MOLECULAR STUDIES OF DRUG RESISTANCE IN *Mycobacterium tuberculosis* AND *Staphylococcus* SPECIES

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RIGA 2004

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RIGA 2004

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Dissertation is accepted for the commencement of the degree of Dr. biol. (in Molecular Biology)

Commencement: May...21 VI..., 2004

Tracevska T. 2004. *Mycobacterium tuberculosis* un *Staphylococcus spp.* zāļu rezistences mehānismu molekulārie pētījumi. Latvijas Universitāte, Rīga, 48 lpp.

KOPSAVILKUMS

Baktēriju izraisītās infekcijas slimības vienmēr ir apdraudējušas cilvēci, un, spriežot pēc mūsdienu situācijas, vēl ilgi netiks uzvarētas. Ķīmijterapijas pirmās veiksmes drīz vien aizēnoja zāļu rezistento mikroorganismu parādīšanās. Piemēram, multirezistentais tuberkulozes izraisītājs *Mycobacterium tuberculosis* ir nejutīgs vismaz pret diviem pirmās rindas ķīmijterapijas līdzekļiem: rifampicīnu un izoniazīdu. Tāpat bīstams ir nozokomiālo infekciju izraisītājs, meticilīna rezistentais stafilokoks, kurš iegūst rezistenci vienlaicīgi pret visiem β-laktāmiem un cefalosporīniem. Tādēļ ātrai un precīzai zāļu jutības noteikšanai ir izšķiroša loma ārstēšanas kursa izstrādāšanā, un līdz ar to baktēriju izraisītās infekcijas apkarošanā.

Šī darba galvenie mērķi bija *M.tuberculosis* un *Staphylococcus* spp. mikroorganismu dažādu rezistences mehānismu pētījumi molekulārajā līmenī. Tā, zāļu rezistence mikobaktērijās ir saistīta ar gēnu spontānām mutācijām. Savukārt, stafilokoki rezistenci pret β-laktāmiem iegūst ar mobilo ģenētisko elementu pārnestiem gēniem. Šai darbā, atkarībā no rezistences mehānisma, tika pielietotas vairākas molekulārās metodes, balstītas uz ģenētiskā materiāla pavairošanu ar PCR. Dažādu metožu izšķirtspēja tika salīdzināta ar *in vitro* zāļu jutības testu rezultātiem. Ir novērtēts molekulāro metožu pielietošanas lietderība zāļu jutības noteikšanai klīniskās laboratorijas vajadzībām.

Mutācijas analizēja 145 *M.tuberculosis* kultūru DNS izolātos, iegūtos Latvijā periodā no 1999 līdz 2002 gadam no plaušu tuberkulozes slimniekiem. Šīs kultūras reprezentēja ap 30% no visām šai periodā Valsts Tuberkulozes un Plaušu Slimību Centrā iegūtām *M.tuberculosis* kultūrām. 126 no 145 kultūrām bija multizāļu rezistentas un 19 bija zāļu jutīgas; pēdējās veidoja kontroles grupu. Ar rezistenci pret pirmās rindas prettuberkulozes ķīmijterapijas līdzekļiem rifampicīnu, izoniazīdu, streptomicīnu, etambutolu un pirazinamīdu saistītas mutācijas izpētīja attiecīgi, *rpoB*, *katG*, *rpsL* un *rrs*, *embB* un *pncA* gēnos. Minēto gēnu mutācijas ir zināmas kā visbiežākais attiecīgo aģentu rezistences cēlonis. Ir parādīts, ka mutācijas vienos gēnos ir biežākais zāļu rezistences cēlonis, tādos kā *rpoB* (rifampīna rezistence), kur mutācijas tika konstatētas 93,6%, un *katG* (izoniazīda rezistence) gēnā – 99,1% gadījumos, un citos, savukārt, bija konstatētas retāk, norādot, ka ir svarīga loma arī citiem, mazāk izpētītiem zāļu rezistences mehānismiem. Tā, *rpsL* un *rrs* gēnu mutācijas kopā bija atrastas 85% streptomicīna rezistentiem, *pncA* – 82% pirazinamīda rezistentiem un *embB* – tikai 52% etambutola rezistentiem paraugiem. Kopumā mutāciju noteikšanai tika pielietotas un izvērtētas vairākās molekulārās metodes, taču to jutība pielietojot uz dažādiem gēniem atšķīrās atkarībā no mutāciju lokalizācijas; visprecīzākā bija nukleīnskābju sekvenēšana.

Lai novērtēt PCR metožu lietderību *M.tuberculosis* kompleksa identificēšanai un zāļu jutības noteikšanai klīniskajā materiālā, tika aprobēta IS6110-PCR metode uz 170 klīniska materiāla paraugiem. Parādīts, ka IS6110-PCR ir ātra un specifiskā *M.tuberculosis* kompleksa identificēšanas metode. Ar citu PCR metodi, spoligotipēšanu, var vienlaicīgi identificēt un analizēt *M.tuberculosis* celmu ģenētisko radniecību. Ar šo metodi ir raksturotas Latvijā dominējošās *M.tuberculosis* ģenētiskās grupas 143 (109 zāļu rezistentu un 34 zāļu jutīgu) *M.tuberculosis* kultūru DNS paraugiem. Ir salīdzināts *rpoB* un *katG* gēnu mutāciju biežums starp 109 multirezistento izolātu spoligotipu veidotām lielākām, t.s. *Beidžing* un *ne-Beidžing*

ģenētiskām grupām, un tas bija līdzīgs abās grupās, izņemot *rpoB* gēna dubultmutācijas, kuras *Beidžing* grupā tika konstatētas biežāk.

Paralēli tika izveidota DNS kolekcija no 102 stafilokoku kultūrām (36 *S.aureus* – 28 meticilīnrezistenti un 8 jutīgi, un 66 koagulāzes negatīvie stafilokoki – 65 meticilīnreizstenti un 1 jutīgs), izolētām Latvijas Traumatoloģijas un Ortopēdijas slimnīcā periodā no 2000. līdz 2003. gadam. Ar PCR metodi analizēja *mecA* gēna klātbūtni, kas, kodējot PBP2a proteīnu, nodrošina baktērijas rezistenci pret visiem β-laktāmiem un cefalosporīniem. PCR rezultātus salīdzināja ar četrām *in vitro* jutības noteikšanas metodēm. Visiem meticilīnrezistentiem stafilokokiem atklāja *mecA* gēna fragmentu, apliecinot augstu metodes jutību. Savukārt, *mecA* gēnu atklāja arī vienam *in vitro* jutīgam *S.epidermidis* paraugam, kas neapšaubāmi liecina par šī parauga rezistenci pret meticilīnu. *mecA*-PCR metode veiksmīgi introducēta klīniskās laboratoratorijas praksē; tā ir sevišķi ieteicama *in vitro* jutīgu kultūru apstiprināšanai.

Šis darbs ir izstrādāts laika periodā no 2000. līdz 2003.gadam. Pētījumi tika veikti LU Biomedicīnas Pētījumu un Studiju Centrā, LU Prof. V.Baumaņa vadītā molekulārās mikrobioloģijas laboratorijā un daļēji Staten Serum Institūtā, Kopenhagenā, Dānijā, Dr. V.O-Thomsen vadītā starptautiskā Mikobakterioloģijas laboratorijā. Pētījumi tika veikti sadarbībā ar Valsts Tuberkulozes un Plaušu Slimību Centra laboratoriju (vad. MD Ģ. Šķenders) un Latvijas Traumatoloģijas un Ortopēdijas slimnīcas laboratoriju (vad. LU Prof. Dr.habil.med. A. Žilēvica). Darbu finansēja EU Inco-Copernicus projekts nr. 15-CT98-0328; LZP projekts nr. 2.0011.15.1 "Infekcijas aģentu multirezistences un ģenētiskās mainības molekulāri epidemioloģiskā izpēte"; šī darba autori personīgi finansiāli atbalstīja LZP doktorantūras grants nr.69/71 un FEBS īstermiņa stipendija.

Tracevska T. 2004. Molecular studies of drug resistance mechanisms in *Mycobacterium tuberculosis* and *Staphylococcus* species. 48 pp.

SUMMARY

Infectious diseases caused by pathogenic bacteria have affected people for many centuries. The invention of chemotherapy was an important step to eradicate pathogens, however, it led to the emergence of drug-resistant forms of bacteria. For instance, multidrug-resistant *Mycobacterium tuberculosis* complex bacteria, the main causative agent of tuberculosis, is resistant at least to rifampin and to isoniazid, the first-line antitubercular drugs. At the same time, methicillin-resistant *Staphylococcus spp.*, accepted as a major agent of nosocomial infections, simultaneously acquire resistance to all β-lactam antibiotics and to cephalosporines. That's why precise and rapid drug susceptibility testing is of great significance for prescribing an adequate treatment course.

An investigation into different drug resistance molecular mechanisms in *M.tuberculosis* and *Staphylococcus spp.* is the main goal of this study. *M.tuberculosis* gain drug resistance via chromosomal gene alterations, which modify target molecules. In contrast, *Staphylococcus spp.* acquire resistance, particularly, to β-lactams, by transfer of the resistance-determining gene. In the present study, various PCR-based methods are evaluated, depending on the drug resistance mechanism. Reliability of the methods is evaluated and the practical value for implementation in clinical laboratory practise is estimated.

The study of antitubercular drug resistance involved 145 *M.tuberculosis* culture DNA isolates (126 multi-drug resistant and 19 drug susceptible) obtained

from pulmonary tuberculosis patients in Latvia during the time period from 1999 to 2002. The cultures represented about 30% of all cultures obtained at the State Tuberculosis and Lung Diseases Centre during the period of study. Mutations were analysed in genes most frequently associated with resistance to the first-line anti-TB drugs: rifampin (*rpoB* gene), isoniazid (*katG* gene), streptomycin (*rpsL* and *rrs* gene), ethambutol (*embB* gene), and pyrazinamide (*pncA* gene). It was shown that mutations in some genes, i.e. *rpoB* and *katG* (93,6% and 99.1%, respectively), are of the highest frequency and can be used as corresponding drug resistance markers. In contrast, mutations in remaining genes were less common. Mutations in *rpsL* and *rrs* genes were found in 85% of streptomycin resistant, in *pncA* gene – in 82% of pyrazinamide resistant and in *embB* gene – in 52% of ethambutol resistant isolates, respectively. Different molecular screening methods were evaluated for the rapid detection of mutations. However, reliability of the methods varied and was dependent on mutation location. The nucleotide sequencing method was the most discriminative and was used as a confirmatory method.

To evaluate reliability of PCR-based methods for the identification and drug susceptibility testing in clinical specimens, initially IS6110-PCR was applied to 170 clinical specimens. IS6110-PCR was shown as a rapid and specific method in comparison with commonly used methods. Another PCR method, spoligotyping, was further used for the simultaneous identification and typing of *M.tuberculosis*. Predominant in Latvia genotype groups have been characterised by spoligotyping on 143 (109 drug resistant and 34 drug suscpetible) *M.tuberculosis* culture DNA isolates. The frequency of *rpoB* and *katG* gene mutations was compared between two major groups, so named Beijing and non-Beijing, formed by 109 drug resistant isolates. The higher frequency of *rpoB* gene double mutations occurred in Beijing group.

In parallel, 102 DNA samples from staphylococcal cultures isolated in Hospital of Traumatology and Orthopedics from 2000 to 2003 were analysed for resistance to methicillin. Among them, there were 36 coagulase-positive *S.aureus* (28 methicillin resistant and 8 methicillin susceptible *S.aureus*) and 66 coagulase negative staphylococci group, including 65 methicillin resistant isolates and one methicillin susceptible isolate. The presence of the *mec*A gene, producing PBP2 protein, indicates resistance to methicillin and other β-lactams. Results of *mecA*-PCR were compared to four *in vitro* susceptibility methods. The method correctly identified all phenotypically methicillin-resistant 28 *S. aureus* and 65 coagulase–negative staphylococci. Additionally, *mecA* gene was detected in one *in vitro* susceptible *S.epidermidis* isolate, giving clear evidence of the method's value in screening phenotypically susceptible cultures. Altogether, this study showed high sensitivity of *mecA*-PCR method, and it has been already implemented in routine laboratory practice.

The present study was carried out during the time period from 2000 to 2003 in the Laboratory of Molecular Microbiology (led by MD, LU Prof. V.Baumanis), Biomedical Research and Study Centre, Riga, Latvia and partially in the Reference Laboratory of Mycobacteriology (Dr. V.O-Thomsen), Statens Serum Institute, Copenhagen, Denmark. The study was conducted in collaboration with the State Tuberculosis and Lung Disease Centre, (MD G.Skenders) and the Hospital of Traumatology and Orthopedy, (MD, LU Prof. Dr.habil.med A. Zilevica), Riga, Latvia. The project was funded by the EU Inco-Copernicus 15-CT98-0328 grant and the Latvian Council of Sciences (nr. 2.0011.15.1). The author was supported by the Latvian Council of Sciences (grant nr.69/71 for doctoral students) and the FEBS short-term fellowship.

Трачевская Т. 2004. Молекулярные исследования механизмов лекарственной устойчивости у *Mycobacterium tuberculosis* и *Staphylococcus spp.* Латвийский Университет, Рига, 48 с.

АННОТАЦИЯ

Человек всегда был подвержен инфекционным заболеваниям, вызываемым патогенными бактериями. Применение антибиотиков на первых порах было успешным во многих областях медицины, но вскоре привело к появлению антибиотик-устойчивых форм микроорганизмов. Наибольшую опасность представляют так называемые мультирезистентные формы, такие, как *Mycobacterium tuberculosis*, который устойчив как минимум к рифампицину и изониазииду, первоочередным противотуберкулёзным средствам. Мультирезистентность есть также у нечувствительного к метициллину и другим β-лактамам стафилококка, источника нозокомиальных инфекций. Поэтому своевременное выявление таких форм бактерий важно для правильного выбора курса лечения.

Главной целью этой работы было исследование механизмов лекарственной устойчивости у бактерий *M.tuberculosis* и *Staphylococcus spp.* Если устойчивость у *M.tuberculosis* связана в основном с появлением спонтанных мутаций, то у *Staphylococcus spp.* устойчивость к метициллину кодируется генами, которые переносится мобильными генетическими элементами. В данном иследовании, в зависимости от механизма резистентности, были использованы разные молекулярные методы, основанные на полимеразной цепной реакции (ПЦР). Точность методов проверялась в сравнении с результатами традиционного тестирования *in vitro* и оценивалась для дальнейшего введения в лабораторную практику.

Анализ мутаций был произведен на 145 *M.tuberculosis* ДНК образцах полученных из культур, выделенных в Латвии 1999 по 2002 год у больных туберкулезом. Эта выборка представляла около 30% всех культур *M.tuberculosis* выделенных в Латвии за период исследования. 126 из 145 культур были мультирезистентны и 19 – чувствительны. Мутации, связанные с устойчивостью к первоочередным противотуберкулезным средствам рифампину, изониазиду, стрептомицину, этамбутолу и пиразинамиду были проанализированы, соответственно, в генах *rpoB*, *katG*, *rpsL* и *rrs*, *embB* и *pncA*. Было установлено, что мутации в одних генах (*rpoB* (рифампин-устойчивость), - 93,6%, *katG* (изониазид-устойчивость) - 99,1%) встречаются с высокой частотой и по большой части определяют устойчивость. В других генах мутации были констатированы реже (*rpsL* и *rrs* (стрепромицин-устойчивость) вместе 85%, *pncA* (пиразинамид-устойчивость) – 82%, *embB* (этамбутол-устойчивость) — только 52%), указывая на наличие других механизмов, требующих дополнительных исследований. Чувствительность использованных молекулярных методов варьировала в зависимости от локализации мутации.

Чтобы оценить пригодность ПЦР методов для детекции *M.tuberculosis* комплекса и определения его чувствительности к лекарственным препаратам прямо на клиническом материале, сначала на 170 клинических образцах был опробован IS6110-ПЦР метод. IS6110-ПЦР оказался быстрым, чувствительным и специфичным к *M.tuberculosis* комплексу. Другой основанный на ПЦР метод, сполиготипирование, был внедрен для детекции и одновременного

генотипирования *M.tuberculosis* штаммов. С помощью этого метода были характеризованы доминирующие в Латвии генетические группы среди 143 *M.tuberculosis* (109 мультирезистентных и 34 чувствительных) ДНК. Также сравнили частоту мутаций в *rpoB* и *katG* генах среди 109 мультирезистентных ДНК изолятов, образующих две большие генетические группы, т.н. Бейджинг и не-Бейджинг. Частота мутаций была схожей в обеих группах, за исключением двойных мутаций в *rpoB* гене, которые чаще встречались в группе Бейджинг.

Параллельно была создана коллекция ДНК из 102 стафилококковых культур (36 *S.aureus*, из них 28 - метициллин-устойчивые и 8 – чувствительные, а также 66 коагулазнегативных, из них 65 метициллин-устойчивые и один – чувствительный) выделенных в больнице Травматологии и Ортопедии (2000-2003 годы). С помощью ПЦР метода анализировали наличие *mecA* гена, который кодирует протеин PBP-2a, отвечающий за устойчивость к метициллину и другим β-лактамам. Результаты сравнили с четырьмя *in vitro* методами олределения чувствительности. У всех *in vitro* метициллин-устойчивых стафилококков обнаружили *mecA* ген, доказав высокую чувствительность метода. Кроме того, *mecA* ген был найден у одного *in vitro* чувствительного образца *S.epidermidis,* указывая на устойчивость к метициллину. *mecA*-PCR метод был успешно внедрен в практику клинической лаборатории и особенно рекомендуется для проверки фенотипически чувствительных культур.

Исследовательская работа была проделана в с 2000 по 2003 год в лаборатории молекулярной микробиологии (рук. Проф. ЛУ В.Бауманис) при Центре Биомедицинских Исследований Латвийского Университета (Рига, Латвия) и частично в *Statens Serum* Институте (Копенгаген, Дания), лаборатории Микобактериологии (рук. Др. В. О-Томсен). Мы вели сотрудничество с Латвийским Центром Туберкулеза и Заболеваний Легких (рук. лаборатории Г.Шкендерс), а также с Латвийским Ценром Травматологии и Ортопедии (рук. лаборатории Проф. ЛУ А. Жилевица). Работу финансировали *EU Inco-Copernicus* проект nr. 15-CT98-0328; проект Латвийского Совета Науки nr. 2.0011.15.1 и лично автора работы - проект Латвийского Совета Науки для докторантов nr.69/71 и краткосрочный проект FEBS для студентов.

ABBREVIATIONS

BCG – *Mycobacterium bovis* BCG
CoNS – coagulase-negative staphylococci
CSF – cerebrospinal fluid
CTAB – cetyltrimetylammonium brmide
DOTS – directly observed therapy short-course
DR - drug resistance
ECL – enhanced chemical luminescence
EMB – ethambutol
ERDR – ethambutol resistance determining region
ETH – ethionamide
FQ – fluorquinolones
HIV – Human Immunodeficiency Virus
HTO – Hospital of Traumatology and Orthopedy
INH – isoniazid
INNO-LiPA – *"Innogenetics"* line probe assay
IS – insertion sequence
LCx – lygase chain reaction
MDR – multi-drug resistance
MET – methicilin
MIC – minimal inhibitory concentration
MRSA - methicillin resistant *Staphyloccocus aureus*
MRSE - methicillin resistant *Staphyloccocus epidermidis*
MSSA – methicillin susceptible *Staphyloccocus aureus*
MTC – *Mycobacterium tuberculosis* complex
NCCLS –National Committee for Clinical Laboratory Standarts
PAAG – polyacrilamide gel
PASS – paraaminosalicylic acid
PBP – penicillin-binding protein
PCR – polymerase chain reaction
PFGE – pulsed-field gel electrophoresis
PZA – pyrazinamide
RAPD - randomly amplified DNA polymorphism
RD – region deleted
RFLP – DNA restriction fragment length polymorphism
RIF - rifampin
SM – streptomycin
SSC*mec* – staphylococcal chromosomal cassette, *mec* determinant
SSCP – single–stranded DNA conformation polymorphism
STLDC – State Tuberculosis and Lung Disease Centre of Latvia
SVA – Public Health Agenture of Latvia
TB- tuberculosis
WHO – Word Health Organisation
WT – wild type

LIST OF ORIGINAL PAPERS

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4) Viesturs Baumanis, Inta Jansone, Lonija Broka, Anda Nodieva, Mihails Bratkovskis, Tatjana Tracevska: Evaluation of the insertion sequence –6110-based polymerase chain reaction of pathogenic mycobacteria. ***Acta Universitatis Latviensis*** 2003, 622:17-24.

5) Tatjana Tracevska, Inta Jansone, Anda Nodieva, Olgerts Marga, Girts Skenders, Viesturs Baumanis: Spectrum of *pncA* mutations in multi-drug resistant *M.tuberculosis from* Latvia. ***Antimicrob Agents Chemother,*** resubmitted after revision.

6) Tatjana Tracevska, Inta Jansone, Anda Nodieva, Olgerts Marga, Girts Skenders, Viesturs Baumanis: Characterisation of *rpsL*, *rrs* and *embB* mutations associated with streptomycin and ethambutol resistance in *M.tuberculosis.* ***Research in Microbiology,*** resubmitted after revision.

"You will begin to touch heaven, Jonathan, in the moment that you touch perfect speed. And that isn't flying a thousand miles an hour, or a million, or flying at the speed of light. Because any number is a limit, and perfection doesn't have limits. Perfect speed, my son, is being there." Without warning, Chiang vanished and appeared at the water's edge fifty feet away, all in the flicker of an instant... "To fly as fast as thought, to anywhere that is," he said, "you must begin by knowing that you have already arrived ...".

Richard Bach. "*Jonathan Livingston Seagull*"

Devoted to my family and especially to my husband Alex making me remember that there are other things in live than science.

