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Research paper

First insight into phylogeography of *Mycobacterium bovis* and *M. caprae* from cattle in Bulgaria

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ABSTRACT

Bovine tuberculosis (bTB) represents a significant economic burden to the agriculture. In spite of decades of the control program, *Mycobacterium bovis* infection levels in cattle in Bulgaria continued to rise over recent years. In order to gain a better understanding of the *M. bovis* diversity, we used spoligotyping for strain differentiation and the data were compared to the international databases Mbovis.org and SITVIT2 for shared type and clade assignment. Study sample included 30 *M. tuberculosis* complex isolates from cattle originating from different regions of Bulgaria. The isolates were subdivided by spoligotyping into 4 spoligotypes: 2 types shared by 20 and 8 isolates and 2 singletons. SITVIT2-defined types SIT645 and SIT647 belonged to the common and classical bovine ecotype *M. bovis* (9 isolates) while types SIT120 and SIT339 belonged to the *M. caprae* ecotype (21 isolates). A certain phylogeographic gradient of the spoligotypes and clades at the within-country level was observed: *M. caprae* was prevalent in the central/southwestern, while classical *M. bovis* in the northeastern Bulgaria. Whereas all four types have global or European circulation, neither was described in the neighboring Balkan countries. *M. caprae* isolates identified in this study mostly belong to the Central/Eastern European cluster. In summary, this study provided a first insight into phylogeography of *M. bovis* in Bulgaria and described, for the first time, *M. caprae* as an important infectious agent of bTB in this country.

1. Introduction

Tuberculosis in animals and humans is caused by different members of *Mycobacterium tuberculosis* complex, whereas *Mycobacterium bovis* is the major etiological agent of bovine tuberculosis (bTB) (Brosch et al., 2002; Brites et al., 2018). *M. bovis* can also infect humans causing zoonotic tuberculosis, clinically and pathologically similar to the human tuberculosis (https://www.oie.int/en/animal-health-in-the-world/animal-diseases/bovine-tuberculosis/). More recently, *M. caprae* (formerly *M. bovis* subsp. *caprae*) was recognized as a separate member of the *M. tuberculosis* complex (Aranaz et al., 2003). Initially described as the main aetiological agent of caprine tuberculosis, *M. caprae* was later reported from wild boar, cattle and sheep (Rodriguez-Campos et al., 2014). Different wild animal species were recognized as TB reservoirs, becoming a source of infection for domestic livestock and hampering the eradication of bTB in cattle (Bachvarova and Tsvetkov, 1995; Delahay et al., 2006; Richomme et al., 2013). Bovine tuberculosis represents a significant economic burden to the agriculture (Delahay et al., 2006; Torres-Gonzalez et al., 2016). In Bulgaria, in recent years, as a result of decreased animal health control and monitoring of transported animals from one place to another and irregular tuberculinization, the bTB increased in many public and private farms in different regions of the country. According to the Bulgarian Agency for Food Safety for the period from 1983 to 2012, 125 epizootological outbreaks of bTB with 607 positive results were reported in the Northeast, Central North and Northwestern regions of Bulgaria (Bulgarian Food Safety Agency, 2014). Timely detection of the vectors of infection and identification of the factors contributing to transmission of *M. bovis* are important to accelerate the recovery of farms. The absence of information about the population structure of *M. bovis* in Bulgaria underline the need for systematic research on genetic variation in *M. bovis*. In addition, one should note that *M. bovis* genotyping data on the Balkan/southeastern region of Europe are scarce in the public databases (www.mbovis.org, www.pasteur-guadeloupe.fr:8081/

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Original article

EVALUATION OF CLASSICAL AND RAPID METHODS FOR ISOLATION AND IDENTIFICATION OF *MYCOBACTERIUM BOVIS* IN CATTLE IN BULGARIA

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Summary

Savova-Lalkovska, T., M. Bonovska, A. Dimitrova, V. Valcheva, Y. Petkov, G. Hadjieva & H. Najdenski, 2019. Evaluation of classical and rapid methods for isolation and identification of *Mycobacterium bovis* in cattle in Bulgaria. *Bulg. J. Vet. Med.* (online first).

Bovine tuberculosis is still a serious problem with major economic impact in many countries. The aim of study was to evaluate the diagnostic capabilities of the classical and some modern, rapid methods for isolation and identification of *Mycobacterium bovis*. In the period 2015–2018 from 29 outbreaks in 10 different regions of Bulgaria, 1193 lymph nodes from slaughtered cattle were examined by pathoanatomical, bacteriological, PCR and immunochromatographic methods. Of the 283 bacterial isolates, 263 were identified as *M. bovis*– member of the *M. tuberculosis* complex.

Key words: bacteriology, bovine tuberculosis, conventional PCR, immunochromatographic test, lymph nodes, pathoanatomy

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic disease characterised by the progressive development of specific granulomatous lesions or tubercles in the lymph nodes, lung tissues or other viscera. The causative agent of bovine tuberculosis is *Mycobacterium bovis*, a member of *Mycobacterium tuberculosis* complex (MTC). All MTC members are phylogenetically related and can cause tuberculosis in several animal species and men (Brosch *et al.*, 2002). At the genome level *M. bovis* shows 99.95% identity with *M. tuberculosis*, the agent of human tuberculosis (Garnier *et al.*, 2003).

Tuberculosis remains a significant health, social and economic problem due to the increasing number of sick people and animals, not only in developing but also in industrialised countries. In the countries where bTB is still common and pasteurisation of milk is not practiced, 10 to 15 per cent of human TB cases are caused by *M. bovis* (Good & Duignan, 2011). Humans are rarely affected by bTB, but people of some professions such

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ISOLATION OF *MYCOBACTERIUM AVIUM* subsp. *PARATUBERCULOSIS* FROM MOUFLON IN BULGARIA

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Abstract. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis (John's disease) mainly in large and small domestic and wild ruminants, and suspected causative agent in human Crohn's disease. In Bulgaria, paratuberculosis is still poorly researched in both groups of ruminants. We present results of the first in-depth study of mouflon, grown free in one hunting reserve in the Western region of the country. The aim was to prove the presence of MAP in diagnostic materials from regularly hunted or dead mouflon suspected for paratuberculosis. Small intestine and mesenteric lymph nodes (MLN) from 12 hunted and 4 dead mouflon and 10 faecal samples (Fc) were studied in the period of 2009–2013. Typical for paratuberculosis pathomorphological lesions were observed in four mouflon (of 16 examined). The intestinal wall was thickened, strongly folded and soft, with severe hyperemia. The MLN were enlarged, soft, with marbled appearance. The affected section of the ileum showed hyperplasia of the mucous corion and submucosa with diffuse infiltration of epithelioid cells. Lymphadenopathy with atrophy of T and B lymphocytes areas was observed in the mesenteric lymph nodes. For bacteriological isolation of MAP, the tissue and faecal samples were decontaminated with NALC-NaOH, cultured in Middlebrook 7H9 Broth and on Herrold's medium. The Ziehl–Neelsen stained smears and isolates were examined microscopically for acid-fast bacteria. Presence of MAP was observed in tissue samples of 4 (25%) mouflon and in 2 (20%) faecal samples. The same samples were confirmed by the IS*900* PCR for the presence of specific for MAP fragments with a commercial amplification kit. The cases of paratuberculosis found at different times in the free-living mouflon in our study prove that the disease exists in Bulgaria and highlight the need for more serious control of the disease among wild and domestic ruminants.

