		URFs (n=19)	
	OR (95% CL)	*p*	OR (95% CL)	*p*	OR (95% CL)	*p*	OR (95% CL)	*p*
Age (years)								
≤20	1.5 (0.4, 5.9)	0.55	1.7 (0.2, 19.1)	0.65	Ref		Ref	
20-44	Ref		Ref		Ref		Ref	
≥45	0.5 (0.1, 2.5)	0.41	0.7 (0.1, 6.7)	0.77	0.7 (0.1, 8.4)	0.8	2.3 (0.6, 8.6)	0.23
Gender								
Male	Ref		Ref		Ref		Ref	
Female	2.5 (1.1, 5.4)	0.02	4.6 (1.2, 17.0)	0.02	5.6 (1.1, 27.9)	0.04	1.4 (0.5, 4.2)	0.49
Route of infection								
Heterosexual, Other	Ref		Ref		Ref		Ref	
MSM	Ref		Ref		Ref		Ref	
IDU	4.0 (1.2, 13.0)	0.02	8.4 (2.0, 34.6)	0.003	1.6 (0.1, 21.8)	0.73	1.4 (0.1, 13.3)	0.78
Presumed country of infection								
Bulgaria	5.3 (0.6, 43.2)	0.12	0.6 (0.1, 4.8)	0.66	0.1 (0.0, 0.5)	0.006	0.2 (0.1, 0.6)	0.007
Abroad	Ref		Ref		Ref		Ref	
Diagnosis period								
1986-1995	Ref		Ref		Ref		Ref	
1996-1999	5.5 (0.6, 51.3)	0.14	0.5 (0.1, 12.0)	0.67	Ref		0.9 (0.2, 5.6)	0.95
2000-2005	4.5 (0.5, 38.0)	0.17	0.4 (0.1, 6.7)	0.56	0.1 (0.0, 1.5)	0.1	0.7 (0.2, 2.9)	0.59
2006-2009	2.3 (0.3, 20.8)	0.45	2.5 (0.2, 30.2)	0.48	0.7 (0.1, 4.1)	0.69	0.2 (0.1, 1.5)	0.13

Odds ratios (OR) are the estimated odds of a subtype occurring in the study population for a given risk factor relative to the referent group (Ref) as compared to occurrence of all other subtypes. *p*, is the p-value for differences in subtype prevalence compared to the referent group; CL, confidence limit.

Discussion

In the present study we describe molecular epidemiological analysis of 82 (40.6%) HIV-1 CRFs and URFs of 202 sequences representing about 20% of infections identified to date in Bulgaria. Our results extend those reported previously by our group that initially identified multiple subtypes in Bulgaria [27]. In addition to the current national data from ongoing HIV and AIDS surveillance suggesting that the HIV/AIDS incidence in Bulgaria remains low, our study shows that the HIV-1 epidemic in Bulgaria has several key characteristics, of which a high genetic diversity of more than 15 different HIV-1 subtypes, CRFs and URFs, including some extremely rare HIV-1 forms like: CRF05_DF, CRF04_cpx CRF36_cpx, CRF14_BG, and a plethora of different URFs that are distinct from all reference sequences. The data of our study suggests the presence of local networks in certain risk groups with specific HIV-1 genotypes [29], an unequal subtype distribution among different risk groups, the lack or low prevalence of certain genotypes in specific groups, and the fluctuation of different HIV-1 genotypes overtime and in certain risk groups.

In our current study we found a broader genetic heterogeneity of HIV-1 circulating in Bulgaria than that previously reported by our group [27]. Most infections were with the major HIV-1 subtypes followed by a variety of CRFs or URFs. Although subtype B was the most prevalent genotype, the second largest genotype in Bulgaria is now CRF01_AE, followed by CRF02_AG. We also identified seven new complex circulating forms not seen before in Bulgaria and which are typically found in Cameroon, Libya, Singapore, Thailand, Saudi Arabia, Greece, Cyprus, Portugal, Spain and France. Notably, two of these CRFs have been associated with outbreaks in IDUs in Greece (CRF04_cpx and CRF14_BG) [32], but only one of these genotypes (CRF14_BG) was present in an IDU in Bulgaria and may repre-