CONTENTS

1. INTRODUCTION

In the last fifty years since chemotherapy was found to be effective against microorganisms many infectious diseases seemed to have won. Partially, recovery of such antibacterials like streptomycin, isoniazid and rifampin have lowered the incidence of tuberculosis caused by *Mycobacterium tuberculosis* complex. Similarly, the introduction of penicillin and its chemically modified analogues was highly effective against *Staphylococcus* species. Staphylococci are known as major causative agents of community-acquired and hospital-acquired infections causing sometimes death of previously healthy people. Unfortunately, rash use of antibacterials led to the emergence and subsequent spread of drug resistant bacteria.

Plasmid- or transposon- mediated transfer of drug resistance genes is the most common mechanism of drug resistance in many bacteria. Therefore, such resistance mechanisms are well-explored in many bacteria, like *E.coli*, the universal object of microbiology. Highly pathogenic nature, slow growth and genomic divergence of *M.tuberculosis* (e.g., unclear function of transposons and absence of plasmids) could not assist to such studies. That's why until the last time little was known about the mechanism of action of many antitubercular drugs and about the ways of gaining resistance. However, the introduction of new molecular techniques allowed scientists to build trustful scenarios. The realisation of complete genome sequencing programmes for such microorganisms as *M.tuberculosis, M.bovis BCG, M.avium, M.leprae, S.aureus,* and *S.epidermidis* was an essential step in deciphering biochemical pathways in bacteria, therefore, highlighting putative genes involved in drug metabolism and resistance.

To investigate molecular basis of drug resistance, two species of microorganisms were selected: *Mycobacterium* and *Staphylococcus* spp. There were various reasons that determined the selection of these particular bacteria. *Staphylococcus* is the most common cause of nosocomial infections, and *Mycobacterium tuberculosis* complex causes tuberculosis, a severe infectious disease. It would be interesting to compare the molecular mechanisms of drug resistance of particular microorganisms from a region where a problem of high-level drug resistance persists, such as Latvia. Moreover, it is important to estimate the frequency of resistance determining mutations or genes and to compare it with that in other parts of the world. High prevalence of some mutations may indicate the spread of particular genotypes, which points at ongoing transmission, e.g. has high epidemiological value. Therefore, genotyping techniques were applied to analyse molecular relatedness of microorganisms.

Knowledge of frequently occurring mutations *M.tuberculosis* at specific codons that correlate with the genetic relatedness of MDR strains should contribute to our understanding of resistance to the first-line anti-TB drugs both at regional and international levels. This information may be used further in order to develop and adopt molecular methods for testing *M.tuberculosis* drug susceptibility and molecular typing directly in clinical specimens. This is a welcome feature of molecular biology due to prolonged (2-6 weeks) cultivation usually required for *M.tuberculosis* complex species. The evaluation of insertion-sequence-based *M.tuberculosis* detection method was an additional objective of this study.

In order to control the spread of resistance among *Staphylococci*, appropriate infection control practices should be applied in hospitals, including precise microbiological diagnosis and detection of antimicrobial susceptibility. Therefore, another objective of this study was introduction and evaluation of molecular methods for testing methicillin susceptibility in *S.aureus* and in coagulase-negative staphylococci culture isolates compared to *in vitro* methods routinely used in clinical

laboratories. This part of the study contributes to the further studies of particular groups of staphylococci strains that may hypothetically spread among Latvian hospitals.

2. REVIEW OF LITERATURE

2.1. Short history of chemotherapy

Paul Erlich is thought to be a founder of chemotherapy. He explored and showed antimicrobial properties of salvarsan, an atoxyl descender, in order to treat syphilis. The first antimicrobial agent widely introduced in medical practice was penicillin discovered in 1929 by A. Fleming by a lucky chance (Fleming, 1929). Natural drugs with bacteria demolishing and inhibiting properties produced by fungi, bacteria, actinomiceta, plants and animals, were called antibiotics. Choice of a particular agent was based on the mechanism of action and on its sterilizing or bacteriostatic effect.

Later, antibacterial activity was found in many chemically synthesized agents, especially when used in combination with natural antibiotics. Particularly, the discovery of antibacterial and antitubercular properties of streptomycin in 1944 (Schatz and Waksman, 1944), and both isoniazid and pyrazinamide in 1952 (Kushner et al., 1952; Middlebrook, 1952), led to effective chemotherapies what decreased tuberculosis mortality rates worldwide. New effective drugs against TB were discovered in the 1970s and are still used for the treatment of TB. Today more than hundred chemotherapeutic agents are known, which are used to treat grampositive and gramnegative bacteria caused disorders, as well as some viral, protozoal and fungal infections. However, the widest application of chemotherapy drugs occurred in mycobacteria and grampositive cocci, particularly, in staphylococci caused infections.

2.2. Acquired drug resistance as a consequence of chemotherapy

Prolonged and inappropriate chemotherapy regimens compounded by non-compliance of patients in completing the prescribed course of treatment led to the appearance of drug-resistant strains. The first alarming sign was the appearance of penicillin-resistant staphylococci and multiple resistant enterococci, which caused serious nosocomial (hospital-acquired) infections in 1960-s (Dalhoff, 1999). In 1977 the appearance of high level resistance to penicillin in *Streptococcus pneumoniae* which cause respiratory diseases such as pneumonia was detected in South Africa (Jakobs et al, 1978). In the mid-1980s the number of tuberculosis cases also increased worldwide accompanied by the growth of multi-drug resistant cases (Blanchard et al, 1996).

Nowadays many bacteria-caused diseases are increasingly difficult to treat because of the emergence of drug-resistant microorganisms. Eradication of such diseases requires profound studies of drug resistance molecular mechanisms in bacteria and determination of bacterial evolution pathways. This important information can be further used in the search for new antibacterial agents.

2.3. Natural and acquired mechanisms of microbial drug resistance

There are various mechanisms of drug resistance acquisition, mostly based on the ability to produce drug-inactivating enzymes and on the modifications in bacterial DNA, RNA or protein structures, which directly interact with the agent. There are natural (inner) and acquired factors of antimicrobial agent resistance.

Natural resistance is based on the bacterial cell limitation for antibacterial agent diffusion or active transport. For example, *Pseudomonas* spp. is naturally resistant to β–lactam antibiotics and aminoglycosides. Recently drug–specific and multi-drug efflux pumps have been identified to play a major role in the resistance of many species to antibacterials (e.g. resistance of Gram-negative bacteria to tetracyclines) (Li & Nikaido, 2004).

At present, two major mechanisms of acquired drug resistance can be distinguished: non-genetically and genetically determined acquired drug resistance.

Non-genetically determined acquired drug resistance may be caused by the lowering metabolitic activity in the bacterial cell. Most antimicrobial agents are active against actively reproducing cells and, therefore, will not be effective against dormant bacteria. Also under pressure of chemotherapy the number of bacteria-agent interaction ligands can decline, this way lowering the inclusion of drug in the host cell.

In contrast, genetically determined acquired drug resistance can be encoded by the chromosomal DNA of bacteria or by the plasmids. This mechanism includes gene mutations, changing structure of target proteins or agent modifying enzymes, acquisition of drug resistance genes, transferred by plasmids. Gene mutations occur spontaneously in bacteria with frequency 10^{-7}-10^{-12} and themselves play a minor role in a huge population of bacteria. However, further selection of viable cells with mutations or gained genes leads to the domination of particular strains in the population. Plasmids or episomes contain one or various genes encoding proteins that inactivate, modify or extract antimicrobial agents from the cell. Multiple-resistance genes may be encoded by the transposons integrated into plasmids. Plasmids have an ability to transfer their resistance factors and to rise epidemic resistance by various mechanisms: conjugation, transfection or transduction.

Two distinct mechanisms of genetically determined resistance will be described further in this paper, generally focusing on two pathogens, *Mycobacterium tuberculosis* and *Staphylococcus spp.* and taking into account different mechanisms of drug resistance development. Although both mechanisms are genetically determined, drug resistance in *M.tuberculosis* is usually determined by chromosomal mutations, whereas staphylococci have various mechanisms. However, an acquisition of drug resistance genes by plasmid transfer remains the main drug resistance mechanism in staphylococci as well as in most microorganisms, which, probably, has more advantages than gene mutations.

2.4. Characterisation of *M.tuberculosis* complex

2.4.1. Tuberculosis in Latvia and worldwide.

M.tuberculosis complex (MTC) remains the leading infectious cause of morbidity and mortality. Tuberculosis (TB) is a chronic infectious disease, affecting mostly pulmonary organs. TB causes about 8 million pulmonary TB cases and more than two million deaths per year worldwide (Espinal et al., 2001). In humans and cattle, tuberculosis is generally caused by *M.tuberculosis* complex including *M.tuberculosis*, *M. bovis*, *M. africanum* and *M.microtti* species. However, in the industrialised world, *M.avium* complex (including *M.avium*, *M.intracellulare* and an intermediate group) has become the major cause of mortality in HIV-infected patients (Horsburgh, 1991). In Latvia, *M. tuberculosis* is the most common pathogen isolated in case of TB.

The incidence of tuberculosis was increasing in Latvia from the 1980ties at about 5% to 10% of annually notified cases. The highest incidence was observed in 1998 (1820 TB patients, with prevalence 74.0 per 100.000 of population). The

invention of directly observed therapy short-course programme (DOTS) in 1995 and DOTS-plus programme in 1998 decreased TB incidence by 13% from 1998 to 2003. In a survey of 1334 registered TB patients in Latvia in 2003 incidence was 57.0 per 100.000 of population (SVA report, 2003). The comparison of TB situation in Latvia with the nearest North-European countries in 2002 is shown in Table 1. As one can see from Table 1, situation with TB incidence in the former US countries is much worse than in Scandinavian countries, where incidence is less than 10 per 100 000 of population.

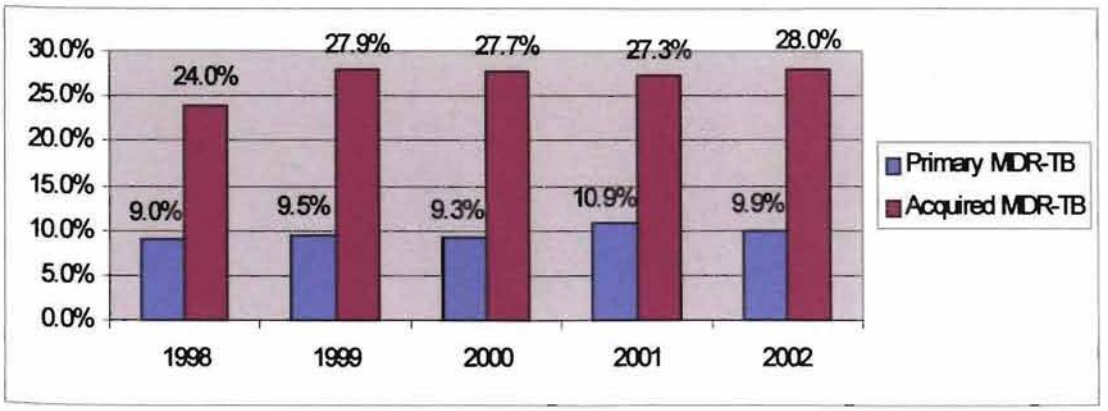
At the same time, the positive tendency in TB treatment in Latvia has been affected by a very high incidence of drug resistant cases. Today a high proportion of TB patients in Latvia hosts multi-drug resistant (MDR) strains, i.e. resistant at least to

Table 1. Epidemiological data on incidence of TB (total) in the Baltic Sea region in 2002.

Country/Region	Total number of a new cases in 2002	Prevalence (per 100.000 population)
Latvia	1535	65.4
Estonia	522	38.2
Lithuania	2097	60.5
Denmark	429	8.0
Norway	256	5.7
Sweden	418	4.7
Finland	475	9.1
Iceland	8	3.0
St.Petersburg Region	1026	65.2
Kaliningrad Region	1042	110.0

rifampin (RIF) and isoniazid (INH). Multi-drug resistance to TB in 2000 in Latvia seemed to be one of the highest in Europe, with primary multi-drug resistance (MDR) in 9.3% of new cases and acquired MDR in 27.9% cases. In comparison, the average primary MDR rate in Europe as a whole is only 4%. Many of the culture isolates from MDR *M.tuberculosis* patients show resistance not only to RIF and INH, but also to other first-line antituberculous agents: streptomycin (SM), ethambutol (EMB) and, less frequently, to pyrazinamide (PZA) (Espinal et al., 2001; EuroTB, 2003). The incidence of MDR TB cases in previous years is shown in Figure 1.

Figure 1. Multi-drug resistant TB in Latvia, 1998-2002 (STLDC data).



2.4.2. Mycobacterial genome

MTC belongs to *Mycobacteriaceae* family, which is a member of the order *Actinomicetales.* These are gram-positive bacteria (according to Bergey's taxonomy) that are distinguished by the high G+C content of their DNA (60-70%) and an unusual cell wall. Besides a slightly atypical peptidoglycan (Rastogi & Barrow, 1994), mycobacteria have a unique polysaccharide component, arabinogalactan (Daffe et al, 1993). Arabinogalactan layer contains covalently bound long, branched lipids, termed mycolic acids, which can be from 40 to 90 carbons long.

Mycobacterial genome of *M.tuberculosis* laboratory virulent strain H37Rv was completely sequenced in 1998, followed by revealing the complete genome sequence of clinical strain CDC1551 in 2002 and *M.bovis* in 2003 (Cole et al, 1998, Fleischmann et al., 2002; Garnier et al., 2003). The total nucleotide sequence determined for *M.tuberculosis* strain H37Rv genome was 4,411,529bp long containing approximately 4000 putative genes (Figure 2). 60% of genes have predicted functions providing *M.tuberculosis* with a wide range of metabolic

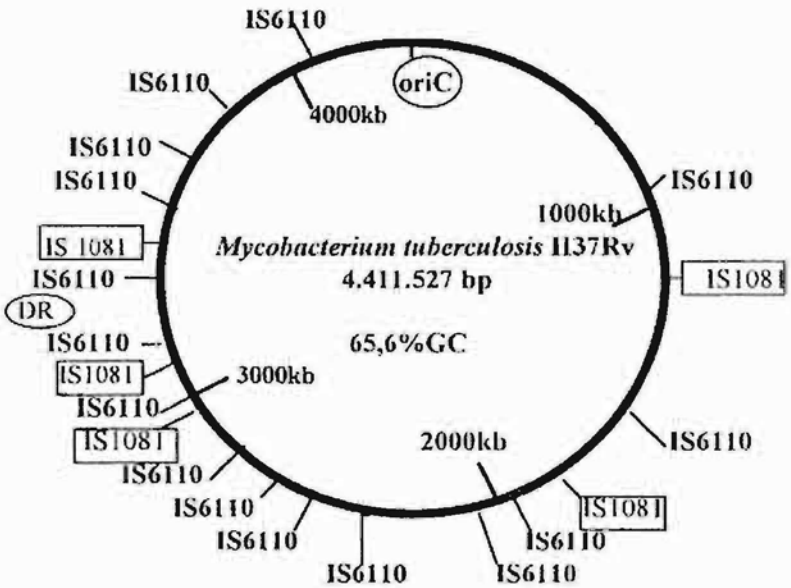


Figure 2. Circular map of the *M.tuberculosis* H37Rv strain genome (Phillip et al., 1998, Cole et al, 1998). OriC – replication origin, IS6110 and IS1081 – insertion sequences, DR – direct repeat region.

pathways. Many genes are involved in lipid metabolism due to the complexity and diversity of lipid content in the mycobacterial cell wall (Cole et al, 1998). A high number of regulatory genes, indicating high adaptability to changing conditions, is consistent with the environmental origins of mycobacteria, which could have become a human pathogen only 15000 years ago. (Sreevatsan et al, 1997). At the same time, genome of different *Mycobacterium* species has a highly clonal nature without detectable lateral gene exchange (Alland, 2003, Supply et al, 2003). High conservation of mycobacterial genome with slowly occurring mutations prevailing above region deletions defines that mycobacteria is an evolutionary young organism. Interestingly, *M.leprae* complete genome sequence showed a critically shortened

genome (reduced to 3,2 Mb) containing around 1600 genes, pointing at the reductive evolutional pathway for pathogenic mycobacteria (Brosch et al, 2002).

Mycobacterial genome contains many repetitive genes and transposons, such as two large families of glycine-rich proteins, Pro-Glu (PE) and Pro-Pro-Glu (PPE), and insertion sequences, mostly of unknown function. Studies of the PE proteins demonstrated their role in mycobacterial virulence (Ramakrishnan et al, 2000). Both PE/PPE and Esat-6 protein (a central T-cell antigen in human) families enter the RD1, a region deleted in *M. bovis* BCG Pasteur vaccine strain, confirming the considerable interest about these proteins as virulence factors (Tekaia et al., 1999; Bange et al., 1999, Brosch et al., 2002). In conclusion, no plasmids were found in MTC species, whereas there are some data about plasmids in nonpathogenic *M.scrofulaceum* and *M.intracellulare* species (Cole et al, 1998). Episomal or transposon-mediated transfer of resistance genes into *M.tuberculosis* has never been demonstrated, although this is a common mechanism for the acquisition of drug resistance in other bacteria (Cole, 1994). Absence of extra chromosomal DNA indicates that phenotypic changes in MTC occur due to genome DNA mutations.

2.4.3. Antitubercular agents and drug susceptibility testing

The main anti-TB chemotherapy agents are so called first-line drugs: INH, RIF, SM, EMB and PZA. The second-line drugs are used mostly as a supplement to main chemotherapy due to their lower efficacy and high toxicity, for example, kanamycin, capreomycin, ethionamide, paraaminosalycilic acid (PASS), cycloserine, ofloxacin, thioacetazone, viomicin etc. Some antituberculars, known as antibiotics, are naturally produced like RIF and SM; others are chemically synthesized (INH, PASS, EMB, PZA). The directly observed short-course chemotherapy prescribed by WHO, consists of a two-month treatment with the four first-line drugs (INH, RIF, PZA and SM or EMB) followed by four-month therapy with two first-line drugs INH and RIF, or, alternatively, a six-month course with INH and EMB in combination with second-line drugs (Vareldzis et al, 1994).

There are various methods for MTC growing and for drug susceptibility testing. Drug susceptibility testing is traditionally performed *in vitro* on cultures grown on solid medium (Löwenstein-Jensen, Middlebrook etc) for several weeks by the absolute concentration method or by the proportional dilution method (Hawkins et al., 1991). In the absolute concentration method, used in Latvia, resistance is defined as growth on solid media containing graded concentrations of drugs greater than a 20CFU at a specific drug concentration. In the last decade, the BACTEC radiometric method (Becton Dickinson, Sparks, Md.) for drug susceptibility testing has been developed. Here, MCT is cultivated for a slightly shorter time on liquid medium with added anti-TB agent at minimal inhibiting concentration (Roberts et al., 1991). Cell growth is estimated automatically.

However, slow growth of pathogenic mycobacteria requiring weeks and even months to obtain drug susceptibility results is the main disadvantage of the mentioned methods. This delays appliance of chemotherapy and, if it was incorrectly started, leads to selection and accumulation of drug resistant clones in population. Also, sometimes weak growth or even absence of growth can be observed, especially for MDR mycobacteria. The reasons for that can be reduced fitness, or low reproductive effectiveness of MDR bacteria due to mutations affecting metabolism, as proposed by Cohena et al. (2002). This is another disadvantage of the *in vitro* methods, because they are based on phenotypic characteristics. The problem could be solved by the molecular methods, based on bacterial genome features, which can be used with various aims like species identification, typing and drug susceptibility testing.

Molecular methods may precisely determine the molecular basis of drug resistance, indicate spread of particular strains and, most significantly, they supply data about possible epidemiological links. Additionally, these methods can be used to study the evolution of particular mycobacteria groups and their virulence levels.

2.4.4. Molecular mechanisms of drug resistance in *M.tuberculosis*

Mycobacteria develop drug resistance via a limited number of mechanisms. Structural and functional changes in the enzymes that either activate antimycobacterial drugs or are themselves the target of drug action are most commonly observed in *M.tuberculosis*. That means that gene mutations in *M.tuberculosis* genes are responsible for resistance to the first – and second–line drugs (Musser, 1995, Sander&Bottger, 1999). Resistance to each drug develops independently except for those with the similar mechanisms of action (e.g. INH and ethionamide). No drug efflux mechanisms have yet been described that can account for drug resistance in *M.tuberculosis*, although diffusion and transport into mycobacterial cells is an extremely important variable in the drug activity. The drug targets, mechanism of action and mechanisms of drug resistance in *M.tuberculosis* have all been extensively studied and partially discovered over the last ten years. Mode of action of commonly used first- and second- line anti-TB drugs and mechanisms of resistance in *M.tuberculosis* are summarised in Table 2.

By the mode of action, anti-TB drugs can be divided into nucleic acid, protein or cell membrane synthesis inhibitors. Taking into account higher therapeutic value of the first-line anti-TB drugs, the mechanisms of action and mechanisms of bacterial resistance to RIF, SM, INH, EMB and PZA will be paid more attention to further in the text.

Inhibitors of nucleic acid synthesis

This group of antituberculars includes agents inhibiting DNA or RNA synthesis. Molecular mechanisms of RIF resistance are well studied (Table 2).

Rifampin (RIF) was introduced in practice in 1972 as an antitubercular drug (Woodley, 1972 no BL). RIF is extremely effective against *M.tuberculosis* (MIC 0.1-0.2 mkg/ml) shortening the course of treatment (Heifets, 9:84-103,1994noBl). Resistance to RIF is used as a molecular marker for MDR. By irreversible binding to the β-subunit of RNA polymerase, RIF is inhibiting transcription reaction at the first stages, as it was shown in RIF-susceptible *M.smegmatis* strains (Fischl et al, 1992). However, some additional mechanisms of action can not be excluded.