Key words: *mouflon, paratuberculosis, pathomorphology, microbiology, PCR.*

ВЫДЕЛЕНИЕ *MYCOBACTERIUM AVIUM* subsp. *PARATUBERCULOSIS* ИЗ МУФЛОНА В БОЛГАРИИ

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Резюме. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) является этиологическим агентом паратуберкулеза (болезнь Джона), главным образом, у крупных и мелких домашних и диких жвачных животных и предполагаемым возбудителем болезни Крона у человека. В Болгарии паратуберкулез все еще плохо

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4

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MEDICINE

Veterinary medicine

CASES OF PARATUBERCULOSIS IN DEER IN BULGARIA

Magdalena Bonovska, Tanya Savova*, Reneta Petrova*, Violeta Valcheva, Hristo Najdenski

(*Submitted on October 3, 2017*)

Abstract

In the period 2009–2013 the first in-depth veterinary study of paratuberculosis (Johne's disease) in deer in Bulgaria was conducted. We present pathomorphological, microbiological and PCR results from examined small intestine and mesenteric lymph nodes of 29 killed during hunting seasons and 5 dead deers free grown in one hunting reserve of western Bulgaria. This study was carried out on 23 fallow deer, 1 red deer, 2 white deer, 4 roe deer and 4 hind. Typical for paratuberculosis pathoanatomical lesions were observed in 2 fallow deer and one roe deer. Thickened intestinal mucosa of the ileum with elastic texture, that looked like a cerebral cortex was found. The mesenteric lymph nodes were increased to varying degrees. Histologically diffuse noncaseating granulomatous enteritis and lymphadenopathy with atrophy of T and B lymphocytes areas in the mesenteric lymph nodes was observed. In microscopic smears and bacteriological culture *Mycobacterium avium* subsp. *paratuberculosis*, the etiological agent of paratuberculosis was found. PCR analysis with amplification IS*900* PCR kit showed the presence of specific 209-bp amplicons. The samples of the other examined animals were negative. Faecal samples directly from the rectum of five of the shot deer were collected and examined too. In two of them acid-fast rods on the Ziehl-Neelsen stained smears were observed. They were also culturally and PCR positive.

The single paratuberculosis cases detected over different periods in free-living deer in our study prove that the disease exists in Bulgaria. The results indicate the need for further more extensive studies in hunting reserves in Bulgaria to determine the prevalence of the disease in wild animals.

Key words: deer, paratuberculosis, pathomorphology, microbiology, PCR

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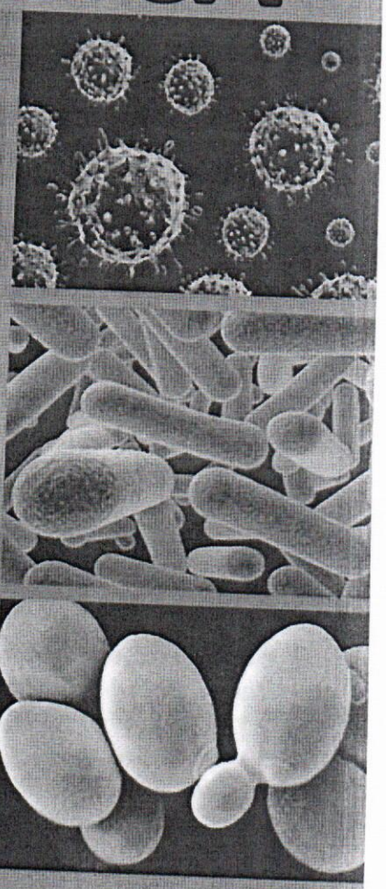

ACTA MICROBIOLOGICA BULGARICA

Review

Paratuberculosis in Animals and Humans – an Actual Health and Economic Problem

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Abstract

Mycobacterium avium subspecies *paratuberculosis* is the etiological agent of paratuberculosis in animals (Johne's disease) and in humans (Crohn's disease). The review summarized the etiology, epidemiology, pathogenesis and diagnosis of the disease in domestic and wild animals, and humans as well as future challenges to diagnostics, control prebiotics promoting the growth of probiotic compounds public health concern. Special attention is paid to the enormous economic losses worldwide of affected farms associated with decreased production of milk, meat and their products, increased incidence of mastitis and infertility in animals, and higher costs of in-patient care, ambulatory care and drugs for humans. The necessity to develop a National program for rapid diagnosis, monitoring and control of paratuberculosis in Bulgaria, similar to such programs in other affected countries, is explained. In addition, this program will enable the conducting epidemiological analyses and assessment of the risk to animal and human health.

Keywords: paratuberculosis, animals, humans, etiology, pathogenesis, diagnosis, control

Mycobacterium avium subspecies paratuberculosis е етиологичният причинител на паратуберкулозата при животните (болест на Джон) и хората (болест на Крон). Този обзор разглежда етиологията, епидемиологията патогенезата и диагнозата на заболяването при домашните и дивите животни и хората, както и новите предизвикателства пред диагностиката, контрола и общественото здравеопазване. Обръща се внимание на големите икономически загуби в световен мащаб, които паратуберкулозата причинява на засегнатите ферми, поради намаленото производство на мляко, месо и продукти от тях, увеличаването на маститите и безплодието при животните и разходите за лечение на заболелите хора – медикаменти и амбулаторно обслужване. Обосновава се и необходимостта от разработване на Национална програма за бърза диагностика, мониторинг и контрол на паратуберкулозата в България, подобно на прилаганите такива в други засегнати от заболяването страни. Тази програма ще позволи провеждане на епидемиологични анализи и съвременна оценка на риска за здравето на животните и хората.

Paratuberculosis in domestic animals

History and world prevalence of paratuberculosis

Paratuberculosis is one of the oldest diseases in cattle described in 1829 in England by Johne, and Frothingham in 1895. For the first time they discovered an acidalcohol-resistant or the first time they discovered an acidalcohol-resistantbacillus in the intestine of a bovine animal affected with chronic diarrhea (Johne and Frothingham, 1895). In the USA the disease was discovered in cattle in the early 1900s, and in New Zealand was first diagnosed in 1912 (Kopecky, 1961). Infectivity of the disease was proved by Bang in 1906. Later, in 1907, McFadyean described the disease in England, and in 1908 Vucovic reported paratuberculosis in sheep in Bosnia (Chiodini *et al.,* 1984). Twort (1910) and Twort and Inggram (1913) obtained a pure bacterial culture and gave a general description of the disease named "Johne's disease" (Huhn, 1965). The bacterium was named *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) at the suggestion of Thorel *et al.* (1990).

Paratuberculosis is a chronic, progressively

6

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Latin-American-Mediterranean lineage of *Mycobacterium tuberculosis*: Human traces across pathogen's phylogeography

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ABSTRACT

Currently, *Mycobacterium tuberculosis* isolates of Latin-American Mediterranean (LAM) family may be detected far beyond the geographic areas that coined its name 15 years ago. Here, we established the framework phylogeny of this geographically intriguing and pathobiologically important mycobacterial lineage and hypothesized how human demographics and migration influenced its phylogeography. Phylogenetic analysis of LAM isolates from all continents based on 24 variable number of tandem repeats (VNTR) loci and other markers identified three global sublineages with certain geographic affinities and defined by large deletions RD115, RD174, and by spoligotype SIT33. One minor sublineage (spoligotype SIT388) appears endemic in Japan. One-locus VNTR signatures were established for sublineages and served for their search in published literature and geographic mapping. We suggest that the LAM family originated in the Western Mediterranean region. The most widespread RD115 sublineage seems the most ancient and encompasses genetically and geographically distant branches, including extremely drug resistant KZN in South Africa and LAM-RUS recently widespread across Northern Eurasia. The RD174 sublineage likely started its active spread in Brazil; its earlier branch is relatively dominated by isolates from South America and the derived one is dominated by Portuguese and South/Southeastern African isolates. The relatively most recent SIT33-sublineage is marked with enigmatic gaps and peaks across the Americas and includes South African clade F11/RD761, which likely emerged within the SIT33 subpopulation after its arrival to Africa. In addition to SIT388-sublineage, other deeply rooted, endemic LAM sublineages may exist that remain to be discovered. As a general conclusion, human mass migration appears to be the major factor that shaped the *M. tuberculosis* phylogeography over large time-spans.

"A knight there was ...
In Latvia raided he, and Russia ...
In far Granada at the siege was he ...
and on the Middle Sea"
[Geoffrey Chaucer *The Canterbury Tales*]

1. Introduction

Mycobacterium tuberculosis is a clonal bacterial species and an important human pathogen that likely accompanied humans in

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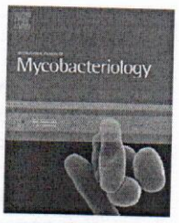

Prevalence of Latin-American-Mediterranean genetic family in population structure of *Mycobacterium tuberculosis* in Bulgaria

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ABSTRACT

Tuberculosis (TB) control remains an important public health priority for Bulgaria. The population structure of *Mycobacterium tuberculosis* is clonal and certain genetic families of this species (e.g., Latin-American-Mediterranean [LAM]) have attracted more attention due to their global dissemination and/or particular pathogenic properties, e.g., association with multidrug resistance (MDR). The aim of this study was to evaluate the prevalence of the M. tuberculosis LAM family in Bulgaria based on the use of different molecular markers. A total of 101 previously spoligotyped M. tuberculosis strains were studied by LAM-specific PCR assay to detect an insertion of IS6110 in the specific genome region. On the whole, clear-cut results were obtained for most strains; spoligotype-based family was reassigned in some of them. At the same time, double bands were amplified in some cases and warrant further validation studies of this method. The higher MDR rate among LAM versus other genotype isolates was observed ($P = 0.04$). In conclusion, these results suggest a low (<4%) prevalence rate of LAM in Bulgaria (that is similar to its Balkan neighbors) and highlight the importance of using robust markers for correct detection of the LAM family.