sent a recent infection. The others were present in non-IDU populations (three heterosexual and one blood product recipient), suggesting that these rare variants may be spreading outside of IDUs. We also identified nine possible new URFs circulating in Bulgaria that may be the result of the mixing of so many genotypes in the population similar to the natural history of HIV-1 in Africa. These URFs were found to be associated with persons reporting travel abroad who may be facilitating the increase of viral diversity in Bulgaria. All of the URFs identified in our study, including the clusters of A-like and F-like sequences, will require further complete genome characterization to determine their genetic composition and final classification [33-36].

The distribution of HIV-1 subtypes in our study population varied by age, sex, geography, and risk exposure. The greatest number of infections and broadest HIV-1 diversity occurred in major cities where most immigrants tend to live. We also observed the largest number of HIV subtypes and hence the greatest amount of HIV diversity in persons over 45 years old despite there being fewer total infections in this age group. Persons infected by heterosexual or IDU transmission had the greatest variety of subtypes, compared to MSM ($p<0.0001$). Furthermore, we also observed that closely related viral clades are increasing in circulation in geographically restricted IDU subgroups. The HIV-1 CRF02_AG seen in one IDU subgroup appears to have been transmitted within separate transmission clusters. Both women and IDUs were more likely to be infected with subtypes CRF01_AE and CRF02_AG, though the trend in IDUs may be due to an overall increase in IDU infections over time in Bulgaria. In contrast, with the exception of two persons, all MSM were infected with subtype B. The high level of HIV-1 diversity and wide distribution among various risk groups and demographic classifications are likely to influence various aspects of the epidemic in Bulgaria, including patient treatment, disease progression, laboratory monitoring, and vaccine development.

Using this strategy by year of HIV/AIDS diagnosis, we found a dissimilar rate of introduction and spread of different HIV-1 clades over time during the history of the epidemic in Bulgaria. Some clades like CRF01_AE and subtype C have declined, and (while) other clades appear to be emerging, like CRF02_AG. Some of these observed changes in the dynamics of HIV-1 infection in Bulgaria can be explained by the initial introduction of specific HIV-1 clades in certain populations spreading afterwards within populations at increased risk for infection. However, in recent years the significant increase in transmission of certain HIV-1 subtypes to and among IDUs and MSM is most likely due to increased risky behavior and spread of subtypes within these groups but also coincided with a decrease in heterosexual infections.

In conclusion, we found a broad level of HIV-1 genetic diversity in Bulgaria, including subtypes, CRFs and novel URFs in different populations of the epidemic. Our data also suggests the extremely dynamic nature of the Bulgarian epidemic is characterized by considerable viral inflow and unequal distribution of different HIV-1 genotypes among high risk populations. These findings emphasize the need for sustained and focused molecular epidemiological surveillance to identify transmission links that can be targeted by prevention strategies to control the HIV-1 epidemic in Bulgaria.

Acknowledgments

Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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ASPERGILLUS SPECIES AS A SYSTEMIC PATHOGEN ISOLATION AND IDENTIFICATION OVERVIEW

K. Dimitrov

Bul Bio-National Centre of Infectious and Parasitic Diseases

ABSTRACT

Systemic Aspergillosis is the most common pathogenic disease in immunosuppressed hosts. Among the patients of highest risk are the ones undergone surgery, bone-marrow transplantation or solid organ transplantation, cytotoxic or corticosteroid chemotherapy, HIV-infection. *Aspergillus fumigatus*, *A. flavus, A. niger*, and *A. terreus* are the most common *Aspergillus* species that cause systemic infection. Infection caused by this species invades generally internal organs and most of the cases have a lethal outcome. This overview is focused on the diagnostic methods and rapid identification of invasive and non-invasive mycosis caused by species of Aspergillus. The rapid detection and identification of Aspergillus in clinical laboratories are extremely important for the management of patients with systemic aspergillosis.