Mutations in the 81bp hypervariable region of the 3.534bp long *rpoB* gene encoding the RNA polymerase β-subunit cause RIF resistance in 96% of cases. Among these, missense mutations in codons S531, H526 and D516V are the most frequent (Telenti et al., 1993; Musser, 1995). RNA polymerase is a typical enzyme for all bacteria cells. Therefore, RIF is not antibiotic specific to mycobacteria only. It is also effective against grampositive (e.g. staphylococci) and gramnegative bacteria inhibiting their growth. Mutations lead to conformational changes in β-subunit preventing it from binding to RIF. In 81bp long hypervariable region, most common

Table 2. Mechanisms of resistance to anti-TB drugs in *M.tuberculosis* (By Musser, 1995; Cole 1998; Sander & Bottger, 1999)

1st or 2nd line anti-TB drug (by the mode of action)	Target in bacterial cell	Gene associated with resistance	Gene product
1. Inhibitors of nucleic acid synthesis			
Rifampin (1st line)	RNA synthesis	*rpoB* (3.534bp)	β- subunit of RNA polymerase,
Fluorquinolones (2nd line)	DNA replication and RNA transcription	*gyrA* (2.517bp)	α-subunit of DNA gyrase,
Ciprofloxacin			
Levofloxacin			
Ofloxacin			
2. Inhibitors of protein synthesis; Aminoglycosides			
Streptomycin (1st line)	Protein synthesis	*rpsL* (372bp) *rrs* (1.464bp)	Ribosomal protein S12 16S rRNA
Kanamycin			
Amikacin			
Gentamicin (2nd line)		*rrs* (1.464bp)	16S rRNA
Capreomycin			
Tobramicin			
Viomicin			
3. Inhibitors of cell wall synthesis:			
Isoniazid (1st line)	Mycolic acid synthesis	*katG* *inhA* *ahpC* *kasA* *ndh*	Catalase-peroxidase, β-enoylACPreductase, Alkyl hydroperoxide reductase, β-ketoacilACPsinthase, NADH dehydrogenase
Thioacetazone (2nd line)	Mycolic acid synthesis	?	?
Pyrazinamide (1st line)	Have to be defined	*pncA*	Pyrazinamidase
Ethionamide (2nd line)	Mycolic acid synthesis	*inhA*	EnoylACPreductase
Ethambutol (1st line)	Arabinogalalactan, lipoarabinomannan	*embB*	Arabinosyltransferase
Cycloserine (2nd line)	Proteoglican synthesis	*DdlA (?)*	D-alanin-alaninlygase
Paraaminosalicylic acid (2nd line)	Mycobactins, folic acids	*Mbt saime (?)* *Rv0802c (?)*, *Rv3700c (?)*	Mycobactins Acetyltransferase

are single point mutations changing amino acid (Figure 3). Deletions or insertions of several nucleotides are less frequent. Double mutations, occurring simultaneously at two codons, are of special interest. According to data presented by J. Musser, about 4% of RIF-resistant isolates don't show mutations in particular region, therefore, an additional mechanism conferring resistance to RIF can exist. These can be mutations in other RNA polymerase subunit coding genes or selective permeability of cell wall (Musser, 1995).

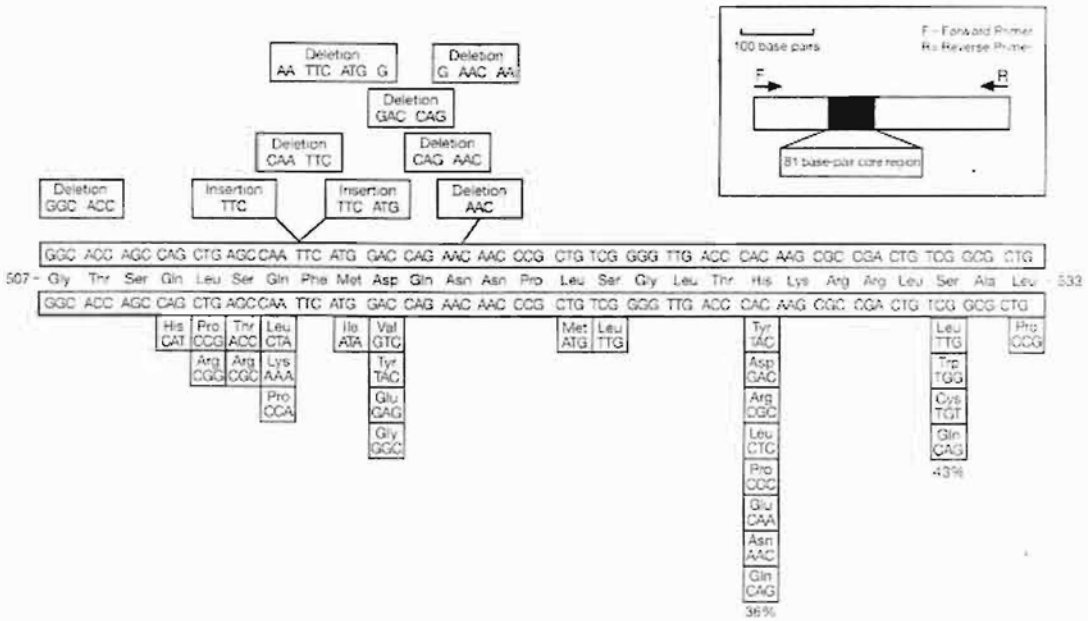


Figure 3. Hypervariable region of the *rpoB* gene corresponding to 507-533 amino acid sequence. The prevalent mutations are shown in boxes (Sander & Bottger, 1999).

Since RIF is a marker for MDR, rapid revealing of RIF resistant strains is of great clinical value. For that aim, molecular testing of RIF susceptibility can be focused on detection of mutations in a single hypervariable region of the *rpoB* gene.

Inhibitors of protein synthesis

SM and related aminoglycosides shown in Table 2, affect bacteria by inhibiting prokaryotic protein translation at ribosomes (Table 2). A common mechanism of resistance to aminoglycoside antibiotics in other bacteria, e.g. to tobramycin and to gentamycin in staphylococci, is drug inactivation by drug-modifying enzymes encoded by plasmids or transposons (Chambers, 1997). In mycobacteria, initiation of mRNA translation appears to be inhibited or translational accuracy is affected (Finken et al., 1993).

SM acts by binding to the small 30S ribosomal subunit instead of aminoacetylated tRNA, thereby interfering with polypeptide synthesis by inhibiting translation (Winder, 1982). Two genes, *rpsL* and *rrs*, encoding the ribosomal protein S12 and the 16S rRNA are known to be responsible for the high level SM resistance in 80% of cases (Douglas et al, 1993). Two thirds of the resistant mutants show mutations in the highly conserved region of the 372bp long *rpsL* gene, converting protein S12 lysine residues K43 and K88 to arginine or threonine. Corresponding mutations have been also previously found in SM-resistant *E.coli* (Finken et al, 1993; Meier et al., 1994). The remaining one third of the SM-resistance conferring

mutations occur in two regions of the 1.464bp long *rrs* gene encoding 16S ribosomal RNA: 530 loop and the 915 region known as SM binding site (Moazed&Noller, 1987). Structural model of 16S RNA with known affected sites is shown in Figure 4. Rearrangements of Watson-Crick base pairs G≡C to wobble pairs G•U or C•U at nucleotide positions 495-GGC-497 and 514-GCC-516 attenuates the tertiary structure of 530 loop inhibiting binding to SM. Those positions are crucial for functioning of the ribosome. Three mutation sites have been detected in the highly conserved 915 region: at nucleotide positions 904 (C→A or G), 905 (A→G) and 906 (A→T), as shown in Figure 4. In contrast to other bacteria, *M.tuberculosis* has a single *rrs* operon increasing the chance for acquiring resistance to SM.

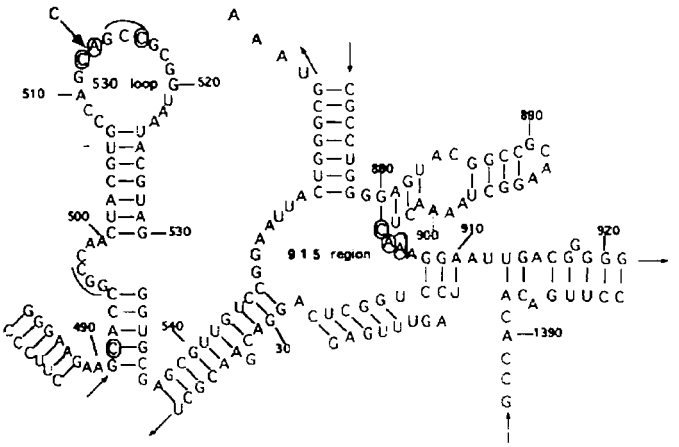


Figure 4. 16S rRNA secondary structure in *M.tuberculosis* (Honore et al., 1995). Nucleotide positions are marked by numbers, nucleotide insertions are shown by arrows, mutated nucleotides are encircled and the underlined nucleotides are involved in Watson-Crick interactions.

Mutations described in *rpsL* and *rrs* genes generally confer to the high or medial level of SM resistance (MIC from 500 to 250-50μg/ml). About 20% of remaining cases of SM resistant isolates had the wild type genes. In those isolates, low level resistance (MIC 25-50μg/ml) is observed, probably, caused by changes in cell wall permeability (Meier et al., 1996). SM resistance is frequently associated with resistance to other aminoglycosides. Nevertheless, these secondary-line drugs can be effectively used for the treatment of polyresistant *M.tubeculosis* and other mycobacteria caused infections due to a different mechanism of resistance. Particularly, analysis of 16S rRNA operon in all isolates resistant to aminoglycosides differentiated a single point mutation G→A at nucleotide position 1408 (Prammananan et al., 1998).

Therefore, to determine SM susceptibility at the molecular level, two genes, *rpsL* and *rrs*, should be analyzed simultaneously. .

Inhibitors of cell wall synthesis

Isoniazid (INH) is most often used for TB treatment because of the very high sensitivity of the *Mycobacterium tuberculosis* to this drug (MIC is 0.02-0.05μg/ml) (Bernstein et al., 1952). It is clarified that INH is acting by inhibition of synthesis of mycolic acids (Sacchetini&Blanchard, 1996), branched alfalipids attached to arabinogalacan polymers (Table 2). Decrease of catalase-peroxidase activity in INH resistant isolates was known already 50 years ago indicating the important role of this enzyme in metabolism of INH. Here we see another mechanism of resistance, where

prodrug transformation to active drug form is prevented by diminishing enzyme activity That mechanism is opposite to those realized in most bacteria via gaining additional biochemical activity, e.g. with drug inactivating enzymes. INH is a prodrug activated after inclusion in mycobacterial cell by catalase-peroxidase encoded by the *katG* gene. An activated drug inhibits enoyl-acylPP reductase encoded by the 810bp long *inhA* gene which product participates fatty acid elongation cycle (Rawat et al., 2003).

As one can see from Table 2, at least five genes, *katG, inhA, ahpC, kasA* and *ndh,* are known to confer INH resistance, but in 60-70% of cases the INH resistance is associated with mutations in the 2359bp long *katG* gene at codon 315 (Ser→Thr) (Heym et al., 1993). Most frequently observed substitutions at codon 315 are ACG change for ACC (Ser→Thr, in 82%) and less frequently ACG change for AAC (Asn), ATC (Ile) and CGC (Arg) (Dobner et al., 1997; Haas et al., 1997). In comparison to RIF resistance, there is a higher rate of INH resistant *M.tuberculosis* mutants occurred *in vitro* (mutation rate 10^{-5}-10^{-7}) partially explaining quick acquisition of resistance to INH. There are several reasons for that. One of them, as it was mentioned before, is a probable summary effect of mutations occurring in any of five known genes, which products are involved in INH metabolism (Telenti et al., 1997; Zhang et al., 1996; Lee et al., 2001). That factor of many genes may be explained by plenty of enzymes involved in synthesis of mycolic acids. The product of the *katG* gene is not essential for mycobacterial cell methabolism and bacteria with mutated *katG* gene, even with lowered vitality and virulence, is able to accumulate mutations in other genes. Additionally, the *katG* gene is localized in unstable region of the chromosome and can easy undergo structural changes (Zhang et al., 1994). Mutations CGG→CTG (Arg→Leu) at codon 463 of the *katG* gene have been also described in INH resistant *M.tubeculsosis* isolates, but later studies showed that mutation occurs in INH susceptible strains as well and is not associated with INH resistance (Rouse et al., 1995; Haas et al, 1997).

There is less known about the association of INH resistance with the *ahpC, kasA* and *ndh* genes. For example, an over-expression of *ahpC* gene which is, probably, caused by mutations in a promoter region, was observed in IHN-resistant isolates with the wild-type *katG* and *inhA* genes (Telenti et al., 1997). AhpC gene codes for an alkyl hydroperoxide reductase that protects from the toxic to bacilli to organic peroxides. Such over-expression is proposed also a compensatory mechanism in case of the loss of catalase-peroxidase activity (Sherman et al., 1996). Lee et al. demonstrated mutations in a ketoacyl-acyl protein synthase coding *kasA* gene in 10% of INH resistant isolates, and recent studies of Lee et al. (2001) showed mutations in *ndh* gene with the same frequency.

So, molecular analysis of the corresponding genes in INH resistant cases supplemented by molecular genotyping is significant due to various reasons. First of all, Van Soolingen et al. (2000) showed correlation of most common S315 single mutation in the *katG* gene with the high level resistance to INH. Secondly, the *katG* mutation R463 is an important marker for deciphering phylogenetic groups of mycobacteria, particularly, for identification of phylogenetic group I, which includes well known Beijing genotype, giving an insight into evolution of more virulent forms of mycobacteria (Brosch, 2002).

Ethambutol (EMB) is another effective and highly specific first-line agent used in chemotherapy in combination with other drugs (British Thoracic Association, 1984). It is believed that EMB is targeting arabinosyltransferases that catalyse the incorporation of arabinan into cell wall polymers arabinogalactan and lipoarabinomannan (Table 2) (Deng et al, 1995, Wolucka et al, 1994). The 12-domen

protein with arabinosyltransferase activity, encoded by the *embB* gene, is integrated into mycobacterial membrane. The *embB* gene is one of the three genes, joined in a single 10kb *embABC* operon. In recent study, mutations associated with resistance to EMB have been detected in about 68% in the *embB* gene and were most common at codon 306 (Met→ Ile, Leu, or Val) (Ramaswamy et al., 2000). It was proposed that Met306 is located in a protein loop in cytoplazma where it is functioning as EMB-resistance determining region (ERDR) as shown in Figure 5. Additionally, Ramaswamy et al. also showed mutations associated with EMB-resistance in eleven other genes, reaching 67% together with mutations in the *embB* gene.

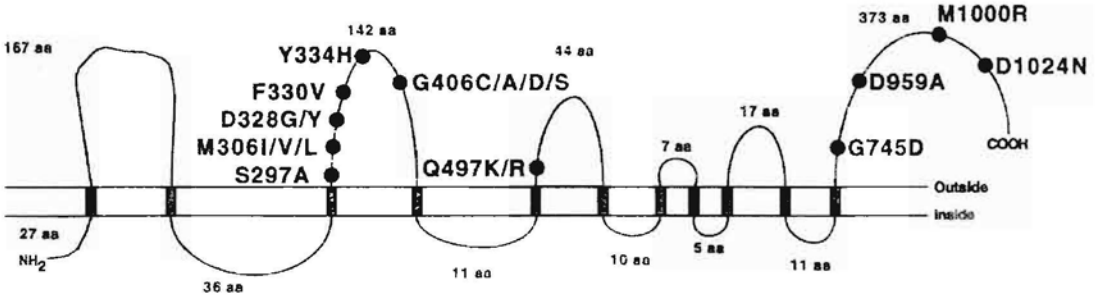


Figure 5. Schematically shown transmembranal protein EmbB encoded by the *embB* gene with resistance-associated amino acid replacements modeled by Ramaswamy et al (2000). Putative ERDR loop includes codon M306 and others.

It is obvious, that molecular testing of EMB susceptibility in *M.tuberculosis* can meet various problems because less than 70% of EMB resistant isolates show mutations in the *embB* gene. In the remaining cases, there may be various genes which can be associated with EMB resistance and which are only partially studied nowadays.

Pyrazinamide (PZA) is also a very effective anti-TB agent particularly when used in combination with RIF and INH shortening the time of chemotherapy from 12-18 to 6 months (British Thoracic Association, 1984). It is effective against semi-dormant pathogenic mycobacteria, which are usually missed by other anti-TB agents. The naturally resistant *M.bovis* is the only exception for PZA. PZA works in mycobacterial cell when bacterial pyrazinamidase converts the inactive PZA to toxic for mycobacteria pyrazinoic acid (Zhang & Mitchison, 2003). PZA resistance in *M.tuberculosis* strains is associated with the loss of pyrazinamidase and nicotinamidase activity due to mutations in different locuses in the 561bp long *pncA* gene that lead to amino acid changes or to premature chain termination. Molecular analysis of the naturally resistant *M.bovis* and *M.bovis* BCG strains revealed mutation His57→Asp in the *pncA* gene (Konno et al., 1967; Scorpio and Zhang, 1996). Recent studies by microarray analysis have showed a whole group of new genes associated with the PZA resistance: *fad*E24, *mab*A, Rv1772, Rv1592 etc (Ramaswamy et al, 2003).

Measuring PZA susceptibility *in vitro* by classical methods on solid medium is problematic since PZA is active in acidic medium, which inhibits growth of *M.tuberculosis*. Therefore, testing of PZA susceptibility by molecular methods is of crucial importance.

Since *M.tuberculosis* is lacking extrachromosomal DNA elements like plasmids, genome alterations play a major role in drug resistance development. Transposons don't transfer resistance in *M.tuberculosis*, however, those were

postulated to provoke genome rearrangements. Moreover, it was hypothesized that different *M.tuberculosis* genotypes develop drug resistance with non-equal intensity. That could depend on the mechanism of drug resistance selected by each particular genotype. Detailed studies of transposable elements and molecular genotyping of MDR *M.tuberculosis* could help to answer the questions posed.

2.4.5. Insertion sequence IS6110 as molecular marker for *M. tuberculosis* complex in clinical specimens

Mobile genetic elements like insertion sequences (IS) and transposons are found in bacteria, archaebacteria and eukaryotes. Insertion sequences are DNA fragments encoding their own ability to transpose within the genome. The function of most mobile genetic elements is unknown and they are usually considered to be genome parasites. In genus *Mycobacterium*, there are 3 groups of IS, which are usually host specific. IS are between 880 bp and 2260 bp long and are found in the chromosome in 1 to more than 20 copies (Guilhot et.al., 1999). One of those, a 1361 bp long insertion sequence named IS6110, is present in MTC only. It was first described by Thierry et al (1990). Insertion sequence IS6110 is a mobile genetic element integrated in *M. tuberculosis* genome and is typical only for species of *M. tuberculosis* complex.

Due to a narrow host range insertion element 6110 can be used for rapid identification of pathogenic mycobacteria. The majority of investigated MTC strains contained between 5 and 20 copies of IS6110 located in various places of genome (van Soolingen et al. 1993). PCR-based methods are sensitive enough to detect a presence of even a few bacteria DNA in a specimen. In this study, evaluation of IS1660-based MTC detection method was done in order to apply PCR-based methods further for drug susceptibility testing and genotyping directly on clinical specimens.

2.4.6. Studies of *M.tuberculosis* complex genotypes

Since *M.tuberculosis* strains can differ by virulence, ability to gain resistance, to transmit and to rise epidemies, it is important to known whether it is associated with certain genotype features. Many outstanding molecular techniques such as IS6110-restriction fragment length polymorphism (IS6110-RFLP), spoligotyping, mixed-linker PCR, typing by using mycobacterial interspersed repeat units (MIRUs) (Figure 6) and others have been developed especially for mycobacteria fingerprinting (Barnes & Cave, 2003).

Standardised genotyping techniques revealed a number of genotype families or groups with more than 70% similarity by IS6110-RFLP, which can predominate in population. For example, the genetically highly conserved genotype family of strains named Beijing have caused large outbreaks of TB worldwide (Bifani et l., 2002). The Beijing genotype strains are often resistant to INH and SM and are associated with multi-drug resistance. In addition, the Beijing genotype is reported to have a selective advantage over the other *M.tuberculosis* genotypes and induces a febrile response to treatment (Soolingen et al., 1995, van Crevel et al., 2001). The Beijing genotype predominates in China and some geographic areas of Asia, and now seems to be spreading to other areas in the world (Anh et al., 2000). The high prevalence of MDR Beijing genotype has been recently shown in the neighbouring countries – Estonia and Russia (Kruuner et al., 2001, Marttila et al., 1998). The comparison of different genotypes of MDR and drug susceptible strains from many countries is available now trough the database created in the frames of EU Concerted Action TB project leaded by the Dick van Soolingen. The database includes genotypes revealed by the IS6110-RFLP method, considered the "golden standard" for typing mycobacterial strains

(Van Soolingen et al, 1991). However, IS6110-RFLP method is time-consuming and requires a large amount of DNA available only after bacteria culturing.