Introduction

Tuberculosis (TB) remains an important public health issue in Bulgaria. Although the number of new cases has declined since 2006 (41/100,000), the TB incidence rate in Bulgaria is still sufficiently high (26.7/100,000 in 2013), as well as multidrug-resistant TB (MDR-TB) rates among new TB cases (5.8%). Clinical and epidemiological characterization of the major *Mycobacterium tuberculosis* (*M. tuberculosis*) subpopulations circulating in different geographic regions is an essential step for developing better diagnostics, therapeutics, and vaccines. The integration of molecular analysis of *M. tuberculosis* with an epidemiological analysis of clinical information provides a new tool to assess possible associations between *M. tuberculosis* strain types and the clinical and epidemiological characteristics of the disease [1].

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8

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Chapter 3

Genetic Diversity of *Mycobacterium tuberculosis* Population in Bulgaria

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Abstract

Tuberculosis remains an important public health issue for Bulgaria, a Balkan country located in the world region with contrasting epidemiological situation for tuberculosis. Here, we present results of the recent studies on the genetic diversity of *Mycobacterium tuberculosis* population in Bulgaria that was evaluated with various DNA fingerprinting methods (spoligotyping, 24-MIRU-VNTR and IS*6110*-RFLP typing). The spoligotype-based population structure of *M. tuberculosis* in Bulgaria was shown to be sufficiently heterogeneous. It is dominated by several worldwide distributed spoligotypes ST53 and ST47 and Balkan-specific spoligotypes ST125 and ST41. The Beijing genotype strains were not found in Bulgaria in spite of close links with Russia in the recent and historical past. Comparison with international database SITVIT2 (Pasteur Institute of Guadeloupe) showed that spoligotype ST53 is found in similar and rather high proportion in the neighboring Greece and Turkey and almost equally distributed across different regions of Bulgaria. Contrarily, ST125 is not found elsewhere and is specific for Bulgaria; furthermore it appears to be mainly confined to the southern part of the country. Novel 15/24-loci format of MIRU-VNTR typing was found to be the most discriminatory tool compared to spoligotyping and IS*6110*-RFLP typing of *M. tuberculosis* strains in Bulgaria. Furthermore, VNTR typing was shown useful for resolving ambiguous

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9

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ACTUAL ASPECTS OF TUBERCULOSIS RESEARCH IN BULGARIA: EPIDEMIOLOGY AND DRUG RESISTANCE

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Abstract: Tuberculosis remains an important public health issue for Bulgaria, a Balkan country located in the world region with contrasting epidemiological situation for tuberculosis. Here, we present results of our recent studies on the genetic diversity of *Mycobacterium tuberculosis* in Bulgaria that was evaluated with various DNA fingerprinting methods. The population structure of *M. tuberculosis* in Bulgaria is sufficiently heterogeneous; it is dominated by several worldwide distributed spoligotypes ST53, ST34, ST47 and Balkan-specific spoligotypes ST125, ST41, ST284. The Beijing genotype strains were not found in spite of close links with Russia in the recent and historical past. Spoligotype ST53 is found in similar and rather high proportion in the neighboring Greece and Turkey and almost equally distributed across different regions of Bulgaria. Contrarily, ST125 is not found elsewhere and is specific for Bulgaria. Novel 15/24-loci format of MIRU-VNTR was the most discriminatory compared to spoligotyping and IS*6110*-RFLP typing. We also investigated molecular basis of drug resistance of the studied strains. *rpoB* and *embB306* mutations may serve for rapid genotypic detection of the majority of the rifampin- and ethambutol-resistant strains in Bulgaria; the results on isoniazid resistance are complex and further investigation of more genes is needed. Comparison with spoligotyping and 24-VNTR locus typing data suggested that emergence and spread of drug-resistant and MDR-TB in Bulgaria are not associated with any specific spoligotype or MIRU-VNTR genotype. A local circulation of the particular clones appears to be an important factor to take into consideration in the molecular epidemiological studies of tuberculosis in Bulgaria.

Key words: *Mycobacterium tuberculosis,* molecular epidemiology, drug resistance, genotypic detection, Bulgaria

Резюме: Туберкулозата остава важен здравен проблем за България, страна разположена в регион с контрастираща епидемиологична ситуация по отношение разпространението на туберкулозата. Представените резултати отразяват нашите изследвания върху генетичното разнообразие на *Mycobacterium tuberculosis* в България въз основа на различни ДНК фингърпринтинг методи. Популационната структура на *M.tuberculosis* е доста хетерогенна и в нея преобладават няколко глобално разпространени международни (ST53, ST47, ST34)

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DRUG-RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*: MOLECULAR BASIS AND GENOTYPIC DETECTION

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ABSTRACT

Emergence of multidrug-resistant Mycobacterium tuberculosis strains and their global dissemination necessitate development, evaluation and comparison of the rapid molecular tests that target genetic determinants of bacterial drug resistance. A wide range of such methods is available at present and the choice of those most appropriate is among the pertinent tasks of the National Tuberculosis Control Programs. Inadequate and/or interrupted therapy allows the selection of spontaneous mutations in favor of resistant organisms while sequential acquisition of these mutations in different genome loci results in the development of resistance to multiple drugs. The standard DOTS course comprises the five first-line drugs: rifampin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB), and pyrazinamide (PZA). Multi-drug resistance (MDR) is defined as resistance to at least RIF and INH. Anti-TB drug resistance is characterized by multigenic (rpoB, katG, inhA, ndh, embB, rpsL, rrs, pncA, gyrA) control and geographic variation of resistance mutations. Correct and rapid detection of drug resistance facilitates the appropriate and timely delivery of anti-TB therapy and reduces overall treatment cost. The prediction of drug resistance of M. tuberculosis by molecular tools presents a rapid alternative to the culture-based phenotypic susceptibility tests. Among the genotypic methods used to date are direct sequencing, microchips technology, PCR-single strand conformation polymorphism, RNA/RNA mismatch, molecular beacons and other assays. Genotypic methods allow rapid prediction of resistance to main anti-TB drugs in the considerable proportion of M. tuberculosis strains circulating in areas with high burden of MDR-TB.

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Keywords. *Mycobacterium tuberculosis;* drug resistance; genotypic detection; real-time PCR; allele-specific PCR; reverse hybridization; Bulgaria

Introduction

The emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* is one of the most critical issues facing tuberculosis (TB) researchers and clinicians today. Patients infected with drug resistant strains are less likely to be cured, and their treatment is more toxic and expensive than the treatment for patients infected with susceptible organisms. Inadequate and/or interrupted therapy allows the selection of spontaneous mutations in favor of resistant organisms while sequential acquisition of these mutations in different genome loci results in the development of resistance to multiple drugs.

The situation with MDR-TB in Bulgaria has slightly improved in recent years, although the country together with Romania remains a hot spot of MDR-TB in the European Union (except for the Baltic states whose special situation is due to their Soviet past, and Cyprus) (24). For example, the rates of MDR-TB among newly diagnosed and retreatment cases for Bulgaria were reported to be 1.7% and 17.1%, respectively (4.3 and 27.2% for Romania).

The standard DOTS treatment course comprises the five first-line drugs: rifampin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB), and pyrazinamide (PZA). MDR *Mycobacterium tuberculosis* strains are defined as those resistant to two major first-line anti-TB drugs, rifampin (RIF) and isoniazid (INH) (45). The mutation rate for RIF is the lowest compared to other drugs' mutation rate (10^{-9} versus 10^{-8}-10^{-7}), and hence RIF resistance is assumed as a surrogate marker of MDR (13). However, a geographical variation in the susceptibility profiles is observed which implies that resistance to other drugs besides RIF and INH should be tested as well.