Systemic mycosis are diseases which affect organs and systems of the patient and can have a sever prognosis. The cause of this diseases can be pathogenic or dimorphic fungi. Dimorphic fungi can be the cause of endemic mycosis with a typical geographical distribution. Such as histoplasma, coccidiomycosis, blastomycosis. This diseases are typical for immune-compromised patient: candidasis, criptococcosis, zygomycosis, fusarium and aspergillus induced infections. (1)

Cryptococcosis of the central nervous system is one of the commonest and most severe mycotic infections in our geographical area. In developed countries of Western Europe cryptococcal and mycobacterial meningitis appear with the same rate (2).

Some species like *Aspergillus* can induce invasive infections only in immune-compromised patient. Other have a lower chance to infect healthy individuals. The risk groups for systemic mycosis are: immune-compromised patients, patients undergoing anti-tumor therapy, organ or bone marrow transplants, surgery intervention, corticosteroid therapy, diabetic and AIDS patients. Also, newborn with lower weight.

ADDRESS FOR CORRESPONDENCE:

Kristian Dimitrov
Bul Bio-NCIPD
26, Yanko Sakazov blvd.
1504 Sofia, Bulgaria
e-mail: blackmesa575@gmail.com

Aspergillus infections can be highly lethal especially in transplant and patients with severe hematologic and oncologic disabilities, undergoing chemotherapy. *Aspergillus* is a very large genus containing over 185 species to which humans are constantly exposed. Only a small number of these species have, however, been associated with disease. Of these, over 95% of all infections are caused by three species: *Aspergillus fumigatus, Aspergillus flavus*, and *Aspergillus niger*. This is mostly contributed by size of the spores – conidia of this species. Several more species have been reported in association with aspergillosis cases, including *Aspergillus nidulans, A. terreus, Aspergillus oryzae, A. ustus*, and *A. versicolor*.

Of the documented species of *Aspergillus*, *A. fumigatus* causes the large majority of cases of both invasive and non-invasive aspergillosis. Inedeed, the allergic forms of the disease appear to be almost exclusively caused by this organism. Both aspergilloma and invasive aspergillosis are also caused by *A. flavus* and *A. niger*. More recently, cases of invasive aspergillosis caused A. terreus and A. nidulans have been reported. (3)

Most human infection are caused by *A. fumigatus*, but *A. flavus*, *A. nidulans, A. niger*, and *A. terreus* have also been implicated. These molds are widespread in the environment. They are common soil inhabitants and are also found in large numbers in dust and decomposing organic matter. Their spores are often found in the outside air.

Other fungi which cause similar infection are Candida sp. Under natural conditions Candida sp. are saprophytic yeasts. Some of the species are known as commensals of human skin and gastrointestinal tract. Some of the species are pathogenic in immunosuppressed host and are the most common causative agents of life-threatening invasive mycoses. Among the patients on higest risk are neutropenic individuals, patient on immunosuppressive or broad-spectrum antibiotic therapy, solid organ transplants (4).

Onichomicosis are another fungal infections caused by dermatophytes, yeasts and molds. The rapid and qualitative diagnosis of onichomicosis and accurate identification of dermatophytes and non dermatophyte molds is necessary because many of the growing number of antimycotic agents have different spectra of activity (5).

A. fumigatus is thermotolerant, able to grow over a temperature range from below 20ºC up to 50ºC, and grows well at over 40ºC (Which contributes to the pathogenicity). It abounds in vegetable matter decomposing in warm environments, such as self-heating hay and compost.

Inhalation of conidia leads to a variety of disease patterns. Atopic subject may develop asthma. In non-atopic subject, the presence of damaged lung tissue may result in the growth of a fungus ball (aspergilloma). Inhalation of massive doses of the conidia may lead to alveolitis. Aspergillosis also occurs in many species of birds and mammals, domesticated and wild, infected primarily by inhalation and starting as a pulmonary disease, although sometimes involv-