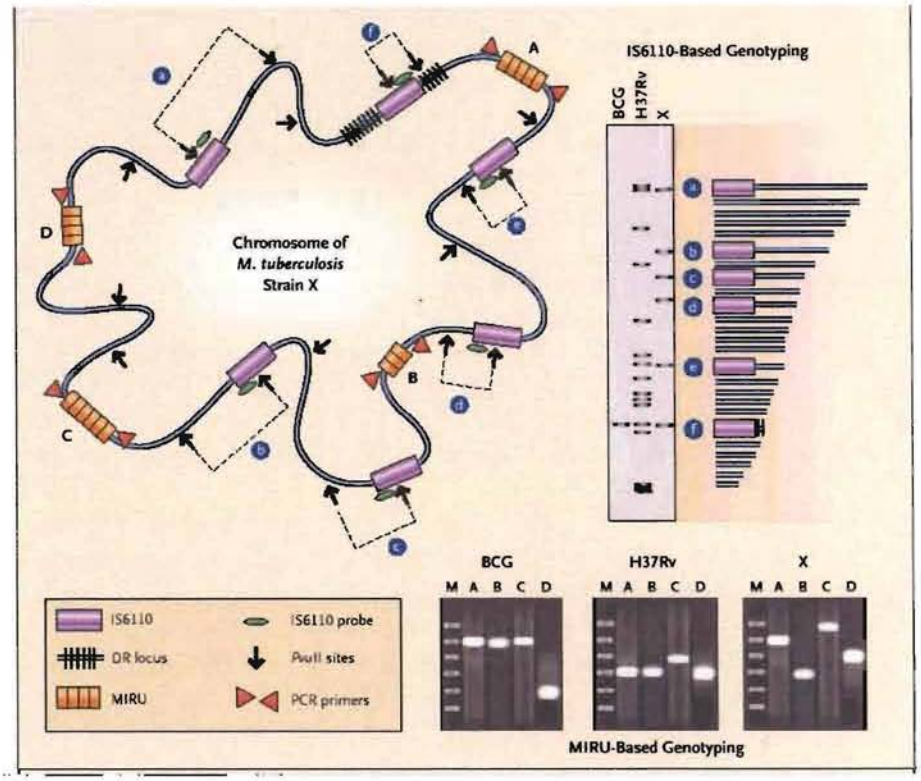


Figure 6. Chromosome of *M.tuberculosis* hypothetical strain X and genotyping of *M.bovis* BCG, the *M.tuberculosis* laboratory strain H37Rv, and strain X on the basis of IS6110 and MIRUs. DR locus is used in spoligotyping. Fragments a through f are the products of DNA digestion with *PvuII*; M is molecular weight marker and A-D are PCR products of MIRU loci (By Barnes & Cave, 2003).

As alternative to RFLP, spoligotyping have been introduced for identification of MTC and for revealing of major genotype groups, especially for Beijing genotype family (Kamerbeek et al, 1997). In comparison to IS-6110-RFLP, PCR-based spoligotyping method is more rapid and can be applied directly on clinical specimens. The major spoligotype families of *M.tuberculosis* are presented today in "SpolDB4" database created by C.Sola and N.Rastogi and containing patterns from more than 30000 individual strains (Filliol et al, 2003). Strain family definition is based on spoligotyping technique and phylogeographic specificity, evaluated subsequently. That way many spoligotypes have been grouped in families: Manila family (Douglas et al., 2003), Ethiopian family (Hermans et al., 1995), Finland families (Puustinen et al., 2003), Haarlem family, Latin American & Mediterranean (LAM) family, X family, already mentioned Beijing family etc.

In this study, spoligotyping was applied to MDR *M.tuberculosis* isolates to reveal the prevalent genotype groups between MDR *M.tuberculosis* in Latvia and to compare the prevalence of MDR-associated mutations between several groups.

2.5. Characterisation of *Staphylococcus* species

2.5.1. Infections caused by *S.aureus* and related species

Staphylococci are opportunistic pathogens that colonise and infect both hospitalised patients with decreased host response and healthy, immuno-competent people in the community. *Staphylococcus* genus species, especially *S.aureus* and *S.epidermidis*, are most frequently isolated from clinical specimens in surgical hospitals besides other gram-positive cocci. The pathogenity of staphylococci is mainly due to their aggression factors: blood coagulase and many toxins. Also staphylococci are characterised by the ability to gain MDR rapidly, which, in contrary to *M.tuberculosis*, can be transferred horizontally by extrachromosomal DNA elements. Drug resistant staphylococci are dangerous by their ability to colonise in healthy people and in medical personal for a long time and to spread when appropriate conditions appear.

S.aureus have been isolated from intensive-care patients, transplant recipients or victims of accidents (Dalhoff, 1999). Coagulase negative *Staphylococci* (CoNS), particularly the *S. epidermidis* and *S.haemolyticus*, have emerged as important cause of nosocomial (acquired due to health-care) infections in recent twenty years. This group is diagnostically distinguished from *S.aureus* by its inability to produce coagulase. The high prevalence of *S.epidermidis* can be explained of by the increased use of indwelling catheters and implanted devices. The pathogenity of *S.epidermidis*, which has been regarded for a long time as relatively innocuous inhabitant of the human skin, is mainly due to ability to form biofilms on indwelling medical devices (Voung&Otto, 2002). Another nosocomial pathogen, *S.haemolyticus*, is characterised by a tendency to develop multi-antibiotic resistance, with a unique predisposition to glycopeptide resistance.

2.5.2. Occurrence and spread of methicillin-resistant staphylococci

Frequent use of penicillin introduced in hospitals in the early 1940s resulted in the occurrence in resistant strains, producing penicillinase (β-lactamase). In the 1960-s, shortly after introduction of semisynthetic penicillinase-resistant penicillins (i.e. oxacillin/methicillin (MET) and other β–lactam ring containing antibiotics) into the clinical practice, first methicillin resistant *S.aureus* (MRSA) were detected in UK (Jevons, 1961). Since then MRSA intrinsically resistant to virtually all β–lactams has become one of the leading causes of nosocomial infections worldwide. In the last decades the multitude of resistant strains of *S. aureus* have developed and spread globally (Fluckiger&Widmer, 1999).

The incidence of MRSA among the clinical isolates of *S. aureus* varies from country to country, but in Europe, a north-south gradient is observed. MRSA is kept at controllable level in Scandinavian countries as well as in Switzerland (< 2%). However, the level of MRSA incidence is more than 30% in Mediterranean hospitals (France, Italy etc) and USA, reaching 60% in Portugal and Japan. Prevalence of particular genotypes gaining resistance to methicillin was shown in epidemic *Staphylococcus aureus* strains. Using molecular typing techniques – primarily pulsed-field gel electrophoresis (PFGE), the five major clonal lineages (the Iberan, Brazilian, Hungarian, New York/Japan and paediatric pandemic MRSA clones), have been now identified. These strains accounted for 70% of over 3000 MRSA isolates recovered in

hospitals mainly of southern and eastern Europe, South America, and the USA (Blanc et al, 2002; Oliveira et al., 2002; Stefani & Varaldo, 2003).

The proportion of MRSE among hospital CoNS has increased from 8 to 30% in the 1980s to 80% and even 90% nowadays (Bouza, 2002, Carratala, 2002).

Analysis of staphylococcal cultures isolated in Hospital of Traumatology and Orthopedics (Riga, Latvia) showed that local situation with MRSA remains stable in five last years with the maximal level 4.64% in 2001, as shown in Figure 7. In opposite, analysis of MET-resistance in CoNS demonstrated that there is a tendency of growing it from 4% in 1998 to 36% in 2002. Among CoNS, the following species have been isolated most frequently in Latvia: *S.epidermidis, S.haemolyticus, S.cohnii, S.warneri* and *S.hominis* (A. Zilevica, 2001). Nevertheless, the number of resistant forms is not as high as reported by authors from other countries. Unfortunately, a complete picture of situation with emergency of MRSA and MRSE in Latvia is still unclear due to lack of centralised data collection from all hospitals of Riga and regions.

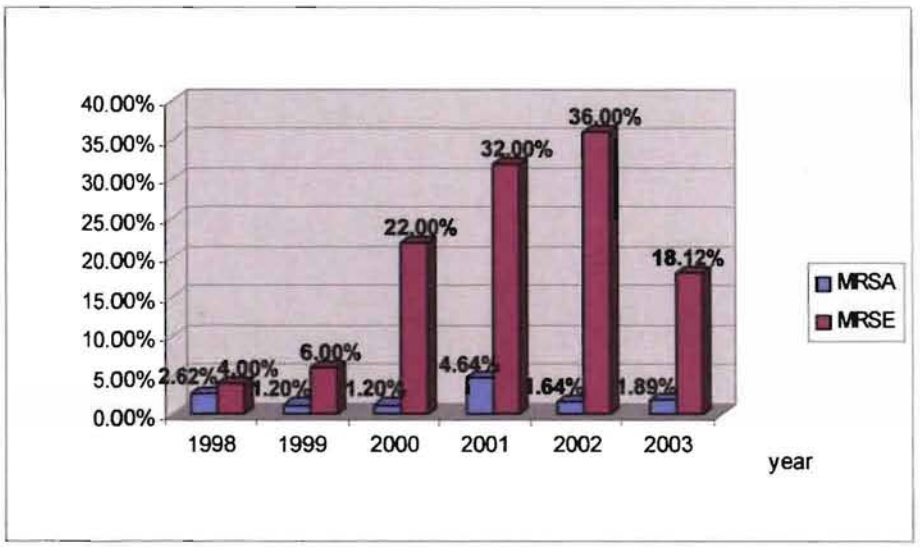


Figure 7. Incidence rate of MET-resistance in staphylococci in Hospital of Traumatology and Orthopedics, 1998-2002.

2.5.3. Characterisation of g. *Staphylococcus* phenotype and genome

Genus *Staphylococcus* belongs to Family *Micrococcaceae*, in which however, staphylococci exhibit a number of different molecular features among other gram-positive cocci (W.Witte, 2000). Most species are natural inhabitants of the mammalian skin and mucous membranes. *S.aureus* can be phenotypically differentiated by the coagulase-positive test-tube reaction. A thick cell wall of staphylocci represents an impermeable barrier to many antibiotics. It is formed a very thick peptidoglycan layer with pentaglycine cross-bridges and teichoic acids. (Schleifer, 1983; Novick, 1990).

A complete *S.aureus* genome sequencing project have been realised by the group of Hiramatsu in 2001 in Japan (Kuroda et al., 2001). The entire chromosome of *S.aureus* standard strain NCTC 8325 is about 2,8 megabases. Compared to mycobacteria, staphylococci has a low G+C content in DNA (32-34%) and well-known virulence factors. As revealed by the Hiramatsu group, *Staphylococcus* genome is composed of a complex mixture of genes, many of which seem to be

acquired by lateral gene transfer. Most of the antibiotic resistance genes are carried either by plasmids or by mobile genetic elements including a unique resistance island. Three classes of new pathogenicity islands were identified in the genome: a toxic-shock-syndrome toxin island family, exotoxin islands, and enterotoxin islands. In the latter two pathogenicity islands, clusters of exotoxin and enterotoxin genes were found closely linked with other gene clusters encoding putative pathogenic factors. The analysis also identified 70 candidates for new virulence factors. Repeated duplication of genes encoding superantigens explains why *S.aureus* is capable of infecting humans of diverse genetic backgrounds, eliciting severe immune reactions.

The genome of *S.epidermidis*, sequenced in 2003, consisted of a single 2.499.279bp chromosome and six plasmids. It contained 2419 protein coding sequences, among which 230 putative novel genes were identified. To compare to the virulence factors in *S.aureus*, aside from two kinds of haemolysins, no other toxin genes were found (Zhang et al., 2003). An important virulence factor in pathogenic *S.epidermidis* is biofilm production in indwelling devices mediated by the expression of the *icaADBC* operon, which is, however, absent in commensal strains (Cho et al., 2002; Otto, 2004).

There is a high heterogeneity between related species and subspecies, indicating that *Staphylococcus* is an evolutionary old microorganism. Low C+G content and high heterogenity allows application of macrorestriction analysis of pathogenic strains with rarely cutting *SmaI* enzyme and subsequent pulse-field gel electrophoresis, which provides a profile composed of 15 to 20 fragments (Weller, 2000).

Plasmids. As it was mentioned above, staphylococci usually contain plasmids that encode various factors: drug resistance, metabolism etc. Plasmids have been detected in about 90% of methicillin-resistant *S.epidermidis* (MRSE) and in 50% of MRSA. There are three major types of plasmids in staphylococci. One group contains small multicopy plasmids ranging in size from 2.5 to 5 K in copy number from 10 to 50. Each plasmid has a single resistance determinant for tetracycline, SM, chloramphenicol, macrolides, kanamycin or cadmium salts. These plasmids are able to replicate in other gram-positive species, and relative plasmids were found in other genera. A second type consists of larger plasmids, 25 to 35Kb in size and about five copies per cell. These usually carry resistance to penicillin and inorganic ions and have not been observed to replicate in other genera. A third type includes larger (40-60Kb) conjugative plasmids, typically carrying resistance to gentamycin, penicillin, neomycin and such compounds as ethidium bromide (Novick, 1990).

Genes can be transferred horizontally by **transposons** and located either on plasmids or integrated in the chromosomal genome and further transferred vertically.

2.5.4. Spectrum of antibiotics and chemical agents used for treatment of staphylococcal infections

Initially, a wide spectrum of chemotherapy agents (β–lactams, aminoglycosides, fluorquinolones, macrolides, streptogamin combinations etc.) was effectively used against staphylococci-caused infections (Witte et al., 1997). However, occurrence of MET-resistant staphylococci, an intrinsically resistant to virtually all β–lactams, lead to MDR, e.i., simultaneous resistance to cephalosporins, erythromycin, ciprofloxacin, gentamicin, trimethoprim and related products. The glycopeptides, vancomycin and teicoplanin, makes the only exception in that group. Resistance to so wide spectrum of agents can be explained by the mechanism of MET- resistance, which will be described later.

It is clear that the number of appropriate chemotherapy agents effective against MRSA and MET-resistant CoNS is not high. The available antibiotics used today are vancomycin, rifampin, fusidic acid, clindamycin, linezolid and quinipristin-dalfopristin (Lowy, 2003). However, rifampin, fusidic acid and clindamycin can be used only in combination with other agents. At the same time, use of linezolid is restricted, at least in Latvia, due to its high cost. Vancomycin is widely used to treat MRSA, however, the emergence of vancomycin-resistant strains has been already reported in USA (Tenover et al., 2004). This shows the clinical importance of extensive molecular studies of MET-resistant staphylococci in order to prevent uncontrollable spread.

2.5.5. Drug resistance mechanisms in *Staphylococcus* spp. Mechanisms of resistance to β–lactams with the special reference to methicillin

The astounding ability to respond in a short time to the antibiotic challenge in staphylococci can be explained by a multiple resistance mechanisms, encoded by plasmids, transposons and by chromosomal genes. The last, chromosome-based mechanism of drug resistance can include acquisition of new DNA fragments with necessary genes or, similarly to mycobacteria, chromosomal gene mutations. Resistance-encoding genes can be horizontally transferred by transformation of intact cells or protoplast, plasmid- or trasposon-mediated conjugation and transduction, even between related species (Berger-Bachi, 1997). In that way, staphylococci realise genetic exchange of determinants of resistance to erythromycin, spectinomycin, penicillin, gentamycin and MET.

Besides drug resistance genes, there is less common mechanism of resistance in staphylococci, which, like in *M.tuberculosis*, is caused by mutations in drug target encoding genes. For example, in that way resistance to rifampin (mutations in the *rpoB* gene) and to fluorqiunolones (mutations in *grlA* and *gyrA* genes) is acquired (O'Neill et al., 2001). At the same time, investigation of fusidic acid resistance showed that, in parallel to resistance-associated mutations in elongation factor G, fitness-compensatory mutations occur at the same region of *S.aureus* genome. This data suggest that compensatory evolution to reduce the fitness costs associated with drug resistance is a frequent occurrence in the clinical environment (Nagaev et al., 2001). This idea correlates with the study of Sherman et al (1996), suggesting that the loss of catalase-peroxidase (KatG) activity in INH-resistant *M.tuberculosis* can be compensated by overexpression of AhpC. Overproduction of that enzyme occurs due to *ahpC* gene mutations and provides ability to withstand oxidative stress.

MET, one of β–lactam antibiotics, is a penicillin descender and has a similar to penicillin mode of action. It is important to know the mode of action and mechanism of resistance to penicillin for better understanding of interaction between staphylococci and MET.

Penicillin acts as analogue of acyl-D-alanil-D-alanin, a substrate of the penicillin-binding proteins (PBP). It covalently binds to the PBP active-site serine, forming a covalent complex and inactivating enzyme. PBPs catalyse penicillin-sensitive transpeptidation reaction of two muropeptide side chains (linkage of glycine and alanine) essential for peptidoglycan polymerization, the final stages of bacterial cell wall biosynthesis (Ghuysen, 1994). Binding of β–lactams by PBP leads to a defective cell wall building and finally to cell death and lysis. The introduction of first penicillins stimulated selection of strains with β–lactamase (penicillase) activity. β–lactamases are encoded by *blaZ* gene, located on plasmids or embedded in

chromosome and are easily transferred by bacteriophages. Today more than 90% of staphylococci produce β–lactamases (Lowy, 2003).

Next generation of β–lactams, such drugs as semisynthetic **methicilin** were resistant to β–lactamase activity. Nevertheless, MET-resistant strains have emerged soon in many areas of high antibiotic pressure, with high ability to spread and to accumulate not related resistance determinants (Berger-Bachi, 1997). MET-resistant strains have acquired an additional 76-kDa penicillin-binding protein, termed PBP2a, exhibiting low affinity to β–lactam antibiotics and therefore protecting cell from antibiotic action. PBP2a differs from other PBPs in that its active site blocks binding of all β–lactams but allows the transpeptidation reaction to proceed (Lim & Strynadka, 2002). PBP2a protein is encoded by the 2kb long *mecA* gene. Since *mecA* gene is not found in MET-susceptible strains, it is molecular marker for MET-resistance. The gene is a part of the MET resistance determinant *mec*, termed also staphylococcal chromosomal casette (SCC*mec*). (Katayama, 2000) This is a 21 to 67kb long DNA element, integrated in a specific site of the chromosome of staphylococci, near the *pur-nov-his* gene cluster, as shown at Figure 8 (Kuhl, 1978). SSC*mec* cassette is bound by inverted repeats suggesting that it might be a transposon. Aside from *mecA* gene, SSC*mec* cassette contains *mecI* and *mecR1*, regulatory elements controlling *mecA* transcription, and 20 to 45kb of *mec*-associated DNA (Hiramatsu et al.,1995). Besides *mec* operon, encoded by approximately 5kb of DNA, *mec*-associated DNA that may contain up to 100 open reading frames, includes transposons, like Tn554 (which contains the *ermA* gene, associated with erythromycin resistance), and insertion sequences, like IS431. The ability of IS 431 elements to trap and cluster resistance determinants with similar IS via the homologous recombination explains the multi-drug resistance that is characteristic for MRSA and MET-resistant CoNS (Chambers, 1997). That's how MET-resistant strains gain additional resistance to other groups to antibiotics. Today, there are known four types of SSC*mec* cassettes, differentiated by size, structure and by carriage of antibiotic resistance genes (Hiramatsu et al., 2001).

mecI and *mecR1* genes represent a negative regulatory center for PBP2a production. They are similar in sequence, structure, function, and mechanism of regulation to staphylococcal β–lactamase regulatory elements, *blaI* and *blaR1* (Gregory et al., 1997). The gene products are the repressor MecI and transmembrane β–lactam sensing protein MecR1, needed for induction of the *mecA* gene. Normally, *mecA* is repressed and no PBP2a is synthesised. Interestingly, MET and oxacillin are poor inducers of the *mec* operon. That's why in experimental studies strains with *mec* determinant may appear phenotypically susceptible. Nevertheless, a majority of clinical strains constitutively produce PBP2a in high amounts due to mutations or deletions in repressor or due to mutations in promoter regions (Suzuki et al., 1993). Simultaneously, *blaI* and *blaR1*, the penicillase (*blaZ*) gene regulatory elements can regulate expression of the *mecA* gene (Figure 8) (Hackbarth&Chambers, 1993).

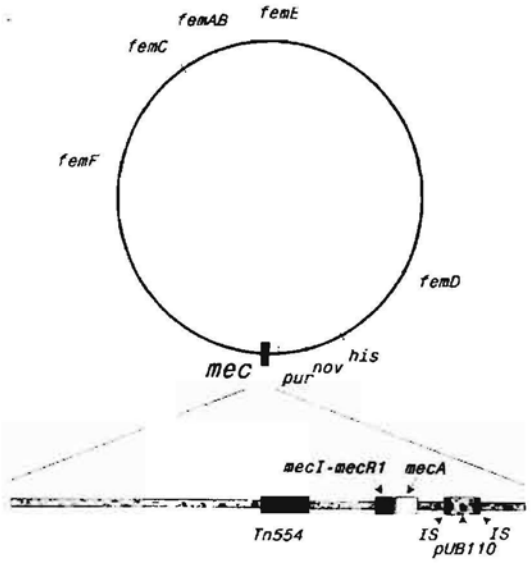


Figure 8. Molecular organization of the approximately 50-kb *mec* region and its chromosomal location relative to *fem* factors. IS indicates IS431 elements flanking the tobramycin resistance plasmid pUB110. Tn554 is a transposon containing *ermA*, encoding erythromycin resistance (by Chambers et al., 1997).

The origins of SSC*mec* are obscure. Today one or several MRSE strains are considered reservoirs of antimicrobial resistance genes that were transferred to other *Staphylococci*, so contributing to the development of methicillin resistance among microorganisms Hypothetically, the *mecA* gene was transferred from "outside" by transposition to *S. epidermidis* and then to *S.aureus*. It was shown, that β–lactamase plasmid of *S.aureus* may provide a temporary insertion site for the *mec*-containing transposon (Hiramatsu, 1995). Cuoto et al. (2003) identified a *mecA* gene in a MET-sensitive *S.sciuri* suggesting that a possible source of the *mecA* element in *S.aureus*. MET resistance was proposed to spread clonally from a few ancestral strains resulting in several lineages of clonal descendants. When acquired, SSC*mec* is permanently included in bacterial genome and transferred vertically (Hiramtsu et al, 2002).

2.5.6. Methicillin-resistant phenotype and susceptibility testing in staphylococci

When SSC*mec* is present, the MET-resistant phenotype in staphylococci can be masked due to chromosomal genes, physically distinct from *mec*, termed *fem* (factor essential for MET resistance), which are present in both susceptible and resistant strains. Mutations in six *fem* genes, *femA, -B, -C, -D, -E* and *-F*, were shown to alter peptidoglycan composition reducing MET-resistance level (Berger-Bachi, 1994).