Molecular basis of *M. tuberculosis* drug resistance

Multiple genes responsible for conferring resistance to the major anti-TB drugs have been identified for *M. tuberculosis*. A majority of RIF resistant strains harbor mutations in the 81-bp hot-spot region (rifampin resistance determining region, RRDR) of the *rpoB* gene (45, 55). INH resistance is controlled by a complex genetic system that involves several genes, *katG*, *inhA*, *ahpC*, *kasA*, and *ndh* (23, 26, 45, 51, 64). Resistance to pyrazinamide (PZA) is usually caused by mutations in the gene *pncA* encoding pyrazinamidase that converts PZA prodrug to its bioactive form (45). Resistance to EMB is often caused by the mutations in the *embB* gene, whose product arabinosyl transferase is involved in mycolic acids metabolism (56). Mutations in particular sites of the *rrs* (16S rRNA) and *rpsL* (small ribosomal protein S12) genes were shown to confer STR resistance in up to 80% of Str^r *M. tuberculosis* isolates (12, 31).

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RESEARCH ARTICLE

Bulgarian specificity and controversial phylogeography of *Mycobacterium tuberculosis* spoligotype ST125_BGR

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Keywords
spoligotyping; MIRU-VNTR; phylogeography; evolution; human migration.

Abstract

The local specificity of bacterial clones may be explained by long-term presence or recent importation/fast dissemination in an area. *Mycobacterium tuberculosis* spoligotype ST125, noticeably prevalent among Bulgaria-specific spoligotypes, has a characteristically 'abridged' profile and an uncertain clade position [Latin-American-Mediterranean (LAM)/S]. A comparison with the SITVIT2 database (Institut Pasteur de Guadeloupe) demonstrated its high gradient in Bulgaria (14.3%) compared with the negligible presence in the rest of the world. Further typing of all available Bulgarian ST125 strains revealed that they: (i) monophyletically clustered in 21-mycobacterial interspersed repetitive units (MIRU)-loci tree of all Bulgarian strains; (ii) grouped closely with the ST34 spoligotype, a prototype of the S family; and (iii) did not harbor a LAM-specific IS*6110* insertion. Comparison of the 21-MIRU-based network with geographic data revealed a complex dissemination pattern of ST125 in Bulgaria. Interestingly, this variable number of tandem repeats (VNTR) network remarkably corroborated with a recent hypothesis of single repeat loss as the primary mode of evolution of VNTR loci in *M. tuberculosis*. In conclusion, *M. tuberculosis* spoligotype ST125 is phylogeographically specific for Bulgaria. This spoligotype was not associated with drug resistance or increased transmissibility; its prevalence in Bulgaria can rather be attributed to the historical circulation in the country, having led, speculatively, to adaptation to the local human population.

Introduction

Local gradients in the prevalence of particular bacterial lineages and sublineages may reflect different events in the past history of the human host. Since early Neolithic, Europe as a whole and Balkans in particular were at the crossroads of human migrations, thereby transmitting human pathogens across the continent. Bulgaria, located near the Europe–Asia border, was in the front of these migrations, which left their imprint on the population structure of human pathogens circulating therein (Calafell *et al.*, 1996; Cavalli-Sforza *et al.*, 1996; Ivanova *et al.*, 2002).

Horizontal gene transfer is assumed to occur negligibly rarely among extant strains of *Mycobacterium tuberculosis*, implying a clonal population structure of this important human pathogen. Genetic families of *M. tuberculosis* are monophyletic clusters of genetically related strains; their evolutionary scenario is unidirectional and phylogenies are hierarchic. Apparently, these families or genotypes originated in well-delimited geographic areas and were usually named according to the geographic, historical or cultural name related to the region/country of their first isolation. Some of them remained circumscribed to their regions of origin, for example, Carabobo cluster in Venezuela (Abadia *et al.*, 2009). Other families have become omnipresent as a result of a likely increased virulence, transmissibility, etc. A natural consequence of clonal divergence might be the acquisition of differential pathogenic characteristics among different lineages. Specific genotypes of *M. tuberculosis* have been shown to dominate in patients, suggesting that these

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Short communication

Penitentiary population of *Mycobacterium tuberculosis* in Kyrgyzstan: Exceptionally high prevalence of the Beijing genotype and its Russia-specific subtype

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ABSTRACT

Here, we present results of the first study of the *Mycobacterium tuberculosis* genotypes circulating in Kyrgyzstan. We focused on the incarcerated population known to be at high-risk for tuberculosis (TB) and with a significant impact on TB incidence in the general population. Beijing genotype was detected in 42 of 56 *M. tuberculosis* sputum-extracted DNA samples from newly-diagnosed adult pulmonary TB patients. RIF and INH resistance was genotypically detected in 28% and 55% samples; 13 of 15 MDR strains belonged to Beijing genotype. 12-locus MIRU-VNTR typing showed 8 of 56 samples to be mixed cases; 7 of them contained a Beijing strain. MIRU analysis demonstrated a high homogeneity of the studied collection (HGI = 0.66) while 28 of 56 strains had a profile 223325153533 corresponding to Beijing/M2 subtype highly prevalent in different Russian settings. Three hypervariable loci, QUB-3232, VNTR-3820 and VNTR-4120, permitted to further subdivide 28 Beijing/M2 strains into 11 subtypes shared by 1 to 9 strains. To conclude, all markers taken together, the penitentiary population of *M. tuberculosis* in Kyrgyzstan exhibited a strong genetic affinity to Russia and a weak relatedness to East Asia.

1. Introduction

Tuberculosis (TB) incidence has reached the epidemic proportions in Kyrgyzstan, a highland Central Asian country of the former Soviet Union although the situation has slightly improved since 2006 (Alisherov et al., 2008). The penitentiary population, albeit negligible compared to the total population (8427 vs 5.4 mln in 2008 [Walmsley, 2009]), visibly influenced the TB burden in the country: incidence 108.8 and mortality 9.6 per 100,000 civilian population; 115.5 and 11.2 per 100,000 total population were reported in 2007 (Alisherov et al., 2008). Incarcerated populations are known to present a special risk group for many communicable diseases, including tuberculosis, in both industrialized and developing world. The probability of transmission is increased by overcrowding, poor ventilation, delays in medical evaluation and treatment. Prisons serve as reservoirs and enhancers of MDR-TB (Carbonara et al., 2005; Habeenzu et al., 2007) and continuous circulation and outbreaks in community of the prison-derived strains have been described in many reports (Ijaz et al., 2004; Ruddy et al., 2004; Martin et al., 2007). Analysis of a population structure of *Mycobacterium tuberculosis* in such high-risk penitentiary population can help detect not only drug resistant but otherwise pathogenic and possibly hypervirulent strains, to understand a dissemination of TB in the general population.

Here, we present results of the first study of the *M. tuberculosis* genotypes circulating in Kyrgyzstan. We focused on the incarcerated population known to be at high-risk for TB and at the same time with a significant impact on TB burden in the general population. We additionally evaluated some recently proposed schemes for *M. tuberculosis* genotyping with DNA extracted from clinical samples.

2. Materials and methods

2.1. Study sample

The study included 56 adult (20–45 years of age) HIV-negative patients with pulmonary TB who were newly diagnosed for TB and

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13

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Chapter V

Drug Resistant Tuberculosis in Bulgaria: Molecular Insights

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Abstract

Tuberculosis (TB) infects a significant proportion of the world population and constitutes a major public health problem, particularly, in the developing regions. A reemergence of TB accompanied by an increasing number of drug resistant *Mycobacterium tuberculosis* strains, including many resistant to multiple drugs, has been noted since the mid-1980s. In Bulgaria, the rate of multidrug-resistant tuberculosis (MDR-TB) was estimated to be 10.7% among newly diagnosed TB patients in 2006 that is much higher as compared to the neighboring countries. An increasing rate of MDR-TB implies importance of surveillance of resistance and its fast detection. Characterization of molecular basis of drug resistance in a survey area is a first step prior to implementation of such methods. Here, we present data on molecular basis of drug-resistance in *M. tuberculosis* strains currently circulating in Bulgaria. We also compared distribution of drug resistance mutations within the main genotypic clusters identified by spoligotyping

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14

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Evolution of Drug Resistance in Different Sublineages of *Mycobacterium tuberculosis* Beijing Genotype

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We compared the population structure and drug resistance patterns of the *Mycobacterium tuberculosis* strains currently circulating in the Beijing area of China. One hundred thirteen of 123 strains belonged to the Beijing family genotypes defined by spoligotyping. The Beijing genotype strains were further subdivided into old and modern sublineages on the basis of NTF locus analysis. A stronger association with resistance to the more recently introduced antituberculosis drugs has been observed for old versus modern strains of the Beijing genotype, suggesting that its different sublineages may differ in their mechanisms of adaptation to drug selective pressure.