ing other organs. *A. fumigatus* is also one of several molds implicated in bovine mycotic abortion, probably initiated by inhalation, but with alimentary infection from spores ingested in moldy fodder as another possible route (3).
Inhaled spherules can go unnoticed by the immune system and than proliferate. They germinate and quickly invade the pulmonary tissue, with growth rate 4-5mm daily. Typical for the genus conidia are formed when hyphae reach bronchial locus rich of oxygen. Most commonly this is a blood vessel. The forming of condida can lead to spreading of the infection and it's distribution. Invasive infection can cause pulmonary arterial thrombosis, hemorrhage, pulmonary necrosis (1). Proteolytic enzymes production is another virulence mechanism which is examined during pathogenesis of the invasive or allergenic disease. The ability of the species *A. fumigatus* to produce elastase is connected to the virulence in mice. In all cases invasive aspergillosis in humans, all isolates have the ability to degrade elastin. Isolated antigen from *Aspergillus* species, which have proteolytic activity can react to a serum from patients with allergenic bronchoalveolar aspergillosis, aspergilom and invasive aspergillosis (1).

Diagnostics
Aspergillosis can occur in two ways depending of the severity of disease. In 90% of aspergillosis patients the following factor is present:
- citotocsical or corticosteroid chemotherapy;
- transplantation of internal organs and bone marrow;
- HIV-infection;
- ongoing neutropenia; (1)

The following samples are used for aspergillosis diagnostics: bronchoalveolar lavage, spinal fluid, urine, blood culture, cutaneous allergenic test with aspergillus antigen (1).
A key factor for the diagnosis is the morphology of the colony on nutrient media. They can be black, brown, yellow, red, white, green or other colors depending on the species and growth conditions. The coloring of the colony depends on microscopic elements of the fungus – staining of vegetative hyphae, conidia tips and sexual structures (if they exist). Coloring of air and substrate mycelium can differ. Other differential diagnostic mark are vesicules. They also differ in shape and size: spherical, hemi-spherical, ellipses or elongated and with stick shape with ball on the one tend (1) .
Gram staining – differential diagnostic method. Most of the fungi with significant medical importance are stained gram positive (1).
Acridine orange – is a method which stains nucleic acids of the microorganisms. In acid pH the fungi illuminated under fluorescent light glow red-orange. Whereas, leucocytes and other body cells in clinical samples glow green (1).
Fluorescent microscopic technique – Calcoflur White and Blancoflur are non-specific fluorochromes which connect with cellulose and chitin of the cell walls. When light up with fluorescent microscope the mycelium glows in the visible spectrum. The method expands the diagnostic possibilities if combined with KOH brightening. This method is applicable to small quality samples from lavage and biopsies (1).
Lactophenol cotton blue - The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: phenol, which will kill any live organisms; lactic acid which preserves fungal structures, and cotton blue which stains the chitin in the fungal cell walls (6).

Materials and media for isolation
The correct identification of any *Aspergillus* species require a specific media. The components of the media can affect the growth of the culture, morphology of the microscopic components, color and size. This can lead to incorrect identification. *Aspergillus* species can colonize almost every media (liquid, solid and hemi-solid). Widely used media in routine practice is Capek Dox agar (syntethic nutrient media). This media is used for comparison study. Primary isolation is used media with malts extract on which the culture can sporulate. For primary isolation is also used blood agar and sabouraud agar with extra supplement – blood (1).

Identification methods and materials
Species identification of fungi is based on form, outer appearance and colony color. The air and substrate thallus of the filamentous fungi can give information about their species appurtenance. The fungi growing on solid media are identifying via microscopically. Most *Aspergillus* species are fast growing. The growth speed is key characteristic for identification of the species. For example the diameter of the colony in normal growth conditions is related to the age. Other main characteristic is the type of the colony edge. Edges can be sharp, thin and diffused, smooth and complete, lobbing and also they can form air and substrate mycelium. (1)

1. Antibody detection versus *Aspergillus* in serum of patients
- immuno-fluorescent detection method for antibodies IgG antibodies are found mostly. Samples are made of 72h culture of *A. fumigatus* – Czapek Dox agar. The suspension in 10^6-10^7 cells per ml is applied on a slide, dried and fixed under heat. The slides after light-microscope control for density are been held in refrigerator of -20º C. The reaction has to states:
First state: the serum of the patient is being diluted with sterile saline solution of 1:10 to 1:640. It's applied on the slide in 8 samples. The slide is been held in moist chamber at 37ºC for 30 min, it's important not melt the samples on the slide. After that the slide is washed vertically under weak stream of water. The samples are dried.
Second state: drops of fluorescent serum versus human globulines suspended with Evanescent blue are applied. The sample held in moist chamber at 37ºC for 30 min. After that the sample is washed and dried.