Additionally, low-level or borderline MET-resistant staphylococci strains lacking *mec* determinant can be isolated. In that case, structural modifications of membrane proteins PBP2 and PBP4 that lower the affinity to MET, may occur for due to multiple mutations (Henze&Berger-Bachi, 1995). Some authors postulate that hyperproduction of β–lactamase or activity of methicillinase encoded by another gene, which is not identified yet, can also contribute to a low-level resistance in staphylococci (Barg et al., 1991; Massida et al., 1994). That studies show that bacteria constantly develop new resistance mechanisms, via the gene mutations or via the acquisition of foreign genes. To note, these MET-resistance mechanisms do not result in development of MDR, in contrary to *mec*-mediated resistance.

MET resistance is strongly influenced by external factors, like temperature, salts, light etc. suggesting that environmental conditions also may affect the expression of resistance. The resistance levels to MET (or oxacillin, which is routinely used as a test substance) and other β–lactams may vary between strains (MIC ranging from 2 to 1000μg/ml), independent from the amount of PBP2a produced. Also, heterogeneous resistance of one strain is specific property of staphylococci. For example, in a low-level resistant population, some cells (between 10^{-4} down to 10^{-7}) express high level resistance (Matthews&Stewart, 1984).

Because of heteroresistance, it is recommended by the National Committee (NCCLS) to use at least two *in vitro* methods for the detection of MRSA and MR CoNS. Traditionally, disc diffusion method is used on agar media. Additionally, an agar screening test is recommended prescribing to incubate bacteria for 24 hours at 35°C on Mueller-Hinton media containing 6μg/ml oxacillin with supplement of 2-4% NaCl (NCCLS, 2000). An example of commercial test systems is E-test, where MIC value is estimated with an oxacillin-saturated strip. The automated systems for rapid detection of MET resistance have been also developed. For example, the BBL crystal system in VITEK II allows a computer-assisted reading of results already after 3-4 hours of incubation. The VITEK II system has a high sensitivity but less specificity for detection of MET-resistant CoNS in comparison to test for presence of *mec* determinant (Cuny et al, 1999).

Studies of MET resistance and susceptibility testing by molecular methods are of special interest because of its clinical importance and resistance transfer mechanisms. Amplification of *mecA* gene in PCR is stated today as a "golden standard" for MET susceptibility testing in *S.aureus* and CoNS. The method was first described by Murakami et al. (1991) and was recommended for routine laboratory use in parallel to standard susceptibility testing methods. Presence of the *mecA* gene always indicates on MET-resistant strain. Therefore, heterogeneous strains can be also revealed by the PCR-based *mecA* gene detection.

In this study, *mecA*-PCR analysis was done in order to evaluate method's correlation with the commonly used susceptibility methods for further application for the laboratory screening needs. Additionally, it was the first step to the further studies of staphylococci strains involved in epidemies in hospitals of Latvia, for example, by the identification of SCC*mec* cassette types and by subsequent genotyping of strains. It could contribute to our knowledge about MET-resistant strains circulating in Latvia and to examine epidemic links between hospitals at local and international level.

3. METHODICAL APPROACH

3.1. Analysis of genes associated with the first-line drug resistance in *M.tuberculosis*

3.1.1. Culturing and isolation of DNA

Cultures of the *Mycobacterium tuberculosis* complex were grown at Latvian Centre of Tuberculosis and Lung Diseases on Lowenstein-Jensen medium for 4-6 weeks. Drug susceptibility was determined both by the absolute concentration method on slants with the H37Rv strain as the positive control and by the BACTEC radiometric method (Becton Dickinson, Sparks, Md.) (Hawkins et al., 1991; Roberts et al., 1991). In the absolute concentration method, resistance was defined as growth on solid media containing graded concentrations of drugs greater than a 20CFU at a specific drug concentration. The MICs on Lowenstein-Jensen medium were 0.2μg/ml and 2.0μg/ml for INH, 40.0μg/ml for RIF, 4.0μg/ml for SM and 2.0μg/ml for EMB. PZA susceptibility was tested by BACTEC method at 100μg/ml concentration of PZA.

Native genomic DNA was isolated from the mycobacterial cultures by an internationally standardised procedure with lysozyme/proteinase K, N-cetil-N,N,N,-trimethyl ammonium bromide and precipitated with isopropanol (Van Soolingen et al., 1999). Purified DNA was dissolved in 20-50μl of TE buffer (10mM TrisHCl, 1mM EDTA [pH 8]). DNA isolated from MT14323, H37Rv and *Mycobacterium bovis* BCG strains was used for controls. DNA concentration method (Boom et al. 1990) was used for extraction of DNA from several clinical specimens (CSF and bronchial washings).

3.1.2 Amplification of fragments of *M.tuberculosis* genes associated with the first-line drug resistance.

The information about sets of primers used for particular gene analysis, gene accession number, the fragment length and the temperature of annealing (T_{an}) are summarised in Table 1.

10-20ng of culture-isolated *M.tuberculosis* DNA diluted in TE buffer or 10μl of prepared clinical specimen was added to the standard PCR mix to a final volume 50μl. The number of amplification cycles was 30-35 for culture-isolated DNA and 40 for uncultivated DNA. In each set of reactions one negative and one positive control (from a drug susceptible strain of *Mycobacterium bovis* BCG) was included. PCR products were analyzed in 1.2-% or 2.0% agarose gels depending of fragment length.

3.1.3. Analysis of the *rpoB* gene (RIF resistance) by the "INNO-LiPA ™ Rif" kit.

Commercial kit INNO-LiPA ™ Rif (*Innogenetics N.V*, Belgium) was used for the detection of *rpoB* gene mutations. Ten μl of each PCR product obtained with biotinylated primers, was used for the INNO-LiPA hybridization, performed according to manufacturer's instructions. Each INNO-LiPA strip contains one probe specific for MTC, five partially overlapping wild-type probes and four probes specific for the most common *rpoB* mutations within the 509-534 amino acid region - D516V, H526Y, H526D and S531L (*Innogenetics N.V*, Belgium). Manual sequencing of *rpoB* gene was performed using Cycle Reader™ DNA sequencing kit (*Fermentas*, Lithuania). Radioactively labeled primers used for manual sequencing were identical to those used for the PCR amplification.

Table 1. The analysed *M.tuberculosis* genes and corresponding primers

Agent	Gene fragment, accession nr.	Set of primers, references	T_{an}
RIF	*rpoB* (256bp) NC000962	71.B - 5'-GGTCGGCATGTCGCGGATGG-3'* 72.B - 5'-GCACGTCGCGGACCTCCAGC-3'* (Rossau et al, 1997)	65°C
INH	*katG* (704bp) X68081	74-5'-CGGGATCCGCTGGAGCAGATGGGC-3' 75-5'-CGGAATTCCAGGGTGCGAATGACCT-3' (Dobner et al., 1997)	62°C
SM	*rpsL* (306bp), L08011; *rrs* (530 loop, 238bp) and *rrs* (912 reg., 240bp), X58890	SM1-5'-CCAACCATCCAGCAGCTGGT-3' and SM2- 5'-ATCCAGCGAACCGCGGATGA-3'; SM3-5'-GATGACGGCCTTCGGGTTGT-3' and SM4-5'-TCTAGCTGCCCGTATCGCC-3'; SM5-5'-GTAGTCCACGCCGTAAACGG-3' and SM6-5'-AGGCCACAAGGAACGCCTA-3' (Honore & Cole 1994; Kempsell et.al., 1992).	61°C
EMB	*EmbB* (103bp), Z80343	46-5'-CTGCTCTGGCATGTCAT-3' 47-5'-AGCGGAAATAGTTGGAC-3' (Ramaswamy at el., 2000)	40°C
PZA	*pncA* (720bp) U59967	P1-5'-GTCGGTCATGTTCGCGATCG-3' P6-5'-GCTTTGCGGCGAGCGCTCCA-3' Scorpio et al., 1997	60°C

* - biotinylated primers (with biotin at 5'end)

3.1.4. Single-stranded DNA conformation polymorphism (SSCP) analysis of the *rpoB* and *embB* genes

SSCP analysis is based on the ability of single stranded DNA to undergo a conformational change when a single nucleotide is altered. Such alterations or mutations then can be detected because of change of DNA mobility in polyacrylamide gel. SSCP analysis was used to analyse point mutations in the *rpoB* and *embB* genes, 256bp and 103bp fragments, respectively.

To achieve better SSCP results, the 256bp fragment of the *rpoB* gene was amplified with one biotinylated (reverse) primer 72.B for better strand separation. The biotinylated DNA strand was separated from the unbiotinylated by "Dynal" streptavidin magnetic beads using the single-stranded DNA purification kit (*Labsystems*, Finland).

After heating for 5 min at 96°C with an equal volume of loading buffer containing 95% formamide, the gene fragments were snap-cooled and immediately loaded onto the 6% polyacrylamide gel gel. The SSCP analysis was performed on a cooled gel plate (*BioRad*, USA). The separated DNA bands were visualized by ethidium bromide and by silver staining.

3.1.5. Analysis of the *katG* and the *rpsL* genes by endonuclease digestion

The *katG* 704bp fragment was analysed for mutations at codon 315 by restriction with endonuclease *AciI* (*New England BioLabs*, USA). *AciI* can be used for determining changes in codon 315. After two hours of incubation at 37°C with the 5U of *AciI* digestion products were separated on 6% PAAG.

306bp fragments of the *rpsL* gene from each of the SM resistant DNA isolates were digested with *MboII* endonuclease recognising changes in codon K43 for 15 min at 37°C and separated on 6% PAAG.

In each set of reaction, drug susceptible *Mycobacterium tuberculosis* sample, BCG and the Mt14323 strain were used as controls.

3.1.6. Automatic nucleotide sequencing

In order to confirm the results of INNO-LiPA, SSCP and endonuclease digestion methods, the relevant PCR products of all analysed genes were applied for automatic nucleotide sequencing. As mutations in *pncA* gene are usually dispersed, PZA resistant and PZA susceptible MDR *M.tubeculosis* isolates were analysed only by automatic sequencing of the 720bp fragment containing the promoter, *pncA* gene and downstream sequence. Also, fragments of the *rrs* gene encoding the 16S rRNA 530 loop and 912 region were analysed by nucleotide sequencing.

The purified PCR products (DNA purification kit, *BioRad*, USA) were applied for automatic nucleotide sequencing. Each of the DNA chains was amplified with fluorescence-labeled dideoxynucleotide terminators using the ABI PRISM® Big Due ™ Terminator Cycle Sequencing Ready Reaction Kit (*Applied Biosystems*, USA) and with the same primers as those that were used for the PCR amplification. The nucleotide sequences were read by the ABI PRISM 3100 DNA Analyser (Applied Biosystems, Inc., Foster City, Calif.). The data were assembled using Applied Biosystems software and nucleotide sequences were compared to the published sequences of the genes in GenBank (www.ncbi.nlm.nih.gov).

3.2. Detection of *M.tuberculosis* complex by IS-6110-based PCR

Since PCR-based methods of TB detection and genotyping are more rapid and sometimes, more precise, it was important to evaluate specificity and sensitivity of such methods. For that aim, prior to application of PCR–based methods like spoligotyping for MTC detection and genotyping directly on clinical material, we have evaluated a more simple, IS-6110-based PCR method, commonly used for MTC detection.

All specimens (bronchial washings, sputum, cerebrospinal fluid, urine, smears and stomach washings) were collected at Latvian Centre of Tuberculosis and Lung Diseases and processed by the standard *N*-acetyl-L-cysteine-NaOH procedure (Kubica et al. 1963), preparing it for LCx (Lygase Chain Reaction) assay. Ready DNA isolates for LCx reaction contained 0.5ml LCx buffer, 27mM $MgCl_2$, NaN_3 and glass beads. After the LCx analysis samples were directly applied to IS6110-PCR. DNA concentration method (Boom et al. 1990) was used for extraction of DNA from several smear-negative specimens of CSF and bronchial washings received in year 2001.

The 245 bp fragment of the IS6110 (from 633 to 877 nucleotide positions) was amplified using primers 5'-CGTGAGGGCATCGAGGTGGC-3' and 5'-GCGTAGGCGTCGGTCACAAA-3' (Hermans et al. 1990). Five µl of DNA isolate in LCx buffer was added to the PCR mixture and amplified according to optimised cycling protocol on the "Progene" cycler (Techne, Cambridge, England).

PCR products were analyzed by electrophoresis in 2 % agarose gel (BioRAD, Hercules, USA) and visualized with ethydium bromide. Positive PCR signal indicated

the presence of MTC genome. In each set of reactions, one negative control and one positive control (DNA from *M. bovis BCG* vaccine strain) were included. Specimens with negative amplification results were repeated with an external addition of control DNA from *M. bovis BCG* strain in order to evaluate the presence of PCR inhibitor.

3.3. Spoligotyping method

Spoligotyping (spacer oligonucleotide typing) method, which is one of the more effective and rapid strategy alternative to IS6110-RFLP for detection and simultaneous typing of *M. tuberculosis* clinical samples. It can be regarded as the golden standard for the identification of strains of *M. tuberculosis*, particularly, for investigation of various genotype groups like Beijing (Glynn et al., 2002). Spoligotyping is based on Reverse Line Blotting (RBL), or hybridization of the polymorphic Direct Repeat (DR) locus amplified products, with 43 spacer oligonucleotides covalently bound to a membrane. Hybridization of *M. tuberculosis* DNA to spacers 35 to 43 is 100% specific for the Beijing genotype. Additionally, spoligotyping can identify some species other than MTC. To compare with RFLP, spoligotyping analysis does not require cultured mycobacteria, it is enough with 10ng of isolate DNA to obtain epidemiologically comparable pattern.

Spoligotyping analysis was performed in the Reference Laboratory of Mycobacteriology at Statens Serum Institut, Copenhagen, Denmark. For spoligotyping, a 20-fold dilution of *M. tuberculosis* culture-isolated DNA was used for PCR and subsequent hybridization reactions as previously described. The H37Rv strain was used as a positive control. Two negative controls were included as well.

Later, spoligotyping was introduced in Biomedical Reasearch and Study Centre (Riga, Latvia) in order to detect and to genotype MTC bacteria on clinical samples. The DNA was isolated at STLDC by the QIA Amp DNA Mini kit ("Qiagen" GmbH, Germany) and 10 µl of isolate was applied to spoligotyping.

PCR. 50µl of the following reaction mixture was used for the PCR:

5µl of the target DNA dilution (10-20ng), 4µl of each primer Dra (biotinylated) and Drb (*Isogen Bioscience B.V.*, Utrecht, The Netherlands), 4µl deoxynucleotide triphosphate mixture (200mM of each dNTP), 5µl of PCR buffer and 0.2-0.4µl Taq+ polymerase. The cycling conditions (Perkin-Elmer PCR system, Norwalk, Conn.) were as follows: 3 min at 96°C, 30-40 cycles [1 min at 96°C, 1 min 55°C, 30 sec at 72°C] and finally, 5 min at 72°C.

Hybridization. The spoligomembrane (*Isogen Bioscience B.V.*, Utrecht, The Netherlands) was activated by washing it for 5 min in 250 ml 2×SSPE/0.1%SDS at 60°C. Afterwards the membrane was placed in a miniblotter apparatus in such a way that the slots were perpendicular to the line pattern of the applied spacers. 35 µl of the PCR products was added to 130-150µl of 2×SSPE/0.1%SDS, heated at denaturing temperature an snap-cooled for 10 min. The denatured product was pipetted to into the channels of miniblotter. The membrane was hybridized for 1 hour at 60°C. After aspiration of PCR products, the membrane was transferred to a hybridization tube and washed twice in 250ml of 2×SSPE/0.5%SDS 10 min at 60°C. The membrane was then incubated in 10ml 2×SSPE/0.5%SDS with 2.5µl of streptavidin-peroxidase conjugate (Boehringer) for 45 min at 42°C. After 10 min wash twice with 2×SSPE/0.5%SDS at 42°C and 5 min with 2×SSPE at room temperature, membrane

was incubated for 1 min in 20ml ECL detection liquid (ECL™ Direct System, Amersham). The membrane was covered by with a transparent plastic sheet and an autoradiograme (Hyperfilm-ECL, Amersham), exposed for 1 to 10 min and developed.

Analysis of results. Autoradiograms were scanned and analysed using the GelCompar software version 4.0 (Applied Maths, Kortritjk, Belgium), and similarity analysis were based on the Jaccard correlation coefficient (max. tolerance 2.0%) and the UPGMA clustering method. All spoligopatterns identified by the GelCompar software as more than 80% identical were controlled visually. A cluster was defined as two or more isolates having spoligopatterns that were 100% identical.

3.4. Detection of methicillin resistance in *Staphylococcus* spp. by amplification of *mecA* gene fragment

3.4.1. *Staphylococcus* spp. culturing and isolation of bacterial DNA

The collection and laboratory analysis of staphylococci strains was carried ot in HTO (Riga, Latvia). Staphylococcal cultures (*S.aureus, S. epidermidis, S. haemolyticus, S. hominis, S. capitis* and *S. warneri*) were isolated mainly from indwelling artificial devices, blood and abscesses and were collected in a HTO. The isolates were identified to a species level by conventional tests such as coagulase reaction, phosphatase activity, haemolysis, susceptibility to novobiocin, etc. and the automated BBL Crystal system (Becton-Dickinson).

Antimicrobial susceptibility was tested by the disk diffusion method according to the NCCLS guidelines using Mueller-Hinton agar (MHA, Oxoid, UK) against the following panel of antibiotics: penicillin, gentamicin, cefazolin, erythromycin, clindamycin, vancomycin, ciprofloxacin, trimethoprim-sulfamethoxazole. MET resistance was also tested by a commercial oxacillin screen plate (potency 1μg of oxacillin). Plates were incubated at 35°C for 24h when zones of inhibition were measured. Plates with *S.epidermidis* cultures were incubated for 48h. *S.aureus* strain ATCC 29213 was used as MET-susceptible control.

Staphylococcal DNA was extracted in Biomedical Research and Study Centre (Riga, Latvia) from the overnight cultures by the lysostaphin-CTAB method as described by Hookey et al. (1998). The DNA was diluted in sterile distilled water and applied to *mecA*-PCR. In addition to that, Slidex MRSA detection kit (*BioMerieux*, Lyon, France), was introduced to detect production of PBP2a encoded by the *mecA* gene. Also, some strains have been analysed by PFGE at Robert Koch Institute, (Wernigerode, Germany) in collaboration with research group of W.Witte to investigate epidemiological links between some patients.

3.4.2. PCR amplification and detection of the *mecA* gene

MecA-PCR was performed with the following primers, previously designed by Geha et al. (1994): *mec*A1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and *mec*A2 (5'- CCA ATT CCA CAT TGT TTC GGT CTA A). The PCR reagent mixture consisted of 200 μM concentrations of dNTPs, 10mM Tris (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, a 0.25 μM concentration of each primer, and 1.25U of *Taq* polymerase (*Fermentas*, Lithuania). DNA amplification thermal cycling profile was as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of amplification (94°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec), ending with the 72°C for 2 min. A positive result was indicated by the presence of the 310-bp

amplified DNA fragment revealed by electrophoresis on a 1.5% agarose gel. In order to ensure the accuracy of the PCR, each isolate was analysed by PCR twice. Each reaction set included MET-resistant strain DNA as positive control and MET-susceptible strain ATCC 29213 DNA as negative control.

In order to approve specificity of primers, an amplified 310bp fragment of the *mecA* gene was sequenced with the same primers as those used for the PCR. Automatic sequencing reaction and analysis of results was done as described above. The similarity of the fragment to the *S.aureus* SCC*mec* type element (Accession number AY271717) was examined.

4. RESULTS AND DISCUSSION

4.1. Final results of the study on mutations associated with the first-line drug resistance in *Mycobacterium tuberculosis*, examined using various methods and confirmed by automatic nucleotide sequencing (APPENDIXES 1, 3, 5 and 6)

This part of the study involved the total of 145 *M.tuberculosis* DNA samples (126 MDR and 19 drug susceptible) isolated in STLDC during the time period from 1999 to 2002 from Latvian pulmonary TB patients, one isolate from each. This set of isolates represented about 30% of all cultures obtained in STLDC during the period of study. Mutations were analysed in genes most frequently associated with resistance to the first-line anti-TB drugs: rifampin (*rpoB)* and isoniazid (*katG),* ethambutol (*embB*), streptomycin (*rpsL, rrs*) and pyrazinamide (*pncA*). A set 109 MDR and 19 drug susceptible *M. tuberculosis* DNA isolates, obtained in 1999-2001, was analysed for the presence of mutations in the *rpoB* and the *katG* genes. Subsequently, a set of 66 MDR isolates obtained in 2001-2002 (all of those were resistant to SM, 33 were resistant to EMB and 28 were resistant to PZA) was analysed for mutations in the *rpsL*, *rrs*, *embB* and *pncA* genes.

Different molecular screening methods such as SSCP (*rpoB* and *embB* genes) INNO-LIPA (*rpoB* gene), endonuclease digestion (*katG* and *rpsL* genes) and, finally, nucleotide sequencing analysis *(rrs, pncA* and all previously mentioned genes) were evaluated for the rapid detection of mutations in the amplified gene fragments. The summary of prevalent mutations in the analysed DNA from resistant isolates, as showed by sequencing, the percentage of mutated genotype cases and of wild type genotype cases (not mutation identified) is given in Table 1.