In some areas of the world, the increased rate of multidrug-resistant (MDR) tuberculosis (TB) appears to be linked to a disequilibrium in the local population structure of *Mycobacterium tuberculosis*, manifested as a predominance of particular genetic lineages and sublineages. For example, strains of the Beijing genotype are endemically prevalent in eastern Asia, South Africa, and northern Eurasia (reviewed in references 1 and 9), and new unexpected routes of their transmission are being uncovered (7). This genotype was first identified in *M. tuberculosis* strains isolated in the Beijing area of China, for which it was named (23). Currently, these strains attract great attention worldwide because they demonstrate important pathogenic features (15, 24) and association with drug resistance (4, 9).

China is one of the MDR TB hot spots, along with Russia and India (6). The increased rate of drug-resistant and MDR strains of *M. tuberculosis* remains a serious problem of TB control in China (14, 20). In the present study, we investigated the current population structure of *M. tuberculosis* in the Beijing area of China and compared it with drug resistance patterns in order to gain new insights into the evolution of drug-resistant TB.

One hundred twenty-three *M. tuberculosis* isolates were recovered from 123 adult pulmonary TB patients admitted to the Beijing Chest Hospital in 2002 to 2005. No epidemiological connection of these patients could be detected by standard investigation. For each patient, only the first available isolate was included in this study. Löwenstein-Jensen medium was used for cultivation of isolates. Testing of susceptibility to rifampin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB), and pyrazinamide (PZA) was done by the method of absolute concentration as previously described (3).

DNA from cultured cells was extracted as described by van Embden et al. (22). Strain differentiation was performed by spoligotyping (11). A PCR approach was used to determine a possible IS*6110* insertion(s) in the NTF region of the *M. tuberculosis* Beijing genotype strains (18). Three NTF variants of the Beijing strains are distinguished on the basis of the presence or absence of the IS*6110* sequence, thus providing a rough subdivision within this genotype. The W branch prevalent in the United States harbors two head-to-tail IS*6110* insertions separated by a 556-bp noncoding spacer (13). Most Beijing strains worldwide harbor only one IS*6110* insertion (1, 13); we previously defined them as the NTF::IS*6110* "modern" branch (16). Finally, the Beijing strains without an IS*6110* insertion in the NTF region (12, 13) were previously defined as atypical (12, 17), ancient/primordial (16), or ancestral (17, 19); here, we assigned these strains to an "old" sublineage of the Beijing genotype.

Odds ratios and *P* values, were calculated with EpiCalc software (8).

Results and discussion. A total of 123 *M. tuberculosis* strains were included in this study. They comprised 40 pansusceptible and 83 resistant isolates randomly selected among those isolated between 2002 and 2005 in Beijing, China. A majority of the resistant strains were multidrug resistant; the distribution of the drug resistance profiles of all of the strains is shown in Table 1.

All strains were subjected to spoligotyping in order to assess their genetic relatedness. This genotyping method subdivided the 123 strains into 14 types (Table 2). The largest cluster

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15

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Exhibition of persistent and drug-tolerant L-form habit of *Mycobacterium tuberculosis* during infection in rats

Research Article

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Abstract: A model for studying mycobacterial L-form formation *in vivo* was established to demonstrate the ability of *M. tuberculosis* to behave as a drug-tolerant L-form persister. Rats were infected by intranasal (i.n.) and intraperitoneal (i.p.) routes with $1x10^8$ cells/ml of *M. tuberculosis*. At weekly intervals during a period of five weeks, samples from lung, spleen, liver, kidney, mesenterial and inguinal lymph nodes, broncho-alveolar and peritoneal lavage liquid were plated simultaneously on Löwenstein-Jensen (LJ) medium or inoculated into specially supplemented for L-forms Dubos broth (drug-free and drug-containing variants). The use of liquid media enabled isolation of mycobacterial L-form cultures during the whole period of experiment including the last two weeks, when tubercle bacilli were not isolated on LJ medium. An unique feature of mycobacterial L-forms was their ability to grow faster than the classical tubercle bacilli. Isolation and growth of L- form cultures in primary drug-containing media demonstrated their drug-tolerant properties. Electron microscopy of liquid media isolates showed that they consisted of morphologically heterogenous populations of membrane-bound and of variable sized L-bodies that completely lack cell walls. The identity of the isolated non-acid fast and morphologically modified L-forms as *M. tuberculosis* was verified by specific spoligotyping test. The results contribute to special aspects concerning the importance of mycobacterial L-form phenomenon for persistence and latency in tuberculosis, phenotypic drug tolerance, as well as for diagnosis of difficult to identify morphologically changed tubercle bacilli which are often mistaken for contaminants.

Keywords: *Mycobacterium tuberculosis* • *L-forms* • *Persistence* • *Drug-tolerance* • *Rat infection*

1. Introduction

By the early 1882-1940s after Robert Koch discovered the cause of tuberculosis and before introduction of chemotherapy against tuberculosis, a series of papers reported about the existence of L-form elements: filterable forms, branching filaments, syncytial growth, large spheres and also "variegated mycelia" characterizing mycobacterial growth [1-3]. Mattman summarized the known data about the ability of *M. tuberculosis* to convert to different forms such as non-acid fast cell wall deficient L-forms and suggested a "L-cycle" for mycobacteria [4]. The nature of this phenomenon is not well understood, but L-forms have been considered to be associated with the ability of bacteria to persist for a long period and resist host immune response and antimicrobial agents [4-12]. Indeed, cell wall defective bacilli have been observed in patient sputum specimens [13,14]. However, although cell wall deficient forms (L-forms) are sometimes reported to be found in patient specimens, their identity as genuine *M. tuberculosis* not due to contaminating bacteria has not been confirmed due to lack of an appropriate method for replicable isolation and cultivation of the variant L-form bacteria.

The present study was initiated in order to establish a rat model for studying the formation of *M. tuberculosis* L-forms and to assess its unusual characteristics.

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Short communication

Cell wall deficiency and its effect on methicillin heteroresistance in *Staphylococcus aureus*

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Abstract

Clinical strains of *Staphylococcus aureus* with different phenotypic methicillin susceptibility characteristics, bearing or lacking the *mecA* gene, were tested for their ability to transform into a cell wall-deficient state under special conditions of cultivation. Conversion to L-form growth with formation of typical L-form 'fried egg' colonies and expression of oxacillin resistance was observed in sensitive (*mecA*-negative) and heteroresistant (*mecA*-positive) strains. Transmission electron microscopy observation of these strains revealed pleomorphic populations of cell wall-deficient cells with ultrastructure morphology similar to that of a control stable L-form strain of *S. aureus*. The results demonstrate that expression of phenotypic methicillin resistance could be associated with cell wall deficiency in *S. aureus* strains and could underlie the phenomenon of heteroresistance.

Keywords: *Staphylococcus aureus*; Methicillin heteroresistance; Cell wall deficiency

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are a common cause of hospital-acquired infections. They are often isolated from patients in intensive care and surgical units [1,2]. MRSA strains were first isolated in the 1960s following the introduction of methicillin for treating staphylococcal infections [3]. Methicillin resistance is due to the production of a low-affinity penicillin-binding protein (PBP2a). PBP2a is encoded by the *mecA* gene, recently found to be harboured on the staphylococcal cassette chromosome *mec* (SCC*mec*), which is considered to be a novel type of mobile element [4,5]. The nature of heterogeneous expression of methicillin resistance is not fully understood. Coexistence of two subpopulations (susceptible and resistant) within a clinical isolate and expression of resistance only in a small number of cells leads to diagnostic problems in clinical laboratories [6]. Studies are underway to determine the factors and genes associated with this phenomenon. Many of these genes are involved in cell wall biosynthesis [7].

Little is known about the population-based morphological variability and cell wall deficiency in heteroresistant strains of *S. aureus* or about their contribution to phenotypic expression of methicillin resistance.