The sample is viewed on fluorescent microscope in immersion – buffer paraffin 7.8 pH, covering glass and non-fluorescent immersion oil. The highest dilution which has clear fluorescence is taken under consideration for the title of the serum. Title bigger then ≥ 1:160 is indicative.

- immunodiffusion for detection of Aspergillus antibodies

Phenol agar is poured in a small petri dish for immunodiffusion. A soon as the agar hardens the petri dish is putted on 4ºC. In the petri dish with harden agar are made small wells in which are inoculated with: control serum; antigen; serum of the patient;

The result is read after the petri dish is held at 25ºC for a few days.

2.1. Detection of *Aspergillus* antigen via latex agglutination

Pastorex Aspergillus is quality and hemi-quality test, which is based on simple technique of agglutination. This method detects polysaccharide antigen of *Aspergillus* (glactomannan) in serum. Latex particles carrying monoclonal rabbit EBA-2 antibody versus *Aspergillus* galactomannan are used. These particles react with the polysaccharide antigen causing agglutination, which can be visualized without the use of equipment. The sensibility of this method range to 15 ng/ml(1).

2.2. Sandwich ELISA

Rat EB-A2 monoclonal antibodies are used. This technique is also known as commercial kit – Platelia Aspergillus (Bio Rad, Marnes-Coquette, France), this method is considered as one of the most sensitive methods for detection of galactomannans (1).

3. Genetic methods for diagnostic of systemic mycosis

Methods for hybridization and amplification of nucleic acids are fundamental for molecular diagnosis. The first group of methods are aimed for identification of a current microorganism. This is accomplished using a DNA probe (artificially synthesized one stranded DNA chain) complementing to a targeted DNA. The polymerase chain reaction (PCR) consists in exponential amplification of a specific DNA fragment, using primer (short artificially synthesized oligo nucletid). A hybridization with the specific sequence of the targeted DNA occurs followed by DNA synthesis via Taq polymerase. After the initial phase of denaturation (thermic dissociation of the two stranded native DNA) comes the hybridization of the primers and the synthesis of a new DNA chain. In the next cycle these newly synthesized DNA fragments will act as a matrix for new DNA synthesis process. This process is repeated in about 25 – 50 cycles, generating millions of molecules DNA.

Pulse electrophoresis of chromosomal DNA (PFGE)

The process consists of electrophoretic division of DNA with consistent switching of the direction of the electric field. This method has been highly regarded for the molecular typing in the near past. The method is developed by Schwartz and Cantor in 1984. Characterization of the organisms, trough electrophoretic division of the intact chromosome DNA is known as karyotyping. PFGE is applied as a reference method for genetical typing in bacteriology for clinically important microorganisms (1).

Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) analysis is a relatively new technique which has a discriminatory power that makes it suitable for identification as well as
for strain typing (7).

AFLP is a whole genome analysis based on restriction enzyme DNA analysis followed by specific amplification of the restriction fragments. The FAFLP typing strategy is based on DNA digestion with two restriction enzymes, ligation of appropriate linkers (adaptors) to the restriction sites and PCR amplification of the polymorphic fragments with fluorescently labeled primer (8).

Rapid detection and identification of causative agents is of extreme importance for the management of patients with systemic mycosis. The routinely used phenotypic methods are time consuming and lack sensitivity and/or specificity. Molecular methods for diagnostics of systemic aspergillosis are mostly PCR-based and use serum or blood specimens. However in the case of poor-quality DNA containing serum sample there are many issues due to insufficient sensitivity or specificity of the method (9).