Table 1. Summary of the mutations in genes associated with the first-line drug resistance, detected in MDR *M.tuberculosis* isolates by nucleotide sequencing

Anti-TB agent	Gene	Mutated genotype (%)	Predominant mutations (%)	Wild type genotype (%)	No of resistant isolates
Rifampin	*RpoB*	93.6%	Ser531Leu –52.2% Asp516Val - 14.7%	6.4%	109
Isoniazid	*KatG*	99.1%	Ser315Thr – 99.1%	0.9%	109
Streptomycin	*RpsL* *Rrs*	together 85%	Lys43Arg - 61% 513.A→C, 516C→T 24%	together 15%	66
Ethambutol	*EmbB*	52%	Met306Val / Ile - 48.0%	48.0%	33
Pyrazinamide	*PncA*	82%	Thr76Pro - 18% Tyr103His - 18%	18%	28

The ***rpoB* gene (Appendix 1, 3)** fragment sequencing results were available for the 109 MDR and 19 drug susceptible *M. tuberculosis* isolates. In total, 16 variants in single and double codon mutations were found in 102 (93.6%) of 109 MDR isolates including the isolate with the combination of codon deletion and two codon mutations at positions 524-527 (see Table 1). The following *rpoB* gene mutations predominate: S531L in 52.2% and D516V in 14.7% of the 109 isolates. Altogether, nine (8.2%) double mutations were detected; six of which were located at different pairs of codons.

Four of the *rpoB* gene double mutations had not been previously described (D516V plus P535S, Q510H plus H526Y, D516V plus K519T and R529P plus S531L). Several isolates (6.4%) did not contain mutations in the region examined. No mutations were detected in drug susceptible isolates.

In parallel, 34 of 109 MDR and 19 drug susceptible DNA isolates were analyzed by the reverse hybridization-based INNO-LiPA to determine RIF susceptibility. The results were in fine agreement with the *in vitro* susceptibility testing. One of the analysed MDR isolates, however, was a mixture of wild-type (WT) and mutant H526Y strains. Altogether, nine different patterns were found for the 34 RIF resistant samples. The most frequent *rpoB* gene mutations were S531L, D516V and H526D. Mutations were clearly demonstrated in 27 of the 34 RIF resistant samples by INNO-LiPA. The remaining seven RIF resistant samples with unclear INNO-LiPA patterns were further examined by manual nucleotide sequencing. Sequencing results showed the double mutations in five cases and mutation L533P in two cases. So, INNO-LiPA was effective in identifying the four most common *rpoB* mutations D516V, H526D, H526Y and S531L. Unfortunately, the method could not identify double mutations which are common in Latvian *M.tuberculosis* DNA isolates.

Additionally, the SSCP method was used to examine 23 of the same DNA isolates, eight from drug susceptible and 15 from RIF resistant *Mycobacterium tuberculosis* cultures. Drug resistant isolates with the most common *rpoB* gene mutations in Latvia were selected to evaluate the discriminative power of the SSCP method including five isolates of the S531L, three isolates of the D516V, two isolates of the H526D and five isolates of the D516Y plus P5335S substitutions. The SSCP analysis showed a strand mobility difference between the RIF susceptible and the RIF resistant samples, except for the five samples with the S531L mutation. Since S531L is the most common *rpoB* mutation in our isolates, application of the SSCP method for rapid *rpoB* screening for RIF susceptibility can not be recommended at least in Latvian MTC isolates.

The *rpoB* mutations located in codon Ser531 (up to 54%) and in H526 (up to 40%) of the RIF resistant isolates are the most frequently described woldwide (Traore et al., 2000; Fan et al., 2003, Yue et al., 2003). A comparison of these results shows that the Ser531Leu mutation predominates also in Latvia. In contrast, the frequency of mutations at codon 516 (25 of 109 (23%), involving both single and double mutations in our region is slightly higher than that of H526 (10 of 109 (9.2%)).

The ***katG* gene (Appendix 1,3)** fragment sequencing showed a single nucleotide change at codon 315 in 108 (99.1%) of the 109 MDR *M.tuberculosis* isolates (see Table 1). In 104 (95.4%) isolates it was a AGC →ACC change, and in 4 (3.7%) isolates it was a mixture of ACC and ACA. In all 108 strains, the nucleotide change from AGC to ACC or ACA resulted in the substitution of Serine (Ser) for Threonine (Thr). In 1 (0.9%) of the 109 isolates the 704bp long *katG* gene fragment did not contain a mutation. No mutations were found in the *katG* gene of 19 drug susceptible isolates.

Additionally, the restriction analysis was performed with *AciI* (NEB, USA) on 704bp fragment of the *katG* gene of 35 of 109 MDR *M.tuberculosis* isolates. The analysis accurately showed a nucleotide change at codon 315 in all cases.

A high percentage of Ser315Thr mutation among INH-resistant *M.tuberculosis* isolates was observed also in St-Petersburg area of Russia (92%), Lithuania (85.7%) and Netherlands (89%) (Marttila et al., 1998, Bakonyte et al., 2003, Van Doorn et al, 2003). However, a wider mutation spectrum was observed in the USA and other

European countries (Nachamkin et al., 1997, Fang et al., 1999). Van Soolingen et al (2000) discuss about codon 315 mutations being associated with the high level resistance to INH as a result of inappropriate therapy. Our results suggest that Serine substition at codon 315 of the *katG* gene is a characteristic of INH resistant strains and therefore can serve as a genetic marker for INH resistance in our region. Surprisingly, substitution at codon 315 was absent only in one INH resistant strain. This can be explained by the observation that there are more genes which can be responsible for IHN resistance – *inhA, oxyR-ahpC, kasA* (Wilson et al., 1996; Lee et al., 1999), *ndh* (Lee et al., 2001), recently described *mabA, Rv1772, fadE24, Rv1592, Rv0340*, and *iniBAC* genes (Ramaswamy et al., 2003). However, mutations in these genes are of lower frequency than in the *katG* gene. A more detailed analysis of the mentioned genes could reveal mutations additional to Ser315Thr at the *katG* gene.

The 306bp fragments of ***rpsL* gene (Appendix 5)** from 66 SM resistant DNA isolates were analysed by digestion with *MboII* endonuclease and by automatic sequencing. Cleavage of the 306bp fragment, observed in 26 (39%) isolates, indicates the presence of the wild type codon K43. In opposite, absence of cleavage in 40 (61%) of the isolates indicated a nucleotide change at codon K43. Nucleotide sequencing of the same 40 isolates confirmed nucleotide change from AAG to AGG (K→R) at position 43 (K43R) (see Table 1). Digestion with *MboII*, therefore, confirmed wild type nucleotide sequence at codon K43. 26 isolates with the wild type *rpsL* sequence were further examined for mutations in two regions of the ***rrs* gene** encoding for the 16S rRNA. Analysis of the 912 region showed the wild type sequence in all the isolates tested. In contrast, 16 or 24% of all tested 26 isolates showed substitutions in the 530 loop region at nucleotide positions 513A→C (n=11) and 516C→T (n=5). The remaining 10 of these isolates or 15% of the all isolates tested showed the wild type sequence in these regions of the *rpsL* and *rrs* genes. In summary, 56 (85%) of the tested 66 SM resistant isolates, had nucleotide substitutions either at amino acid position K43 of *rpsL* gene or at nucleotide positions 513A or 516C of the *rrs* gene.

A percentage of the *rpsL* (61%) and the *rrs* (24%) gene mutations in part correlates with the previous reports, where alterations in the *rpsL* gene have been detected in 52.0 to 56.8% and mutations in the *rrs* gene detected in 8.0-15.6% of the analysed SM-resistant isolates (Morris et al., 1995; Sreevatsan et al., 1996). Mutations in the *rrs* gene could confer SM resistance in the isolates lacking *rpsL* gene mutations. Mutations have been very rarely found in both genes simultaneously; possible interplay of mutations was proposed in several such examples (Meier et al., 1996; Sreevatsan et al., 1996). By Meier et al (1996), a high level of phenotypic resistance to SM *in vitro* (>500μg/ml) is associated with mutations in the *rpsL* gene, whereas mutations in the *rrs* gene occur only in isolates with the low-level resistance to SM (MICs, 10μg/ml). Interestingly, no one of our isolates showed mutation either at *rpsL* codon K88 or at *rrs* gene 912 region, both of those have been widely described by other investigators (Kempsell et al., 1992; Musser, 1995).

A slightly higher prevalence of K43R mutations may be explained by the high prevalence of Beijing genotype among Latvian MDR *M.tuberculosis* strains, as described later in Section 4.3. It is also based on the observation that in our study only strains with the Beijing genotype (confirmed by RFLP analysis and spoligotyping) had the K43R mutation, whereas isolates with non-Beijing genotype showed the wild type sequence in the analysed fragment of the *rpsL* gene (data not shown).

The single-stranded DNA conformation polymorphism (SSCP) method was applied to the ***embB* gene (Appendix 5)** fragment of the 33 EMB-resistant and 33 EMB-susceptible isolates. Using 103bp fragment SSCP analysis substitutions in *embB* gene were found in 15 (45%) and by sequencing in 17 (52%) of 33 EMB resistant isolates (Table 1). Surprisingly, SSCP revealed nucleotide mutation at codon Met306 in five (15%) of 33 *in vitro* EMB susceptible MDR isolates. Similar results have been reported from Northwestern Russia, where mutations at codon Met306 were found in 14 (48.3%) of 29 EMB-resistant strains and in 48 (31.2%) of 154 EMB susceptible strains (Mokrousov et al., 2002). Like in our study, the discrepancy between the results of phenotypic and genotypic EMB resistance tests was found in MDR strains only. Mokrousov hypothesise that there could be a drug target in tubercle bacilli, different from the one encoded by the *embB*; it could be affected by the EMB during the treatment with other first-line anti-TB drugs.

Compared to nucleotide sequencing, SSCP accurately showed mutations ATG →GTG (Met306Val) and ATG →ATC (Met306Ile) but not the change in codon 306 from ATG to ATA, also resulting in Ile. It is not surprising, that only 52% of the analysed isolates showed nucleotide alterations in the *embB* fragment, as at least 12 candidate genes were shown to confer EMB resistance (Ramaswamy et al., 2000). Therefore, nucleotide sequencing or SSCP analysis of alterations in the *embB* gene used to detect EMB susceptibility are of limited value and should not be recommended for rapid screening of clinical specimens.

In present study, 28 of 66 MDR *M.tuberculosis* isolates were resistant to PZA. Therefore, the ***pncA* gene (Appendix 6)** was analysed including upstream and downstream regions in 28 PZA-resistant and 10 PZA susceptible (control group) MDR isolates by the nucleotide sequencing. Point mutations in the *pncA* gene were found in ten different codons in 23 (82%) of 28 PZA resistant isolates (see Table 1). No mutations were detected in 10 of the PZA susceptible MDR *M.tuberculosis* isolates. All the mutations found lead to an amino acid change. In addition, one mutation resulted in the premature termination of pyrazinamidase synthesis. In PZA resistant isolates, codons T76 and Y103 were the most frequently affected (43%). One isolate showed a mixture of the wild type and a mutant sequence, with mutations at two codons, C14 and Y103. Since double mutations have not been previously described in the *pncA* gene, we propose that each of the mutations arose independently in this strain. Five (18%) of the isolates showed the wild type *pncA* gene sequence indicating another mechanism for PZA resistance. It might be a change in cell wall permeability or evolving pyrazinamide metabolism pathways, for example, due to mutations leading to the modification of pyrazinoic acid target (Huang, 2003). Such strains provide an opportunity for further studies of alternative PZA resistance mechanisms.

From the analysis of 28 PZA resistant Latvian *M.tuberculosis* isolates, mutations found at 7 of the 10 codons have been previously described in seven studies (Cheng et al., 2000; Lemaitre et al., 1999; Marttila et al., 1999; Mestdagh et al., 1999; Morlock et al, 2000; Scorpio et al., 1997; Sreevatsan et al., 1997). Mutations at three other codons (C14Y, D63G and V180F) are first reported in this study. A survey of data from seven previous studies from different parts of the world show that mutations in the *pncA* gene are predominantly found at codons R140 (n=21, 13%), L85 (n=15, 9.1%) and T47 (n=12, 7.3%). On the other hand, in the present study, the most commonly occurring mutations were found at codons T76 and Y103. Seven of the mutated codons (Q10, C14, P62, D63, C72, T76 and C138) detected in our study, were located in these hypothetically hot regions (Scorpio et al., 1997) while three other

codons (L85, Y103 and V180) were located outside these regions, partially confirming that hypothesis.

Since the loss of the pyrazinamidase activity leading to PZA resistance is mostly caused by mutations randomly dispersed in *pncA* gene and touch the coding region or gene expression regulating part, nucleotide sequencing remains the most appropriate tool for the analysis of the *pncA* gene at the molecular level.

To summarise, this study was focused, first of all, on *M. tuberculosis rpoB* and *katG* genes, as mutations in these genes mean resistance to RIF and IHN, e.i. multi-drug resistance. The results showed a good correlation of mutations in particular genes that in more than 96% was associated with *in vitro* determined resistance results. Therefore, molecular screening of these two genes can be recommended for reliable and rapid drug susceptibility testing in specimens prior culturing. The remaining isolates with wild type sequence should be evaluated according to results of *in vitro* susceptibility testing. Mutations in the *rpsL/rrs, embB* and *pncA* genes, associated with resistance to SM, EMB and PZA, respectively, were found at lower frequency, especially in *embB*, than in *rpoB* and *katG* genes. That fact can be explained by the additional mechanisms of resistance, associated with other candidate genes, not involved in this study, or such as a change in membrane penetration or drug efflux. Therefore, rapid screening of the mentioned genes may be of lower clinical value, except for special cases when the presence of point mutation can be employed as an obvious marker of drug resistance. However, that way susceptibility results will be unclear for the remaining *M.tuberculosis* isolates with wild type sequences. Of course, an extended study of other candidate genes could solve this problem.

In general, the proportion of mutations found in the analysed genes correlates with the previous findings. However, some exceptions took place. For example, in the *katG* gene, codon 315 was affected in all studied Latvian INH-resistant *M.tuberculosis* isolates, except one. That demonstrates the importance of codon 315 in development of INH-resistance in Latvia. Not surprisingly, three novel mutations have been shown in the *pncA* gene, as mutations have a disseminated character in that gene. Some genes (*rpoB, pncA*) had mutations at already known codons at higher frequency when reported from other studies. Particularly, higher occurrence of double mutations has been shown in the *rpoB* gene including four double mutations not previously described. This fact, altogether with high prevalence of mutations at particular codons, may be explained by the history of chemotherapy in Latvia (like INH or RIF monotherapy regimes in past), as well as by ongoing transmission of particular *M.tuberculosis* strains. However, it is obvious that in genes like *katG*, *rpsL* and *embB*, where mutations were shown to affect a single codon only, particular codon mutations could be selected as more effective to withstand antibacterial pressure and, at the same time, not to be fatal for bacteria. A hypothetical effect of some mutations on mycobacterial virulence will be discussed later, proceeding from spoligotyping results.

Various molecular methods, commercial INNO-LIPA kit (*rpoB* gene mutations), SSCP (*rpoB* and *embB* genes) and endonuclease digestion (*katG* and *rpsL* genes) analyses have been evaluated for rapid detection of mutations. Nucleotide sequencing was used to confirm the obtained results. So, INNO-LiPA was accurate for the detection of most common *rpoB* gene mutations, but failed to reveal double mutations as well as unusual mutations. However, in the two last cases kit indicated on RIF-resistant genotype. SSCP method was not effective for the most common Leu531 mutation in the *rpoB* gene fragment; it was precise for the Met306Val/Ile alteration in the *embB*, but not for ATG306ATA change, also resulting in aminoacid Ile. Site-

specific endonuclease digestion reaction was shown as a reliable and rapid method when the location of mutation is well known, e.g. Ser315 in the *katG* gene or Lys43 in the *rpsL* gene. However, it is not effective in the case of multiple mutations or when mutation affects other locus. A dispersed nature of *pncA* gene mutations proved true selection of nucleotide sequencing as a reliable method to study PZA-resistance. To conclude, nucleotide sequencing, used for the identification of mutations, and evaluating the methods described above, was shown as the most precise. It was also rapid and less cumbersome since the automatic reader of sequencing results was applied.

4.2. Identification of *M.tuberculosis* complex by IS-6110-based PCR (APPENDIX 2)

In this study, 170 specimens obtained at STLDC during the time period from 1998 to 2000, were analysed by IS6110–PCR. IS6110–PCR revealed the highest number of MTC positive specimens (40.6 %) in comparison with commercial LCx assay (27.6 %) and with commonly used methods as bacteria growth in BACTEC system (20.6 %) and on Lowenstein-Jensen medium (15.3 %). The smear microscopy (bacterioscopy), a method reliable to detect MTC bacteria only if it is present in a high amount, was the least sensitive. This group of 13 BACTEC-positive specimens was also positive for MTC by all the remaining methods (100 % specificity for smear-positive specimens).

IS6110-PCR was the most sensitive method in comparison with LCx assay and with other clinical laboratory methods. Sensitivity and specificity of PCR was estimated on the basis of BACTEC results. Thus, 91 PCR negative results and 26 PCR positive results (together 68.8 %) were in accordance with BACTEC system, and were considered as true negative and true positive, respectively. In 44 (25.8%) cases PCR was positive instead of negative BACTEC results. 12 of 44 PCR-positive results correlated with LCx assay, but were discordant to the results of BACTEC.

Instead of it, nine (5.2 %) discrepant PCR-negative and BACTEC-positive results were observed, thus an estimated overall PCR sensitivity was 72 %. False positive results in LCx or BACTEC may be caused by laboratory contamination. False negative results may be explained by the absence of IS6110 fragment in the *M. tuberculosis* pathogen also.

As PCR is able to detect both dead and live MTC bacteria, some patients may remain PCR-positive for mycobacterial DNA several months after treatment. Therefore, 44 discrepant PCR-positive and BACTEC–negative samples were further evaluated in the context of the clinical findings.

Clinical material isolated from CSF was studied as well. Seven smear and culture-negative specimens of CSF showed negative results by both amplification methods (IS6110-PCR and LCx). The results correlated with the clinical findings. Several specimens were received from patients with suspicion of TB or of tuberculous meningitis, though having smear-negative results. The specimens were processed using Boom (Boom et. al. 1990) method to increase DNA concentration. In three cases (CSF-3024, CSF-3025 and L-202) our search results were in fine agreement with the clinical findings and were used for the elaboration of the therapeutic strategy. In one case (L-204), PCR methods showed a weak amplification signal and atypical genetic pattern in Mixed Linker PCR. The reliability of *rpoB* and *katG* gene was shown on several CSF specimens, however, it should be additionally evaluated in a larger scale study. However, according to our preliminary observations, PCR-based methods such

as INNO-LiPA and *rpoB/katG* gene sequencing are highly sensitive and specific. Particularly, INNO-LiPA is already implemented in laboratory practice in SCTLD to detect RIF-resistance on clinical specimens.

The amplification of nucleic acids gives a good chance of detecting MTC in smear negative samples. IS6110-PCR is a rapid and relatively simple method; PCR results are available in 1 or 2 days instead of several weeks needed to grow mycobacteria in BACTEC system. IS6110-PCR was shown to be able to detect mycobacterial genome in variable clinical material – sputum, bronchial lavage, CSF etc. The method correlated with several studies evaluating PCR for diagnosis of (mainly pulmonary) TB, which reported sensitivity and specificity ranging between 70-100 % when culture of MTC was taken as the golden standard (Forbes 1997). However, in practice, many problems occur due to inhibition or due to cross-contamination with the products from previous PCRs (Nordhoek et al., 1994).

4.3. Molecular typing of *M.tuberculosis* by spoligotyping (APPENDIX 3)

To evaluate the predominant genotype groups in Latvian MDR *M.tuberculosis* and to compare prevalent mutations in the *rpoB* and *katG* genes among the different genotype groups, 109 MDR isolates of *M. tuberculosis* were genotyped by the spoligotyping method. In addition to that, a set of 34 drug susceptible isolates were analysed by spoligotyping. To note, random selection of our samples reflected the rate of primary (33%) and relapsed (61,5%) MDR TB in general population with *M. tuberculosis* infection between 1999 and 2001. Selected cultures represented about 25% of all MDR cultures obtained annually at STLDC.

20 variants of spoligopatterns were distinguished on 109 MDR isolates. In total, 95 out of the 109 isolates were located in 6 different clusters having 2 to 63 isolates, of which the two largest clusters accounted for 63 and 19 isolates each. The remaining 14 isolates did not show clustering. 109 isolates could be subdivided into two main groups, one containing 63 and the other 46 isolates. The largest one, called "Beijing genotype" group was formed by 63 (58%) isolates, all showing Beijing-characteristic nine-spacer spoligopatterns SP1 (Appendix 3, Figures 1 and 2). Another main group called "non-Beijing" was formed by 46 isolates with 19 variants of spoligopatterns. Within this group, the second largest cluster after Beijing consisting of 19 isolates (SP12) appeared. All, except one, non-Beijing isolates shared >72% pattern similarity within the group. Genotyping of drug susceptible *M.tuberculosis* isolates was not the main aim of this study, however, it is important to note, that Beijing genotype was identified in 12 of 34 tested drug susceptible isolates, and the predominant spoligotype in that group was SP12 (Appendix 3, Figures 1 and 2).

In this study, the Beijing genotype of *M. tuberculosis* was not associated with the younger age of patients, as it was previously reported. The average age of Latvian patients hosting the Beijing genotype was 46 years; most (35.2%) of these patients being between 41-50 years of age. It is obvious that the distribution of age groups for the Beijing and non-Beijing genotypes follows the general trend of TB in Latvia, where the highest incidence is found among the population of 44-54 years of age (160 per 100.000 in 2000). The ratio of females to males in our study in both Beijing and non-Beijing groups was 1:3, which reflects the incidence of TB in females and males in the general population.