The purpose of the present study was to investigate the ability of clinical *S. aureus* isolates with different methicillin susceptibility characteristics to convert into a cell wall-deficient state under special condition of cultivation and to express oxacillin resistance.

2. Methods

2.1. Strain characteristics

Clinical isolates of *S. aureus* were obtained from patients in intensive care units (Plovdiv University Hospital, Bulgaria) and were identified using standard laboratory techniques.

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Synthesis, antimycobacterial activity and docking study of 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives and related hydrazide-hydrazones

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ABSTRACT

A new convenient method for preparation of 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b–g** and coumarin containing hydrazide-hydrazone analogues **4a–e** was presented. The antimycobacterial activity against reference strain *Mycobacterium tuberculosis* H37Rv and cytotoxicity against the human embryonic kidney cell line HEK-293 were tested *in vitro*. All compounds demonstrated significant minimum inhibitory concentrations (MIC) ranging 0.28–1.69 μM, which were comparable to those of isoniazid. The cytotoxicity (IC_{50} > 200 μM) to the "normal cell" model HEK-293T exhibited by 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b–e**, was noticeably milder compared to that of their hydrazone analogues **4a–e** (IC_{50} 33–403 μM). Molecular docking studies on compounds **4a–e** and **5b–g** were also carried out to investigate their binding to the 2-*trans*-enoyl-ACP reductase (InhA) enzyme involved in *M. tuberculosis* cell wall biogenesis. The binding model suggested one or more hydrogen bonding and/or arene-H or arene-arene interactions between hydrazones or pyrazole-fused coumarin derivatives and InhA enzyme for all synthesized compounds.

A reemergence of tuberculosis accompanied by an increasing number of drug resistant *Mycobacterium tuberculosis* strains highlights the urgent need of searching and developing of new antitubercular drugs, capable of bypassing the resistance mechanisms. In the last decades, major advances in molecular biology have increased the knowledge of the mechanisms of resistance to the main anti-TB drugs, with the identification of specific gene mutations that are associated with drug resistance[1,2]

Isoniazid (INH), an essential antitubercular agent recommended by the WHO, is a prodrug that penetrates the tubercle bacilli by passive diffusion and is bio-activated by the bacterial anti-oxidant enzyme (KatG).[3,4] It exerts its anti-tubercular activity *via* interference with the synthesis of mycolic acids, which comprise crucial elements of the mycobacterial cell wall. Even with the clinical success of isoniazid, severe adverse effects, especially peripheral neuropathy and hepatotoxicity, are associated with INH-based treatment protocols; moreover its usefulness is further limited by the occurrence of resistance.[5] To overcome the resistance,[6] the drug design strategies frequently employ a combination of the INH molecule with other pharmacophores, rendering antitubercular activity. The novel INH hydrazide derivatives appeared to be promising anti-tubercular agents - more effective and less hepatotoxic than isoniazid.[7–16] In the meantime Ellis *at al.*,[17] have described the mechanism of action of the pyridoxal isonicotinoyl hydrazones (PIC) and suggested that hydrazones act as a lipophilic vehicle for the transport of its intact INH moiety into the mammalian cell and the mycobacterium (Fig. 1). The mechanism of antimycobacterial activity of INH[18] and isonicotinoyl hydrazone derivatives passes through formation of electrophilic intermediate species (i.e. a hydrazyl radical or ion) (Fig. 1). The acyl radical being coupled to NADH or NAD + seems to be crucial in yielding adduct responsible for the inhibition of 2-*trans*-enoyl-ACP reductase (InhA), and in restraining the mycobacterial cell wall synthesis. InhA catalyze the final step in the elongation cycle of the bacterial

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18

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Antimycobacterial activity of novel hydrazide-hydrazone derivatives with 2*H*-chromene and coumarin scaffold

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ABSTRACT

This study reports the synthesis of new 2*H*-chromene or coumarin based acylhydrazones, which were evaluated for their *in vitro* antimycobacterial activity against reference strain *Mycobacterium tuberculosis* H37Rv and compared to the first-line antituberculosis drugs, isoniazid (INH) and ethambutol (EMB). The most active compounds **7m** (MIC 0.13 μM), **7o** (MIC 0.15 μM) and **7k** (MIC 0.17 μM) demonstrated antimycobacterial activity at submicromolar concentration level and remarkably minimal associated cytotoxicity in the human embryonic kidney cell line HEK-293T. Structure-activity relationship for this class of compounds has been established.

Despite notable progress in the treatment of mycobacterial infections, tuberculosis (TB) is still a public health concern worldwide.[1,2] The dramatic increase in number of cases of multidrug-resistant (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB)[3–5] require development of novel low toxic compounds with improved efficacy[6–8] Current treatment of TB with first and second line drugs require a minimum six months of therapy to prevent relapse. This long-term chemotherapy certainly increases the risk of drug resistance. Although isoniazid (INH) is still the most effective antituberculosis drug, recommended by WHO, it has two major drawbacks which need to be overcomed: hepatotoxicity and its deactivation via the genetically polymorphic *N*-arylaminoacetyltransferases (NATs). Indeed hydrazones, isoniazid derivatives wherein N^2 is blocked towards acetylation by NATs, appeared to be more effective and less hepatotoxic than isoniazid.[9–12] Various classes of pyridine, pyrimidine, coumarin, quinoline, benzoxazole, indole, purine, pyrrole, furan, benzofuran, triazoles etc. based hydrazones[9,10,12–16] have been evaluated for their antimycobacterial potential. Also, 2*H*-chromenes, coumarins and their derivatives have gained great therapeutic importance for the discovery of novel antimycobacterial compounds.[17–24] Keri et al. (2015)[25] have emphasized the strategic place of coumarin derivatives in medicinal chemistry particularly on their antituberculosis effect.

Therefore, we focused on the synthesis of 2*H*-chromene or coumarin compounds with various substituted hydrazide-hydrazone pharmacophore attached to the 3rd position in the chromene ring. The antimycobacterial activity of the synthesized compounds against *Mycobacterium tuberculosis* H37Rv strain and the toxicity against human embryonal kidney cell line HEK-293T were evaluated.

The synthesis of 2*H*-chromane and coumarin based hydrazones is presented in Scheme 1. Compounds **3a–c** were prepared from the condensation of salicylaldehyde **1** and α,β-unsaturated aldehydes **2a–c** under base-promoted conditions.[26] For the preparation of 2*H*-chromene-3-carbaldehyde **3a** and 2-methyl-2*H*-chromene-3-carbaldehyde **3b** we applied the synthetic strategy of Azizmohammadi et al.,[27] while the synthesis of 2-phenyl-2*H*-chromene-3-car-

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19

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Original article

Antimycobacterial activity of chiral aminoalcohols with camphane scaffold

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ABSTRACT

A series of aminoalcohols were synthesized by reaction of aminolysis of camphor derived oxiranes with chosen amines. The compounds were evaluated for their *in vitro* activity against *Mycobacterium tuberculosis* H37Rv. Ten of the new structures show much higher activity than the classical anti-TB drug ethambutol. Some of the most active compounds were tested against MDR strain 43, and four of them demonstrated excellent activities with MICs 0.27–0.72 μM. The cytotoxicity of representative exerting antimycobacterial activity compounds was assessed. Quantitative structure–activity relationship (QSAR) model is derived to estimate the contribution of each structural fragment to the activity. The camphane-based aminoalcohols are promising lead compounds for further development of novel antimycobacterial agents.

1. Introduction

Mycobacterium tuberculosis (MTB) infects latently one-third of the world's population causing approximately 9 million cases of active disease each year [1]. The WHO-recommended anti-TB therapy involves four drugs: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB), but the emergence of multi drug-resistant bacteria (MDR TB) against which the first-line drugs have become ineffective, requires treatment for up to two years with more toxic, less active and more expensive drugs. The latter usually involve any first-line drugs to which the strain is still susceptible and alternative or second-line drugs [2]. The long current drug regimen, the emergence of drug resistant strains and HIV co-infection necessitate the urgent development of new and effective anti-TB drugs.

The synthesis and activity of EMB (Fig. 1. **I**) was first reported by Wilkinson and coworkers [3]. Despite the relatively modest MIC of 10 μM, EMB is a useful addition to tuberculosis chemotherapy, in part because of its very low toxicity and relatively few side-effects. Based on structure–activity relationship (SAR) studies it appeared that crucial for its activity is the distance between the two nitrogens, the presence of β-aminoalcohol motifs, and the small side chains [4]. Lately, the occurrence of a 'better ethambutol' has been systematically investigated through virtual screening or a combinatorial approach [5,6]. Several 1,2-diamines, such as SQ 109 (Fig. 1. **II**), displaying improved antimycobacterial potencies and promising pharmacokinetic properties have thus been reported [7]. It is very likely that the highly lipophilic adamantane structure was integral for the antitubercular activity.