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CANDIDA SPECIES AS A SYSTEMIC PATHOGEN. ISOLATION AND IDENTIFICATION OVERVIEW

D. Dimitrov

Bul Bio-National Centre of Infectious and Parasitic Diseases

ABSTRACT

Fungi can be the cause of many nosocomial infections. Systemic fungal infections in many cases are targeted at patients or individuals with weak immune system suffering from neutropenia, human Immunodeficiency virus (HIV) or undergoing chemotherapy and etc. One of the most common systemic fungal infection is caused by the genus Candida from which Candida albicans takes the most significant part. Systemic fungal infections are very dangerous because they cannot be diagnosed by a common trait that characterizes the current pathogen. This is because the fungi can travel through blood and disseminate in any organ and therefore cause a variety of symptoms linked to the affected organ. The early identification of the fungus is critical for an appropriate treatment. In that regard we need to know how to isolate properly the pathogen avoiding false positive results, so that we can identify it. In this overview we will discuss these steps focusing on systemic infections caused by Candida albicans. The genus and is widely spread in all our surroundings, including places that are supposed to be free of contamination as hospitals and others. The genus *Candida* comprises about 200 species, close to 20 of which are associated with pathology in humans or animals. Various pathological conditions were shown to be associated with yeasts. The fungus was isolated by Bennett in 1844 in the sputum of tuberculosis patients, by Wilkinson in 1849 from vaginal candidiasis, by Robin in 1853 from a systemic infection and by Zenker in 1861 from a brain infection in a debilitated patient in whom the fungus spread hematogenously from an oral infection (1). Similar disease is caused by Geotrichum candidum. Geotrichosis affects mainly patients with systemic diseases like diabetes mellitus, leukoses, neoplasms etc. Clinically, it is similar to candidiasis and may occur as an oral, vaginal, skin, or systemic infection (2). Onichomicosis are fungal infections caused by dermatophytes, yeasts and molds. The rapid and qualitative diagnosis of onichomicosis and accurate identification of dermatophytes and non dermatophyte molds is necessary because many of the growing number of antimycotic agents have different spectra of activity (3).

ADDRESS FOR CORRESPONDENCE:

Dilko Dimitrov
Bul Bio-NCIPD
26, Yanko Sakazov blvd.
1504 Sofia, Bulgaria
e-mail: dilkodimitrov@gmail.com

Yeasts from the species Candida can cause candidiasis. Candida albicans appears to be the most commonly recovered ethiological agent of candidiasis. Mostly affected are children, immune compromised patients or patients continuously treated with antibiotics. The typical morphology and determination of the biochemical features are of major importance for a relevant antimycotic treatment course. The most reliable tests are based on utilisation of carbohydrates but unfortunately they are time consuming and too expensive. The microscopic detection of the typical morphology is rapid, easily applicable method that is sufficient to differentiate among Candida species.

The systemic candida infection can be severe or even lethal (4). Disseminated candidiasis can be defined as a multiorgan infection including possible candidemia although blood cultures do not always yield the fungus. The disease may include involvement of the central nervous system, kidneys, heart, eyes, or other organs and systems. Once Candida enters the bloodstream, weather from exogenous or endogenous source, the microorganisms have to adhere to the endothelial surfaces of the blood vessels, before dissemination into tissue. This process involves endocytosis, endothelial invasion, and response to the invading pathogen expressed in production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF) or interleukine (IL)-6 (1). It was also noted that germ tubes are better endocytosed than blastospores (1).

Physiology. Candida spp. metabolizes glucose via the hexose mono-phosphate pathway under aerobic conditions (assimilation), or via the Embden-Mayerehof pathway in anaerobiosis (fermentation). Additional metabolic mechanisms such as mitochondrial oxidative phosphorylation and the Krebs cycle and protein synthesis by 80S ribosomes (composed by 60S and a 38S subunit) do not differ from those described in other eukaryotic cells(1). Among catabolites secreted by some Candida spp. are acids, fatty acids, and alcohols, including ethanol (1).