The *rpoB* and *katG* gene fragment sequencing results on MDR *M.tuberculosis* isolates were compared between Beijing and non-Beijing genotype groups. In both

genotype groups, the following *rpoB* gene mutations predominate: Ser531Leu in 52.2% and Asp516Val in 14.7% of 109 isolates. 63 Beijing isolates showed a higher percentage of double mutations (eight, 12.7%) in comparison to the group of 46 non-Beijing isolates (one, 2.2%) ($P<0.05$). Notably, IS6110-RFLP analysis showed different patterns for isolates with the same location of double mutations (data not shown). All isolates with the Beijing genotype had the same the *katG* gene substitution: Ser315Thr.

In addition, a genetic polymorphism at codon 463 of the *katG* gene was detected. All 63 isolates comprising in the Beijing group had Leucine (Leu) (CTG) at position 463 of the 704bp fragment of the *katG* gene, whereas 45 of 46 isolates from the non-Beijing group showed Arg (CGG) at position 463. That mutation is not associated with INH resistance, but together with neutral polymorphism present at codon 95 of the *gyrA* (a subunit of DNA gyrase) gene can be used to assign strains of *M.tuberculosis* to three phylogenetic groups, as shown by Sreevatsan et al (1997). Therefore, all our isolates of the Beijing genotype having Leu at codon 463 could be assigned to the phylogenetic Group 1. The remaining strains that constituted the non-Beijing group with Arg at codon 463 could be assigned either to Group 2 or to Group 3 on the basis of polymorphisms at codon 95 of the *gyrA* gene.

So, the spoligotyping method correctly identified the major genotype families in Latvian MDR *M.tuberculosis* isolates, as compared with IS6110-RFLP results obtained from the same isolates in our laboratory, Biomedical Research and Study Centre (data not shown). The prevalence of a particular genotype group, named Beijing, in MDR isolates can be explained by the active transmission of TB in Latvia as well as by more virulent nature of pathogens from that genotype group. Since the prevalence of Beijing genotype was identified, spoligotyping results were further used for comparison of mutations in the *rpoB* and in *katG* gene between Beijing and non-Beijing genotype groups. In this study, a possible impact of particular codon mutations on virulence was evaluated for Beijing genotype, which was shown to be more able to spread (Bifani et al, 2002; Glynn et al., 2002). However, it was recently shown that Beijing genotype strains do not have ability to gain drug resistance more rapidly than other genotype strains (Werngren & Hoffner, 2003). In the present study, a comparison of two genotype groups showed that there is no difference in the *rpoB* and *katG* gene mutations. However, Beijing type isolates showed a higher prevalence of double mutations than non-Beijing isolates. It is not known yet, how long Beijing genotype has been present in Latvia, but the occurrence of double mutations in *M.tuberculosis* isolates of that genotype may be commented only by the prolonged exposure to RIF chemotherapy. In the present study, spoligotyping was mainly done on culture-isolated DNA. However, it has been already introduced in our laboratory on clinical specimens as reliable method for simultaneous MTC detection and typing.

4.4. Detection of methicillin resistance by *mecA* gene PCR (APPENDIX 4)

Altogether, 102 staphylococcal cultures isolated in HTO from 2000 to 2003 were analysed for the presence of the *mec*A gene. Among them, there were 36 coagulase-positive *S.aureus* (28 MRSA and eight MSSA) and 66 CoNS, including 65 MET-resistant isolates (44 *S. epidermidis*, 15 *S. haemolyticus*, one *S. hominis*, one *S. capitis* and one *S. warneri)* and one MET-susceptible *S.epidermidis* isolate.

Nucleotide sequencing identified the nucleotide sequence of the 310bp *mecA* PCR fragment as a part of the *S.aureus* SCC*mec* type element, containing *mecA* gene. According to PCR results, all phenotypically Met-resistant 28 *S. aureus* and 65 CoNS showed the presence of the 310-bp fragment of the *mec*A gene, thereby confirming MET resistance (the method's sensitivity = 100%). For 8 of the 9 control strains, respectively, phenotypically MET-susceptible eight MSSA and one *S.epidermidis*, the PCR response was negative – the *mec*A gene was absent. One MET-susceptible *S. epidermidis* isolate, exhibiting oxacillin MIC 1 μg/ml, proved to possess the *mec*A gene and should be recognized as methicillin-resistant. That fact could be explained by higher sensitivity of the PCR method in comparison with standard methods, as PCR allows "to touch" bacteria at the genome level. Therefore, the discordance of PCR and disk diffusion methods could be caused by the heteroresistant nature of the staphylococci or by absence of *mecA* gene expression on phenotype level (Berger-Bachi, 1997; Frebourg et al., 1998). Our results correlated well with the previous studies, where detection of *mecA* gene was shown as more reliable than the disk diffusion method, agar screen plate and some commercial MRSA tests (Louie et al., 2000; Ferreira et al., 2003; Kipp et al., 2004).

There is no doubt that at least two methods should be applied in parallel for the detection of MET resistance. In addition to oxacillin disk diffusion method, MET resistance in *Staphylococci* can be detected by two gold standard methods recommended by the NCCLS, namely oxacillin screening plate test and detection of the *mec*A gene by PCR (Goettsch et al., 2000). At the same time, *mecA*-PCR may be especially useful to reveal heteroresistant or borederline resistant strains, which appear phenotypically MET-susceptible.

Mechanisms of drug resistance in *S.aureus* and CoNS raise special interest due to occurrence of MET-resistant strains in hospitals, causing nosocomial and community-acquired infections. In comparison with *M.tuberculosis*, staphylococci have various ways to gain drug resistance. Particularly, the mechanism of resistance to β-lactams in staphylococci provides more simple molecular detection in comparison with the methods required for the detection of mutations associated with drug resistance in *M.tuberculosis*. This study showed high sensitivity of *mecA*-PCR detection, and it is already implemented in routine laboratory practice for the detection of MET-resistance in both coagulase-positive and –negative staphyloccci.

Transposon and plasmid mediated horizontal transfer of drug resistance gene remains predominant in many bacteria. However, resistance of staphylococci to several antibacterials, e.g., rifampin and fluoroquinolones occurs due to chromosomal gene mutations and will require other detection methods.

CONCLUSIONS

1) Molecular mechanisms of *M.tuberculosis* resistance to the first-line anti–tubercular drugs (RIF, INH, EMB, PZA and SM) were studied. Resistance-associated mutations were characterised by several methods in the *rpoB, katG, embB, pncA, rrs* and *rpsL* genes of the Latvian MDR *M.tuberculosis* DNA isolates. Novel mutations were found in the *pncA* and *rpoB* (double mutations) genes. Most of the examined molecular methods were shown reliable and contributing to the further use in clinical laboratories.
2) The high level of double mutations was detected in the *rpoB* gene (12.7%) among RIF-resistant isolates, possibly, indicating the consequences of the prolonged monodrug chemotherapy and different evolutional pathways of MDR strains in Latvia.
3) A single point substitution Ser315Thr in the *katG* gene (99.1%) was found to be typical for Latvian MDR isolates, therefore, it can be used together with *rpoB* mutations as a local molecular marker of MDR resistance.
4) The molecular analysis of PZA resistance is of special clinical value taking into account problems of the classical susceptibility testing. For that aim, nucleotide sequence analysis of the *pncA* gene could be recommended, being aware on the fact that in 12% of PZA-resistant *M.tuberculosis* isolates mutations were not found.
5) The low percentage of mutations detected in the *embB* gene should contribute to the development of EMB-resistance determining methods based on simultaneous screening of various associated genes, e.g. macroarrays.
6) The prevalence of Beijing genotype (58%) among Latvian MDR *M.tuberculosis* isolates from primary and relapsed TB cases was shown. The prevalence occurred, more likely, due to recent transmission, therefore, partially confirming the hypothesis about higher virulence of Beijing genotype. This underlines the need for rapid detection and typing of MTC bacteria from uncultured specimens, e.g., by spoligotyping.
7) The mechanism of drug resistance different from *M.tuberculosis* was studied in the genus *Staphylococcus*. Chromosome-integrated *mecA* gene, determining the resistance to methicillin and other β-lactams, was detected by the PCR. *mecA*-PCR showed high specificity and could be recommended as confirmatory analysis in addition to the standard laboratory methods. Further understanding of the pathways of molecular evolution of particular methicillin-resistant strains requires more thorough studies including genotyping and evaluation of epidemiological links.

ACKNOWLEDGEMENTS

I am deeply grateful to my first teachers Valdis Berzins, Elita Avota and Jurij Vyshnevsky, who introduced me to the field of molecular biology science.

I wish to express my gratitude to Professor Viesturs Baumanis, who endorsed me to start the studies on mycobacteria and helped a lot with his creative mind and huge experience. The most intensive and productive research was done in Laboratory of Molecular Microbiology, Biomedical Research and Study Centre. This could not be possible without help and support of the people working here: Inta Jansone, Liana Pliss, Valentina Capligina and many others, who sheared the experience during my studies.

I did my research on staphylococci in collaboration with Hospital of Traumatology and Orthopedy (Riga, Latvia), laboratory of Microbiology, leaded by Professor Aija Zilevica. I wish to acknowledge Aija Zilevica for giving me opportunity to uptake this significant study and for her excellent coordination of the project. At the same time, I am grateful to the people from this laboratory for providing me culture samples. I am also indebted to Professor Wolfgang Witte and co-workers from Robert Koch Institute (Wernigerode, Germany) for opportunity to learn staphylococci typing methods.

I partially did my research in Reference Laboratory of Mycobacteriology, Statens Serum Institut (Copenhagen, Denmark), and I would like to acknowledge professor Eskild Petersen, Vibeke Ostergaard-Thomsen, Troels Lillebaek, Isik S.-Johansen and the laboratory staff. In particular, I want to mention technical personnel Pia, Jette and Sushada. Indeed, I got an excellent experience stimulating my scientific growth through the time of my staying in your laboratory.

This research would not be possible without participation of colleagues from State Tuberculosis and Lung Disease Centre, L.Broka, A.Nodieva, G.Skenders and O.Marga, who are also acknowledged.

I was able to meet outstanding scientists and to enjoy thoughtful discussions through the meetings and courses organised by the European Society of Mycobacteriology, Institute Pasteur (Veronique Vincent) and Federation of European Biochemical Societies.

And, finally, I wish to thank my family who stood by and, especially, my husband Alex, for his support and for his excellent technical help in preparing posters.

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APPENDIXES 1- 6 (original papers)

APPENDIX I

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2002, p. 3789–3792
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.10.3789–3792.2002

Vol. 40, No. 10

NOTES

Mutations in the *rpoB* and *katG* Genes Leading to Drug Resistance in *Mycobacterium tuberculosis* in Latvia

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Received 29 March 2002/Returned for modification 21 June 2002/Accepted 12 July 2002

To characterize the genetic basis of drug resistance in *Mycobacterium tuberculosis* in Latvia, mutations involved in rifampin (*rpoB* gene) and isoniazid (*katG* gene) resistance in DNA from 19 drug-susceptible and 51 multidrug-resistant *M. tuberculosis* complex isolates were analyzed. The most frequent *rpoB* gene mutations found by the Line Probe assay were the S531L (14 of 34 isolates), D516V (7 of 34), H526D (4 of 34), and D516Y plus P535S (4 of 34) mutations. Direct sequencing of seven isolates with unclear results from Line Probe assay showed the presence of the L533P mutation and the Q510H plus H526Y (1 of 34) and D516V plus P535S (4 of 34) double mutations, neither of which has been described previously. Single-strand conformation polymorphism analysis showed strand mobility differences between the rifampin-susceptible and -resistant samples for the D516V, H526D, and D516Y plus P535S mutations but not for the S531L mutation. Nucleotide substitution at codon 315 (AGC→ACC) of the *katG* gene was found in 48 of 51 multidrug-resistant samples by sequencing. Furthermore, *katG* gene restriction fragment length polymorphism analysis with endonuclease *Aci*I confirmed the nucleotide change in codon 315.

The incidence of tuberculosis (TB) in Latvia, as in the rest of the world, has followed the same increasing trend (3). From 1991 to 1998, the incidence of TB in Latvia increased, reaching a rate of 74 per 100,000 inhabitants. Since then, it has remained at about this level. At the same time, drug resistance among TB patients in Latvia seems to be the highest in the world (12), with primary multidrug resistance found in 8.6% of new cases and in 34.5% of all TB patients in the year 2000. In comparison, the average primary rate of multidrug resistance in Europe as a whole is only 4%. Mutations in the 81-bp hypervariable region of the *rpoB* gene encoding the RNA polymerase β-subunit are the cause of rifampin (RIF) resistance in 96% of the cases. Among these, missense mutations in codons S531, H526, and D516 are the most frequent (11, 19). Isoniazid (INH) is most often used for TB treatment because of the very high sensitivity of *Mycobacterium tuberculosis* to this drug (2). In 60 to 70% of the cases, INH resistance is associated with mutations in the catalase-peroxidase-coding *katG* gene at codon 315 (Ser→ Thr) (7). In this study, we characterized the *rpoB* and *katG* gene mutations that are dominant in the Latvian isolates of multidrug-resistant (MDR) *M. tuberculosis* (16) by commercial Line Probe assay (LiPA), direct sequencing of PCR products, and single-strand conformation polymorphism (SSCP) and PCR-restriction fragment length polymorphism analyses. We suggest that this approach using molecular markers could be useful for predicting multidrug resistance in *M. tuberculosis*.

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Seventy patients from different regions of Latvia admitted to the State Centre for Tuberculosis and Lung Diseases between 1999 and 2000 with clinical symptoms of lung or renal tuberculosis or tuberculous meningitis were included in this study. Diagnosis was confirmed by microscopy and by culturing (18). Patients with a primary infection were preferably selected. They were typical of TB patients in Latvia in that they ranged in age from 25 to 60 years and in that two-thirds of them were male. Cultures of the *M. tuberculosis* complex were grown on Löwenstein-Jensen medium for 4 to 6 weeks. Drug susceptibility was determined by using the absolute concentration method on slants with the H37Rv strain of *M. tuberculosis* as the positive control and by using the BACTEC system (6, 13). With the absolute concentration method, resistance was defined as growth on solid media containing graded concentrations of drugs with more than 20 CFU at a specific drug concentration. The breakpoints for INH were 0.2 and 2.0 μg/ml on Löwenstein-Jensen medium and 0.1 μg/ml on the BACTEC system; for RIF, they were 40.0 μg/ml on Löwenstein-Jensen medium and 2.0 μg/ml on the BACTEC system. Of the 70 strains examined, 19 were drug susceptible and 51 were MDR, i.e., resistant to RIF and INH at least. Due to the small amount of DNA from some of the isolates, it was not possible to perform all of the analyses on all 70 isolates. Therefore, only 19 drug-susceptible and 34 MDR *M. tuberculosis* isolates were analyzed by LiPA. All 51 MDR isolates, however, were analyzed for the *katG* gene mutations by nucleotide sequencing. High-molecular-weight genomic DNA was isolated from the 70 mycobacterial cultures by the lysozyme/proteinase K cetyltrimethylammonium bromide procedure and precipitated with isopropanol (20). Purified DNA was dissolved in 20 to 50 μl of TE buffer (10 mM Tris HCl–1 mM EDTA [pH 8]).

DNA isolated from *M. tuberculosis* MT14323 and *Mycobacterium bovis* BCG strains was used for controls.

A commercial INNO-LiPA Rif kit (Innogenetics NV, Ghent, Belgium) was used for the detection of *rpoB* gene mutations. First, a 256-bp fragment of the gene was amplified with biotinylated primers 71.B (5′-GGTCGGCATGTCGCGGATGG-3′) and 72.B (5′-GCACGTCGCGGACCTCCAGC-3′) flanking the 81-bp region of the *rpoB* gene (14). Ten to twenty nanograms of DNA diluted in TE buffer was added to the PCR mixture to a final volume of 50 μl [containing PCR buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 200 μM concentrations of each dNTP, and 1 U of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania)]. Ten picomoles of each primer was used for one reaction mixture. The optimized cycling protocol was used in a Progene thermal cycler (Techne, Cambridge, England). PCR products were analyzed in 1.2% agarose gel. In each set of reactions, one negative control and one positive control (from a sensitive strain of *M. bovis* BCG) were included. Ten microliters of each PCR product was used for the LiPA hybridization, which was performed according to the manufacturer's instructions. Each of the LiPA strips used contained one probe specific for the *M. tuberculosis* complex, five partially overlapping wild-type probes, and four probes specific for the most common *rpoB* mutations within the 509- to 534-amino-acid region (D516V, H526Y, H526D, and S531L) (Innogenetics NV). Manual sequencing of the *rpoB* gene was performed by using a Cycle Reader DNA sequencing kit (Fermentas). Radioactively labeled primers used for manual sequencing were identical to those used for the PCR amplification.

SSCP analysis is based on the ability of single-stranded DNA to undergo a conformational change when a single nucleotide is altered. Such alterations or mutations can then be detected because of the change in DNA mobility in polyacrylamide gel. Here, the 256-bp *rpoB* fragment was used for SSCP analysis. For better strand separation, we used one biotinylated (reverse) primer, 72.B (17). The biotinylated DNA strand was separated from the unbiotinylated strand by Dynal streptavidin magnetic beads by using the single-stranded DNA purification kit (Labsystems, Helsinki, Finland). In brief, 80 μl of the beads, suspended in 40 μl of binding buffer, was mixed with an equal volume of the PCR product at room temperature for 15 min. After washing the beads with 80 μl of washing buffer, the bound DNA was denatured and incubated for 10 min at room temperature in 80 μl of 0.1 M NaOH. The alkaline solution containing the unbiotinylated strand was aspirated and used for SSCP analysis. The beads with the bound biotinylated strand were washed once with washing buffer and then suspended in 20 μl of concentrating solution. After being heated for 5 min at 96°C with an equal volume of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, and 95% formamide), the biotinylated and unbiotinylated DNA samples were snap-cooled and immediately loaded onto the gel. The SSCP analysis of DNA fragments was performed in 6% polyacrylamide gel containing 5% glycerin at 35 mA and 500 V on a 17- by 28-cm gel plate cooled to 5°C (Bio-Rad, Hercules, Calif.). After 8 h of electrophoresis, the separated DNA bands were visualized by silver staining.

A 704-bp fragment of the *katG* gene (GenBank accession no. X68081) (23) was amplified by PCR using direct primer 74 (5′-CGGGATCCGCTGGAGCAGATGGGC-3′), targeting the 52 terminal nucleotides before codon 315, and reverse primer 75 (5′-CGGAATTCCAGGGTGCGAATGACCT-3′), positioned 158 nucleotides downstream from codon 463 (4). The 50 μl of the PCR mixture that was used contained PCR buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 200 μM concentrations of each dNTP, and 1 U of *Taq* DNA polymerase (Fermentas). A 12.5-pmol portion of each primer was used for one reaction mixture. The standard cycling protocol was applied in a Progene thermal cycler (Techne). Amplification products were visualized in 1.5% agarose gel, purified, and used for sequencing and for digestion reactions.

TABLE 1. Mutations in the *rpoB* gene of 34 RIF-resistant isolates

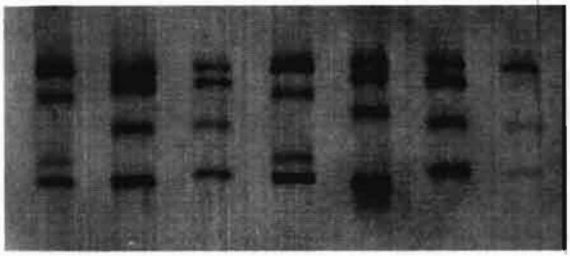
INNO-LiPA pattern	Mutation(s)	No. of isolates
ΔS5, R5, (plus R4a[a], R4b[a])	S531L	14
ΔS2, R2	D516V	7
ΔS4, R4b	H526D	4
ΔS2, ΔS5, S4[a]	D516Y, P535S[b]	3
ΔS2	D516Y, P535S[b]	1
ΔS5, R4a[a], R4b[a]	L533P[b]	2
R4b	Wild type plus H526D	1
ΔS1, ΔS4, R4a	Q510H, H526Y[b]	1
ΔS4, R4a	H526Y	1

[a] Zone is faint.
[b] Detected only by nucleotide sequencing.

Sequencing of the 704-bp *katG* fragment was performed with fluorescence-labeled dideoxynucleotide terminators by using an ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif.) and the same primers as those used for the PCR amplification. Nucleotide sequences were analyzed by an ABI PRISM 310 genetic analyzer (Applied Biosystems). PCR was performed on the relevant 704-bp *katG* fragment, followed by restriction with endonuclease *Aci*I (New England BioLabs, Beverly, Mass.). One INH-sensitive *M. tuberculosis* sample, BCG, and the MT14323 strain were used as controls. *Aci*I is a rare endonuclease that can recognize 5′-C′CGC-3′ and 5′-GCG′G-3′ sequences and can therefore be used for determining changes in codon 315. Four microliters of PCR product was added to the restriction mixture containing 1 μl of 10× restriction buffer 3 (New England BioLabs), 1 μl of *Aci*I endonuclease (5 U/μl), and 4 μl of H_2O. After 2 h of incubation at 37°C, the *Aci*I digestion products were separated in 6% polyacrylamide gel and stained with ethidium bromide.

Nineteen drug-susceptible and 34 MDR DNA isolates were analyzed by the reverse hybridization-based LiPA to determine RIF susceptibility. The remaining 17 RIF-resistant samples from a total of 51 MDR patients were not analyzed by LiPA because of insufficient amounts of DNA.