Camphor and its derivatives are of particular importance among the numerous monoterpenoids. Camphor is a readily available and inexpensive chiral source for the synthesis of a variety of structurally diverse compounds [8,9]. Inspired by the β-aminoalcohol fragment in the molecule of EMB and the analogy of the camphane scaffold as a compact lipophilic moiety to the adamantyl fragment in SQ 109, we dedicated our efforts towards the development of camphor derived structures. Recently, we have accomplished a practical synthesis of new β-amido-alcohols and amido-diols on the base of 3-*exo*-aminoisoborneol (Fig. 1. **III**) and isobornylamine (Fig. 1. **IV**) [10,11]. Some of the compounds show 25 times higher antimycobacterial activity than EMB.

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20

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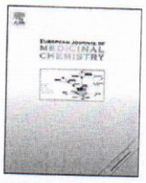

Original article

Efficient synthesis of new (*R*)-2-amino-1-butanol derived ureas, thioureas and acylthioureas and *in vitro* evaluation of their antimycobacterial activity

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ABSTRACT

The synthesis of 22 structurally diverse urea, thiourea and acylthiourea derivatives containing the (*R*)-2-amino-1-butanol motif has been performed. The evaluation of their *in vitro* activity against *Mycobacterium tuberculosis* ($H_{37}Rv$ and strain 43) showed promising results in the case of the acylthiourea derivatives (MIC range 0.36–7.46 μM for $H_{37}Rv$ strain).

1. Introduction

Tuberculosis (TB) is one of the most devastating diseases primarily due to several decades of neglect, HIV infection, immigration and globalization [1]. Approximately one-third of the world's population has been infected with the causative organism *Mycobacterium tuberculosis* (MTB), eight million become sick with TB and globally it accounts for approximately two million deaths per year. The spreading of collaborative TB/HIV infections [2] and the reemergence of TB accompanied by an increasing number of drug resistant and multidrug-resistant (MDR) strains MTB (i.e. resistant to at least rifampin [RIF] and isoniazid [INH]) has been noted since the mid-1980s [3–5]. Thus, management of tuberculosis is complicated, which has become a serious health problem worldwide.

The frontline drugs INH, RIF, pyrazinamide (PZA) and ethambutol (EMB) are currently recommended by the World Health Organization (WHO) for the treatment of TB [6]. The problems with current TB treatment are complex and include: a prolonged standard course regimen of six months, which often result in patient noncompliance; emergency of extremely drug-resistant tuberculosis (XDR-TB) strains; lack of effective drugs against the latent state. One approach to decrease treatment time is improvement of potency of currently used anti-tuberculosis drugs [7], mainly through discovery of more effective combinations with newer, more potent and less toxic active compounds [8,9]. There is a clear trend toward gradually increasing partition of new active compounds, including derivatives of known anti TB drugs [10] and natural products [11]. Except of few new chemical entities [12,13] no other anti MDR-TB drugs with proved novel mechanism of action are available in clinical use since last 40 years, but many classes of new potent compounds [13–15] are currently in different steps of their anti TB evaluation.

EMB is a simple (*S*)-2-amino-1-butanol derived 1,2-diamine, clinically used as primarily bacteriostatic anti-tuberculosis agent (Scheme 1) with not fully known mechanism of action. It targets the arabinosyl transferases responsible for arabinogalactan biosynthesis, a key component of the unique mycobacterial cell-wall [16–18]. Despite modest antimycobacterial activity and due to its synergy with other drugs and lower toxicity, EMB is used in combination with more potent frontline antimycobacterial agents. The configuration of

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21

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Original article

Novel camphane-based anti-tuberculosis agents with nanomolar activity

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ABSTRACT

A series of new amidoalcohols and amidodiols were designed on the base of the camphor scaffold and evaluated for their in vitro activity against *Mycobacterium tuberculosis* H37Rv and MDR strain 43. Some of the new compounds show 25 times higher activity than the classical anti-TB drug ethambutol. Small structural changes in the side chain shift the activity from micromolar to nanomolar inhibitory concentrations. Quantitative structure–activity relationship (QSAR) model is derived to guide the further lead optimization. Two hydrogen bond donors and up to three rings in the molecules are optimal for nanomolar activity. The camphane-based amides present novel promising scaffolds for antimycobacterial agents.

1. Introduction

Tuberculosis (TB) is a global health problem causing substantial morbidity, mortality, negative socioeconomic impact, and human suffering. One-third of the world's population is latently infected with *Mycobacterium tuberculosis* and approximately 9 million cases of active disease occur each year [1]. The recent widespread emergence of multidrug resistant (MDR) strains of *M. tuberculosis* to clinically available drugs puts further impetus to the urgent need for the discovery of new and effective anti-TB agents. Much progress has been done in drug development over the past decade. Currently, there are at least nine compounds in clinical development: two in phase III, four in phase II, and three in phase I trials [2]. Among these, four are existing drugs redeveloped for a TB indication and five are new chemical entities. More than 30 new anti-TB drugs are in preclinical development [3,4].

Monoterpenoids have long been widely used as chiral, enantiopure starting materials in natural product synthesis. Among the numerous monoterpenoids, the camphor derivatives are of particular importance because of their widespread occurrence in plants [5]. In spite of the fact that the chemistry of camphor is as old as the chemistry itself, this natural product and its derivatives still remain attractive as inexpensive source of enantiopure building blocks for organic synthesis [6]. Molecules possessing bornyl fragment exhibit a variety of pharmacological activities, including antibacterial, antifungal, anti-inflammatory, and anesthetic. Although a number of biologically active camphor derivatives are isolated from plants, almost no efforts are dedicated to the development of synthetic analogues [7–12].

Wilkinson and coworkers first reported the synthesis and activity of ethambutol (EMB) (Fig. 1**I**) [13]. EMB was a useful addition to tuberculosis chemotherapy, despite a relatively modest MIC of 10 μM, in part because of very low toxicity and relatively few side-effects. Based on structure–activity relationship (SAR) studies it appeared that the distance between the two nitrogens, the presence of β-aminoalcohols, and the small side chains were critical for determining activity. The configuration of the molecule is decisively important for the activity, since EMB (with *S,S*-configuration) is approx. 200–500 fold more potent than its (*R,R*)-enantiomer. Removal or significant alteration of the basicity of either amino group resulted in a loss of potency, with the exception that the corresponding amides retained activity in some analogues (Fig. 1**II**) [14].

Reports concerning the structural optimization of EMB have remained rather scarce for many years [15]. Lately, the occurrence of a 'better ethambutol' has been systematically investigated

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SYNTHESIS AND ANTIMYCOBACTERIAL ACTIVITY OF NOVEL MANDELIC ACID DERIVED DIAMIDE

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Summary: A new mandelic acid derived diamido-diol was synthesised on the base of (1*R*,2*R*)-1,2-diaminocyclohexane scaffold and evaluated for its in vitro activity against *M. tuberculosis* H37Rv. The compound shows activity comparable with the one of the classical anti-TB drug ethambutol.

Key Words: mandelic acid, 1,2-diaminocyclohexane, *M. tuberculosis* H37Rv

Introduction

Increasing drug resistance and poor activity of existing therapies towards the latent stage of *Mycobacterium tuberculosis* infection has produced a clear need to develop novel therapeutics to treat tuberculosis [1]. Thus fast-acting drugs with novel mechanisms of action that are not cross resistant to existing drugs are being sought actively.

Wilkinson and coworkers first reported the synthesis and activity of ethambutol (EMB) (Fig. 1. **I**) [2]. EMB was a useful addition to tuberculosis chemotherapy, despite a relatively modest MIC of 10 μM, in part because of very low toxicity and relatively few side-effects. Based on structure-activity relationship (SAR) studies it appeared that the distance between the two nitrogens, the presence of β-aminoalcohols, and the small side chains were critical for determining activity. The configuration of the molecule is decisively important for the activity, since EMB with (*S,S*) -configuration is approx. 200-500 fold more potent than its (*R,R*)-enantiomer. Removal or significant alteration of the basicity of either amino group resulted in a loss of potency, with the exception that the corresponding amides retained activity in some analogues (Fig. 1. **II**) [3].