The virulence traits of Candida spp., particularly of the major pathogen, C. albicans, to which possible roles in pathogenicity of candidiasis have been attributed, are believed to be associated primarily, with:

- The ability of the fungus to bind (attach, adhere) to host tissue as an initial step in the recognition and interaction with the host. Special attention has been given more recently to an associated phenomenon – biofilm formation, as a significant factor in the pathogenesis of candidiasis, affecting adversely the host's response to infection and causing difficulties in therapy.
- The production of specific enzymes that could facilitate tissue penetration and invasion, such as secretory aspatryl proteinases (SAPs) and phospholipases.
- Yeast-hyphal morphogenetic transformation,

which could also facilitate penetration and, in addition, assist the microbe to evade the host's defense system

- Phenotypic switching
- Various immunomodulatory effects of fungal determinants, which could contribute to reduced activity of the host's defense system (1).

As a sequel of attachment, microorganisms can grow in colonial communities and produce the so called "biofilms", instead of single entities – the "planktonic forms". Biofilm production was shown for Candida spp., particularly for C. albicans (1). The biofilm contains extracellular materials (ECM), composed of proteins, carbohydrates, and other substances. Other significant characteristics of biofilm to antimicrobials and the difficulties of the host's defense system to cope with the microbe result in difficulties of eradication of infection. Candida biofilm formation has important clinical ramifications as biofilms are formed on inert surfaces of medical devices such as catheters, artificial dentures, prosthetic valves, the use of which is known to be associated with increased risk for development of systemic infection (1).

Phenotypic switching associated with morphological colony changes from smooth to rough or fuzzy type, white to opaque, and other morphological changes is a phenomenon described first in the 1980s (1). Later studies associated phenotypic switching with difference in antigenicity, pathogenic potential, and indicated genetic regulation (1). Phenotypic switching is considered as a potential virulence characteristics as it may express the fungal plasticity, contributing to its adaptability to various anatomic sites of the human body and thereby possibly enabling the variety of clinical entities it causes, as well as it plays a role in the transmission from commensalism to pathogenicity (1). Furthermore , phenotypic switching may assist the microbe to evade the host's defense systems, in analogy to the bacterial "phase transition" phenomenon (1).

Isolation. Due to the nature of the different clinical forms, collections of clinical specimens may be problematic. It is difficult to obtain a sample from the infected organ in some clinical forms. In others, such as in the case of samples from the respiratory tract, the demonstration of Candida in sputum, a sample which is easily obtained . The specimen that is most easily obtained, which leads to the highest isolation rates, is blood cultures. However even these are characterized by a relatively high proportion of failure to isolate the fungus. The most recommended methods for blood cultures include hydrolysis centrifugation isolation system which apparently yields the highest rates of positive cultures (1). Other specimens from normally sterile sites with lesser rates of positive cultures may include CSF, tissue biopsies, or bronchial washings. Such specimens are inoculated on Sabouraud's agar with antibiotic but without cycloheximide (1).

Identification. The conducted identification of the species of fungus is based on shape, outer appearance and coloring of the colonies. Important for the species identification is the thallus of the filamentous fungi. Fungi growing on solid surfaces can be identified via microscopic observation. For that purpose can be used CHROMagar Candida. This is a selective solid media for isolation and preliminary identification of C. albicans (forms green colonies), C. tropicalis (red colonies), C. krusei (with pink colonies). Other alternative is the non-selective Corn meal agar (MERCK, Germany) based on extract of corn or rice flour. This media is primarily used for microscopic identification on the morphology of the fungi. Quick identification test can be conducted – germ tube test. In this test the production of germ tubes when in contact with serum indicates the presence of C. albicans. This test must be confirmed with Corn meal agar test. The procedure includes inoculation of sterile serum with 0,5 ml of the sample and it takes 2-4h. (5). Although fine differences between the species can be noted , they are, generally, very difficult to interpret and influenced by subjective judgment, and thus are not believed to be a valid criterion for routine Candida speciation (1).

Clearing microscope samples with KOH. For the preparation of microscope samples of smooth skin, mucosa and nails is used 10%, 15% and 20% KOH. The KOH melts cells and organic compounds for better observation of the fungi.(5)

Direct examination. If samples from normally sterile sites are obtained, demonstration of the presence of yeasts and/or hyphalelements in such samples, indicative of Candida, is important for the diagnosis. Direct demonstration of Candida in specimens from deep-seated candidiasis can be done both by wet and fixed mounts, aided by calcofluor white. Tissue biopsies can be prepared and stained by histopathological techniques, using the periodic acid-Schiff (PAS) or the Gomorimethamine silver (GMS) stain. (1)

Identification of the Candida isolated is done as in the case of mucocutaneous candidiasis. It is based primarily on macroscopic and microscopic morphology and on physiological/biochemical characteristics of the isolates.

Immunodiagnosis of candidiasis is based primarily on detection of the humoral immune response as in antibody production. As a consequence two types of assays are involved in immunodiagnosis :

- Tests for detection of antibodies, primarily in serum.
- Tests for detection of the fungal antigen(s) in body fluids, again, primarily in serum.(1)

The increased incidence of yeasts infections has stimulated the development of a commercial kits of rapid latex-agglutination tests for antigen detection (6).

Proving Candida antigen via latex agglutination. Diagnostics of systemic mycoses are confirmed when we have isolated biological material (normally sterile) from the yeast, such as blood or urine. The

result shown by the fungi culture are hard to interpret. It is possible for the hemoculture to be negative in the case of a disseminated infection. It is possible for circulating antigens or antibodies to be found in immune compromised patients. This principle is based on detection of polysaccharide antigens or mannans of the Candida species circulating in the serum. The mannan is the backbone of the cell wall of yeast. It circulates and can be a circulating antigen. Latex particles loaded with monoclonal rabbit's antibody (EBCA-1) against Candidaβ-١,٥-oligomanosyl are used. The particles react with the Candida antigen causing agglutination which can be observed. Problems in this current test are related to small amount of circulating mannans (5).

Other none culture identification methods may include molecular techniques such as the use of electrophoretic patterns of DNA. RNA profiling, restriction enzyme analysis, and PCR (1). Common molecular probes used by a number of investigator groups for identification of Candida at genus or species level are ribosomal DNA primers, including the large or small rRNA subunit (1). Additional probes described include SAP DNA, the ERG11 gene involved in ergosterol biosynthesis (1), and others. However, once again, these two do not yet generally constitute part of routine diagnosis (1). Identification of some yeast species remains problematic due to the high degree of phenotypic similarity between some of them. The use of Amplified Fragment Length Polymorphism (AFLP) analysis as an identification and typing method was investigated. In result it is shown that the AFLP patterns are highly specific for each species (7). A similar technique is also applied to a pathogen with a common trait of the Candida spcies which cause systemic infections. Cryptococcus neoformans is a pathogenic fungus and can cause life-threatening infections in humans, especially in immunocompromised patients.In such a case Fluorescent Amplified Fragment Length Polymorphism (FAFLP) genotyping has been applied to analyze clinical isolates (8).

Rapid detection and identification of causative agents is of extreme importance for the management of patients with systemic candidosis. The routinely used phenotypic methods are time consuming and lack sensitivity and/or specificity. Molecular methods for diagnostics of systemic candidiasis are mostly PCR-based and use serum or blood specimens. However in the case of poor-quality DNA containing serum sample there are many issues due to insufficient sensitivity or specificity of the method (9).

Methods for hybridization and amplification of nucleic acids are fundamental for molecular diagnosis. The first group of methods are aimed at identification of a current microorganism. This is accomplished using a DNA probe (artificially synthesized one stranded DNA chain) complementing to a targeted DNA. The polymerase chain reaction (PCR) consists in exponential amplification of a specific DNA fragment, using primer (short artificially synthesized oligonucletid). A hybridization with the specific sequence of the targeted DNA occurs followed by DNA synthesis via Taq polymerase. After the initial phase of denaturation (thermic dissociation of the two stranded native DNA) comes the hybridization of the primers and the synthesis of a new DNA chain. In the next cycle these newly synthesized DNA fragments will act as a matrix for new DNA synthesis process. This process is repeated in about 25 – 50 cycles, generating millions of molecules DNA (5).

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