Inspired by the two β-amino-alcohol fragments in the molecule of EMB we dedicated our studies towards the development of camphane based structures and evaluation of their antimycobacterial activity towards *M. tuberculosis* H37Rv. A series of β-amido-alcohol structures were synthesized using 3-exo-aminoisoborneol (Fig. 1. III) and isobornylamine (Fig. 1. IV) as key starting compounds [4]. Some of the new compounds show 25 times higher activity than the classical anti-TB drug ethambutol. Noteworthy, although that the carbon atom at the nitrogen in all camphane structures possesses (*R*)-configuration, most of the molecules are extremely active. This is opposite to the fact that (*S,S*)-EMB is approximately 500 fold more active than (*R,R*)-EMB. In the present study we describe the synthesis and antimycobacterial activity of a novel, mandelic acid derived diamido-diol containing (1*R*,2*R*)-1,2-diamidocyclohexane scaffold (Fig. 1. V). Thus, we had the opportunity to investigate both the effect of a second amide function and a different type of aliphatic skeleton.

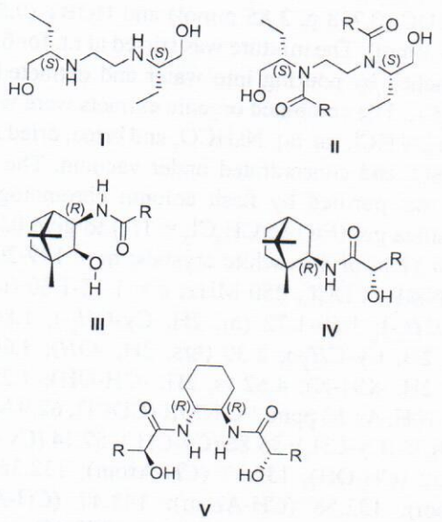

Figure.1

ANTIMYCOBACTERIAL ACTIVITY OF NOVEL CAMPHANE BASED ISOINDOLINE

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Summary: A new isoindoline containing amino-alcohol was synthesised on the base of the camphor scaffold and evaluated for its in vitro activity against *M. tuberculosis* $H_{37}Rv$. The compounds shows activity comparable with the one of the classical anti-TB drug ethambutol. The camphane-based structures present novel promising scaffolds for antimycobacterial agents.

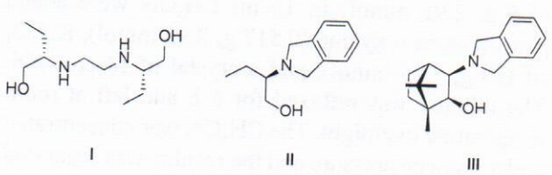
Key Words: camphane, isoindoline, antimycobacterial activity, *M. tuberculosis* $H_{37}Rv$

Introduction

Tuberculosis (TB) is a global health problem causing substantial morbidity, mortality, negative socioeconomic impact, and human suffering. One-third of the world's population is latently infected with *Mycobacterium tuberculosis* and approximately 9 million cases of active disease occur each year [1]. The recent widespread emergence of multidrug resistant (MDR) strains of *M. tuberculosis* to clinically available drugs puts further impetus to the urgent need for the discovery of new and effective anti-TB agents. Much progress has been done in drug development over the past decade. Currently, there are at least nine compounds in clinical development: two in phase III, four in phase II, and three in phase I trials [2]. Among these, four are existing drugs redeveloped for a TB indication and five are new chemical entities. More than 30 new anti-TB drugs are in preclinical development [3,4].

Wilkinson and coworkers first reported the synthesis and activity of ethambutol (EMB) (Fig. 1. **I**) [5]. EMB was a useful addition to tuberculosis chemotherapy, despite a relatively modest MIC of 10 μM, in part because of very low toxicity and relatively few side-effects. Based on structure-activity relationship (SAR) studies it appeared that the distance between the two nitrogens, the presence of β-aminoalcohols, and the small side chains were critical for determining activity [6].

Inspired by the two β-aminoalcohol fragments in the molecule of EMB we dedicated our studies towards the development of mono-aminoalcohols bearing different pharmacophore fragments and evaluation of their antimycobacterial activity [7]. Most of the compounds containing the (S)-2-amino-1-butanol motif are showing similar but not significantly higher activity than EMB. For example, the isoindoline containing structure (Fig. 1. **II**) gave MIC of 10.46 μM towards the referent strain of *M. tuberculosis* $H_{37}Rv$, which is comparable with the MIC of EMB (7.22 μM).



I II III

Figure 1.

In the present study we describe the synthesis and antimycobacterial activity of a novel anti-TB compound, containing camphane moiety. Molecules possessing bornyl fragment exhibit a variety of pharmacological activities, including antibacterial, antifungal, anti-inflammatory, and anesthetic. We were intrigued to combine the camphane scaffold with the β-aminoalcohol isoindoline containing fragment (Fig. 1. **III**) and evaluate the antimycobacterial activity towards *M. tuberculosis* $H_{37}Rv$.

Materials and Methods

1. Chemistry

Reagents were commercial grade and used without further purification. Thin layer chromatography

24

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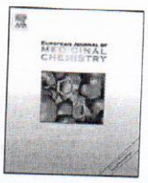

Original article

Synthesis and *in vitro* antimycobacterial activity of compounds derived from (*R*)- and (*S*)-2-amino-1-butanol – The crucial role of the configuration

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ABSTRACT

The synthesis of 47 structurally diverse compounds incorporating the (*R*)-2-amino-1-butanol motif has been realized. Ten of these compounds were found to exhibit *in vitro* specific activity against *Mycobacterium tuberculosis* H37Rv in a MIC range of 0.65 μM–14.03 μM. Five of the most active compounds **11**, **22**, **23**, **31** and **42** (5.7–11.1 fold more active than ethambutol) can be outlined with very low cytotoxicity towards human embryonal kidney non-tumour cells (SI ranging from 91.2 to 375.4). For the purpose of comparison the (*S*)-enantiomers of these most active compounds have been synthesized and evaluated towards *M. tuberculosis* $H_{37}Rv$ showing no activity even at 20–32 fold higher concentrations.

1. Introduction

Tuberculosis (TB) is one of the most wasteful diseases primarily due to several decades of neglect, HIV infection, immigration and globalization [1]. Approximately one-third of the world's population has been infected with the causative organism *Mycobacterium tuberculosis* (MTB), eight million people become sick with TB and globally it accounts for approximately two million deaths, per year. The synergy of TB/HIV infections [2] and the emergence of multi drug resistance (MDR-TB) and extensively drug resistance tuberculosis (XDR-TB) pose a threatening challenge to chemotherapy of tuberculosis with significant problems and complications [3–5]. One fifth of all deaths of adults in developing countries are due to TB and the problem is particularly re-emerging in many industrialized countries mostly because of the free movement of people in the globalized world. In affected regions, the disease is recognized as serious hindrance to economic and social development.

The leading drugs isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) are currently recommended by the World Health Organization (WHO) for the treatment of TB [6]. The problems with current TB treatment are complex and include: a prolonged standard course regimen of six months, which often result in patient noncompliance; emergency of XDR-TB strains; lack of effective drugs against the latent state. One approach to decrease treatment time is improvement of potency of currently used anti-tuberculosis drugs [7] and discovery of new active compounds [8–10].

The simple diamine EMB (Scheme 1) was synthesized by reacting 1,2-dihaloethane with (*S*)-2-amino-1-butanol [11,12]. An alternative synthetic method was also described [13]. The EMB is primarily a bacteriostatic anti-tuberculosis agent with not fully known mechanism of action. It targets the arabinosyl transferases responsible for arabinogalactan biosynthesis, a key component of the unique mycobacterial cell-wall [14–16]. Despite of modest anti-mycobacterial activity and due to its synergy with other drugs, and lower toxicity, EMB is used in combination with more potent front-line antimycobacterial agents. Early SAR study indicates that the distance between the two nitrogens, the presence of two hydroxy groups, and the small side chains in the molecule are key pharmacophore elements [17]. The configuration of the molecule is decisively important for the activity, since EMB (with *S,S*-configuration) is approx. 200–500 fold more potent than its (*R,R*)-enantiomer [17].

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