The LiPA confirmed that the 19 drug-susceptible isolates were RIF susceptible and that the 34 MDR isolates were RIF resistant. The presence of *M. tuberculosis* complex was confirmed by identification of the oligonucleotide zone specific for this complex. The results obtained with LiPA correlated completely with the in vitro susceptibility results obtained from the culturing. One of the analyzed isolates, however, was a mixture of wild-type and mutant (H526Y) strains. Nine different LiPA patterns were found for the 34 RIF-resistant samples (Table 1). The most frequent *rpoB* gene mutations were S531L (found



1 2 3 4 5 6 7

FIG. 1. SSCP analysis of the 256-nucleotide *rpoB* gene fragments from RIF-resistant *M. tuberculosis* isolates. Lanes: 1, 3, 4, 5, and 7, *rpoB* gene fragments with the D516Y plus P535S (lane 1), S531L (lanes 3 and 7), D516V (lane 4), and H526D (lane 5) mutations; 2 and 6, fragments from the RIF-sensitive H37Rv strain of *M. tuberculosis*.

in 14 of the 34 isolates), D516V (7 of 34), and H526D (4 of 34). One isolate showed the H526Y mutation in *rpoB*. Mutations were clearly demonstrated in 27 of the 34 RIF-resistant samples by LiPA. The remaining seven RIF-resistant samples with unclear LiPA patterns were examined further by manual nucleotide sequencing (Table 1). The sequencing results showed the D516V plus P535S double mutation in four of these isolates and the Q510H plus H526Y double mutation in another isolate; these double mutations have not been described previously (11). Altogether, five double mutations of the *rpoB* gene were found in the 34 MDR isolates examined. Sequencing results for the remaining two (of the 34) samples with unclear but similar LiPA patterns (ΔS5, R4a, and R4b; faint zones) showed the L533P mutation.

As an alternative to LiPA, the SSCP method was used to examine 23 of the DNA isolates, 8 from drug-susceptible and 15 from RIF-resistant *M. tuberculosis* cultures. Drug-resistant isolates with the most common *rpoB* gene mutations in Latvia, including five isolates with the S531L, three isolates with the D516V, two isolates with the H526D, and five isolates with the D516Y plus P5335S substitutions, were selected to evaluate the discriminative power of the SSCP method. Computer modeling (DNA Star) was used for the approximate calculation of the folding of a 256-nucleotide *rpoB* plus-strand DNA fragment at 37°C for both the wild-type and mutant variants. Three virtually designed folding models of this fragment containing the most frequently observed mutations worldwide, S531L (TCG→TTG), D516V (GAC→GTC), and H526D (CAC→GAC), were analyzed. Double mutations were not included in this modeling. Computer modeling altered the folding of single-stranded DNA in the fragments with the D516V and H526D mutations but not in the fragment with the S531L mutation, where the TCG→TTG change hypothetically should not influence loop interaction (data not shown). The SSCP analysis showed a strand mobility difference between the RIF-susceptible and RIF-resistant samples, except for the five samples with the S531L mutation (Fig. 1). Since S531L is the most common *rpoB* mutation in our isolates, the employment of the SSCP method for rapid *rpoB* screening for RIF susceptibility cannot be recommended, at least in Latvia.

Fifty-one MDR *M. tuberculosis* complex isolates showing INH resistance in addition to RIF resistance were analyzed for *katG* gene mutations. Since mutations in the *katG* gene have not been observed previously in IHN-susceptible *M. tuberculosis*, only MDR isolates were included in this study. Automatic sequencing of the 51 INH-resistant samples showed a nucleotide substitution at codon 315 in 48 of the 51 cases, of which 46 cases had a codon change from AGC to ACC and 2 cases had a mixture of AGC changes (to ACC and to ACA). In two other cases, a mixture of AGC and ACC from wild-type and resistant strains was found (Table 2). In all 48 cases, the nucleotide change to ACC or ACA resulted in a change of the amino acid from serine to threonine. In one case, no mutation was found in the analyzed fragment. Initially, two methods, automatic sequencing and *Aci*I restriction analysis, were used to examine the 704-bp *katG* gene fragment. Later, however, only sequencing, which was found to be more informative and precise for the detection of mutations, was done. Restriction analysis performed with *Aci*I on 35 samples showed a nucleotide change at codon 315 in all samples.

TABLE 2. Mutations in codon 315 of the *katG* gene detected by nucleotide sequencing of 51 INH-resistant samples

Substitution	Amino acid resulting from substitution	No. of samples
AGC→ACC	Ser→Thr	46
AGC→ACC and ACA mix	Ser→Thr	2
AGC→AGC and ACC mix	Ser (wild type) and Thr mix	2
None	Ser (wild type)	1

The aim of our study was to determine the frequency of mutations underlying drug resistance in *M. tuberculosis* isolates in Latvia, where a rapid increase in the numbers of TB cases and MDR *M. tuberculosis* isolates has been seen over the last 10 years. Previously, the most frequently described mutations in the *rpoB* gene were S531L (47% of isolates) and H526Y (12%) (19). A previous study has shown the presence of the S531L, H526Y, and H526D mutations in 44.5, 10, and 10% of RIF-resistant isolates, respectively (14). Comparison of these results with the results of our study shows that the S531L mutation also predominates in Latvia. In contrast with the results of the previous reports (14, 19), the frequency of the D516V mutation (7 of 34, or 20%) is now higher in Latvia. A high frequency of the D516V mutation (37.9%) has also been described recently in MDR *M. tuberculosis* isolates from East Hungary (1). Our findings correlate well with this study from a region where less common or novel mutations also occur more frequently. The LiPA was effective in identifying the four most common *rpoB* mutations: D516V, H526D, H526Y, and S531L. Unfortunately, the method did not identify the types of mutation in the five isolates with double mutations and in the two isolates with the rare L533P mutation, all of which required nucleotide sequencing for identification. Therefore, in a region with a high percentage of double mutations of the *rpoB* gene, LiPA results may be insufficient. Also, LiPA is relatively expensive (approximately 10 times more expensive than SSCP), and only RIF-resistant isolates were selected for LiPA toward the end of this study.

The high percentage of mutations at position 315 of the *katG* gene (48 of 51 isolates, or 91%) demonstrates the importance of this codon for the development of INH resistance in *M. tuberculosis* in Latvia. A high percentage of mutations

at codon 315 has also been observed in Russia, where 22 of 24 INH-resistant isolates carried a mutation at codon 315 (Ser→Thr), 1 had a mutation at codon 88 (Gly→Arg), and 1 had a mutation at codon 155 (Tyr→Ser) (10). According to another study, the Arg→Leu mutation at position 463 in the *katG* gene was found in 30 to 40% of the INH-resistant isolates (15). Further studies of the mutations at codon 315 seem to hold more promise for understanding INH resistance, since the mutation at codon 463 was also found in *M. bovis* BCG, a strain that is inherently INH sensitive. One MDR isolate showed a lack of mutations in the *katG* gene fragment analyzed here. According to previous studies, there are more genes responsible for INH resistance, such as the *inhA*, *ahpC*, and *kasA* genes (8, 22) as well as the recently described *ndh* gene (9). Therefore, mutations responsible for INH resistance in this isolate may be present in any of these genes. The restriction enzyme *Aci*I was used on the 704-kb PCR product and confirmed the high frequency of nucleotide change at codon 315 in the INH-resistant isolates. The AGC→ACC (Ser→Thr) nucleotide change is the mutation most often observed, but other substitutions at codon 315 [AGC→AAC (Ser→Asn), AGC→ATC (Ser→Ile), and AGC→CGC (Ser→Arg)] have been found in some studies (5). The AGC→CGC (Ser→Arg) substitution cannot be distinguished by *Aci*I restriction analysis. Therefore, nucleotide sequencing of the 704-kb *katG* gene fragment seems to be a more reliable method for detecting the gene responsible for INH resistance. Taking into account the high prevalence of substitutions at codon 315, we propose the use of a shorter *katG* fragment for the detection of resistance to INH. The high prevalence of the S315 mutation in the *katG* gene in INH-resistant isolates is clearly associated with multidrug resistance, since all of the isolates with this mutation were also found to be MDR in in vitro susceptibility tests. These data are consistent with the results of a study from The Netherlands where the S315 mutation was found in more than 50% of the INH-resistant isolates (21).

We speculate that one of the reasons for the high level of drug resistance in *M. tuberculosis* isolates in Latvia may be the selection of strains that develop drug resistance more rapidly. This may explain the high percentage of certain *katG* gene mutations usually associated with a high level of multidrug resistance in our samples. More-detailed molecular studies with strictly defined patient groups may further elucidate this hypothesis. Molecular markers are a valuable tool for predicting drug resistance in epidemiological studies. At present, unfortunately, most molecular typing and mutation detection methods can only be applied to cultivated bacteria [about 10^5 copies of the gene(s) are needed to obtain good results in these assays]. Development of methods for uncultivated clinical material will accelerate their introduction in clinical practice throughout the world.

This work was supported by grants from the European Community, INCO-Copernicus Project ERBIC15-CT98-0328 (Sven Hoffner, Coordinator), and the Council of Sciences of Latvia (program no. 12-9-1410.

We thank Ilva Pole for the DNA isolation and Liana Pliss for technical assistance. We are also grateful to Petras Stakenas and Daiva Bukonyte (Institute of Biotechnology, Vilnius, Lithuania) for advice and to Eskild Peterson (Statens Serum Institut, Copenhagen, Denmark) and Vitauts Kalnins (University of Toronto, Toronto, Canada) for their help in preparing the manuscript.

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APPENDIX 2

Acta Universitatis Latviensis, 2003, Vol. 662, pp. 17–24

Evaluation of the insertion-sequence-6110-based polymerase chain reaction for detection of pathogenic mycobacteria

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Abstract

The insertion sequence IS6110 is a mobile genetic element typical for *Mycobacterium tuberculosis* complex (MTC) bacteria. Due to its narrow host range, this insertion element can be used for rapid identification of pathogenic mycobacteria. The aim of this study was to evaluate the diagnostic value of the IS6110 element in comparison with common molecular and microbiological methods. A total of 170 clinical specimens from tuberculosis (TB) patients were tested for the presence of IS6110 by polymerase chain reaction (PCR). Detection of MTC by IS6110–PCR revealed the highest number of MTC positive specimens (40.6 %) in comparison to commercial LCx assay (27.6 %) and to the commonly used methods for estimating bacteria growth: BACTEC system (20.6 %) and on Lowenstein-Jensen medium (15.3 %). IS6110-PCR was shown to be a rapid and specific method for identification of the *Mycobacterium tuberculosis* complex. It was found to be especially useful for confirming diagnosis in cases of smear- and culture-negative results, when the clinical expansion of tuberculosis was obvious.

Key words: Identification, insertion sequence 6110, *Mycobacterium tuberculosis* complex, polymerase chain reaction.

Introduction

Mobile genetic elements like insertion sequences (IS) and transposons are found in bacteria, archaebacteria and eukaryotes. Insertion sequences are DNA fragments encoding their own ability to transpose within the genome. The function of most mobile genetic elements is unknown and they are usually considered to be genome parasites. In the genus *Mycobacterium*, there are three groups of IS, which are usually host specific. IS are between 880 bp and 2260 bp long and are found in one to more than 20 copies in the chromosome (Guilhot et. al. 1999). One of those, a 1361 bp long insertion sequence named IS6110, first described by Thierry et al (1990), is present in the *Mycobacterium tuberculosis* complex (MTC) only. MTC contains four genetically and serologically related pathogens causing disease in human and in cattle: *M. tuberculosis*, *M. bovis*, *M.*

africanum and *M. microti*. The majority of investigated MTC strains contained between 5 and 20 copies of IS6110 located in various places of the genome (van Soolingen et al. 1993).

Recent achievements in molecular microbiology such as complete genome sequencing of the *M. tuberculosis* H37Rv strain opened new perspectives in the study of mycobacteria (Cole et al. 1998). At the same time, a dramatic increase of tuberculosis (TB) incidence worldwide in the last decades induced an urgent need for new molecular markers enabling rapid detection of MTC, a mean causative agent for TB. It is known that smear examination may not be effective in non-pulmonary, pediatric or paucibacillary disease, or where HIV is prevalent (Rigouts, Portaels 2001). Recently developed molecular methods based on detection of mycobacterial proteins (protein antigen B), 16S rRNA or DNA fragments, are dissolving this problem. Of these methods, the amplification of insertion sequences is most commonly used. IS6110-based PCR enables detection of nucleic acids from MTC directly in clinical specimens: sputum, bronchial washings, urine etc. (Eisenach et al. 1988; Hance et al. 1989). IS6110-PCR is a more rapid, sensitive and specific in comparison to other methods routinely used in clinical laboratories; such as smear microscopy, and culturing on solid and on liquid media (American Thoracic Society Workshop 1997). Detection of MTC in cerebrospinal fluid (CSF) from patients suspected to have tuberculous meningitis is of great value since cultivation and microscopy may often fail to reveal a low amount of bacteria.

In this study, IS6110 was chosen due to several reasons. Firstly, a narrow host range of the IS6110 allows use of this element for precise identification of MTC. Secondly, it is usually present in more than five copies and can be used as a molecular marker for strain differentiation in epidemiological studies (van Soolingen et al. 1993). The aim of our study was to evaluate the sensitivity and specificity of the IS6110 amplification method on smear-negative samples, in comparison with smear microscopy and culturing on solid Lowenstein-Jensen medium. We expected a higher sensitivity of IS6110-PCR in comparison to commercially available systems for TB detection and MTC culturing, such as the Abbott LCx assay and BACTEC. LCx assay is a DNA-related TB diagnostic system based on protein antigen b gene fragment amplification (Andresen, Hansen 1989), but perhaps, is not as sensitive as IS6110-PCR since only one copy of the antigen b gene is present in the MTC genome. BACTEC is a precise radiometric system for mycobacteria growth detection, but it usually requires several weeks to grow a sufficient amount of bacteria.

Materials and methods

In total more than 250 specimens of bronchial washings (76 %), sputum (11 %), cerebrospinal fluid (4.3 %), urine (3.5 %), smear (2.6 %) and stomach washings (2.6 %) were obtained from Latvian patients admitted in 1998-1999 to the State Centre of Tuberculosis and Lung Diseases with suspected and confirmed TB. The patients had clinical symptoms of lung diseases or had suspicion of pulmonary/extrapulmonary tuberculosis shown by clinical and/or X-ray examination. Each patient was represented by one specimen or, in several cases, by various specimens from different locations. The specimens were directed for TB detection by the Abbott LCx (Ligase Chain Reaction) commercial kit (Abbott laboratories, Illinois, USA), mostly in cases when bacteria growth

was negative on the BACTEC system (Becton-Dickinson, Sparks, USA) or with repeated smear-negative bacterioscopy results. In our study, 157 smear-negative specimens were selected, allowing to test whether IS6110-PCR is more sensitive in comparison to the commonly used methods. A small control group of 13 specimens with smear-positive bacterioscopy results was included. The results of our study were interpreted in the context of the clinical findings, TB history and the efficiency of chemotherapy.

All specimens were collected and processed by the standard *N*-acetyl-L-cysteine-NaOH procedure (Kubica et al. 1963). Smears were analyzed by fluorescent microscopy and the corresponding samples were inoculated on solid (Lewenstein-Jensen) and liquid (BACTEC) mediums. The LCx assay was performed at the Centre of Tuberculosis in accordance with the manufacturer's recommendations (Ausina et al. 1997). DNA isolates for LCx reaction contained 0.5 ml LCx buffer, 27 mM $MgCl_2$, NaN_3 and glass beads. After the LCx analysis IS6110-PCR was directly applied to samples. The DNA concentration method (Boom et al. 1990) was used for extraction of DNA from several smear-negative specimens of CSF and bronchial washings received in year 2001.

The 245 bp fragment (from 633 to 877 nucleotide positions of the IS6110 sequence) was amplified in house using primers 5'-CGTGAGGGCATCGAGGTGGC-3' and 5'-GCGTAGGCGTCGGTCACAAA-3' (Hermans et al. 1990). Initially, the nested PCR method was used. At the first step, a 1224 bp fragment (from 47 to 1270 nucleotide positions) of IS6110 was amplified and was used as a reaction template at the second step, where a 245 bp fragment was produced. Later, the first step of nested PCR was omitted since it did not show any advantages and the 245 bp fragment was directly amplified from specimens. Five μl of DNA isolate in LCx buffer was added to the PCR mixture [containing PCR buffer with $(NH_4)_2SO_4$, 200 μM of each dNTPs, 10 pmoles of each primer and 1 U of Taq DNA Polymerase 9MBI Fermentas, Vilnius, Lithuania)] to a final volume of 25 μl. The optimized cycling protocol was used a ***Progene*** cycler (Techne, Cambridge, England) with initial denaturation at 95 °C for 2 min, followed by 40 cycles (95 °C for 15 s, 65 °C for 20 s, 72 °C for 1 min) and a final extension (72 °C for 10 min). PCR products were analyzed by electrophoresis in 2 % agarose gel (BioRAD, Hercules, USA) and were visualized with ethydium bromide. A positive PCR signal indicated the presence of the MTC genome. In each set of reactions, one negative control and one positive control (DNA from the *M. bovis* BCG vaccine strain) were included. The reaction specificity was confirmed by digestion with PvuII (MBI Fermentas, Vilnius, Lithuania) and by hybridization with an appropriate ^{32}S-labeled fragment. Testing was repeated on specimens with negative amplification results, with an external addition of control DNA from the *M. bovis* BCG strain in order to evaluate the presence of a PCR inhibitor.

Results

The detection limit of IS6110-PCR, estimated by amplification of serial dilutions of DNA from the *M. bovis* BCG strain containing only one copy of IS6110, was 5 fg per probe. This amount of DNA can be isolated from two bacterial cells, meaning that our PCR method is able to detect at least two bacteria, each containing one copy of IS6110. The fact that MTC usually contains more than one IS6110 copy increases the detection level of our in house method.

The comparative results of MTC detection in 170 specimens are shown in Table 1.

Table 1. Comparison of the in house IS6110-PCR method and TC clinical laboratory results obtained for 170 specimens. *, number of PCR false positive after adjustment with LCx results and samples suspected in cross contamination

Method used	MTC positive (total n=170)	Discrepancy (PCR-positive and 1-4 method-negative)
Bacterioscopy	13 (7.6 %)	56 (32.5 %)
Growth on Lowenstein-Jensen medium	26 (15.3 %)	39 (22.9 %)
Growth in BACTEC system	35 (20.6 %)	44 / 25* (25.8 % / 14.7 %*)
LCx assay	47 (27.6 %)	32 (18.8 %)
IS6110-PCR	69 (40.6 %)	-

Detection of MTC by IS6110-PCR revealed the highest number of MTC positive specimens (40.6 %) in comparison to LCx assay (27.6 %) and to the commonly used methods for estimation of bacteria growth: on BACTEC (20.6 %) and on Lowenstein-Jensen (15.3 %) mediums. Smear microscopy (bacterioscopy), which is reliable in detecting MTC bacteria only when present in high amounts, was the least sensitive with 13 (7.6 %) MTC positive specimens. This group of 13 specimens was MTC positive by all of the remaining methods (100 % specificity for smear-positive specimens).

IS6110-PCR was the most sensitive method in comparison to LCx assay and to other clinical laboratory methods. However, the lack of a "golden standard" for amplification methods does not exclude false positive results. Therefore, cultivation on BACTEC radiometric liquid system is still considered to be the most reliable tool for detection of MTC (Nordhoek et al. 1994). Sensitivity and specificity of PCR was estimated on the basis of BACTEC results (Elder et al. 1997). Thus, 91 PCR negative results and 26 PCR positive results (together 68.8 %) were in accordance with BACTEC system, and were considered as true negative and true positive, respectively. PCR gave positive results in 44 (25.8 %) cases. Of 44 PCR-positive results 12 were correlated with LCx assay results, but not with BACTEC results, a method internationally considered as a standard method for detection of *M. tuberculosis*. Therefore, these PCR results should be considered as false positive, and the overall specificity for IS6110-PCR estimated as 67.0 %. After charging of 32 PCR and LCx discrepant results plus seven suspected cross-contaminated samples, the total number of false positive results was 25 and the PCR specificity was 79.0 %. Nine (5.2 %) discrepant PCR-negative and BACTEC-positive results were observed, thus the estimated overall PCR sensitivity was 72 %. There were nine (5.2 %) discrepant PCR-negative and LCx-positive cases and four (2.3 %) discrepant PCR negative and Lowensten-Jensen culture-positive cases. False positive LCx or BACTEC results may have been caused by laboratory contamination. False negative results may be explained also by the absence of IS6110 fragment in the *M. tuberculosis* pathogen also. Inhibition of amplification reaction by remains of clumps, blood and urine present in a specimen is another probable cause for false negative PCR (Forbes 1997).

Clinical material isolated from CSF was studied as in this case there was an obvious requirement for rapid and precise diagnostics. In our study, seven smear and culture-negative CSF specimens showed negative results by both amplification methods (IS6110-

Table 2. Evaluation of PCR-based methods applied on clinical material isolated by Boom method. SCF, cerebrospinal fluid; L, bronchial washing; -, negative PCR signal; +, positive PCR signal, indicating the presence of MTC genome; ++, strong positive signal; +w, weak positive signal; WT, wild type (no resistance encoding mutation found). H37Rv strain was used as a PCR positive control. B and C types are different groups of genetic patterns, revealed by molecular typing of MTC bacteria

Clinical number	IS6110-PCR	Rifampin suscept. by *Inno-LiPA* kit	Rifampin suscept. by *rpoB* gene sequencing	Isoniazid suscept. by *katG* gene sequencing	Mixed linker PCR typing pattern	Evaluation based on PCR results	Clinical diagnosis
CSF 3024	+	WT	WT	WT	B type	Drug suscept. TB	Tuberculous meningitis
CSF 3025	++	WT	WT	WT	B type	Drug suscept. TB	Tuberculous meningitis
L-202	+	WT	WT	WT	C type	Drug suscept. TB	Lung TB
L- 204	+w	WT	WT	-	Atypical	False positive	Atypical pneumonia
H37Rv strain	++	WT	WT	WT	Typical	-	-

PCR and LCx). The results were correlated with the clinical findings. Later, several specimens were received from patients with suspicion of TB or tuberculous meningitis, although they had smear-negative results. The specimens were analyzed using the Boom (Boom et. al. 1990) method, by increasing the DNA concentration. All of these samples showed positive IS6110-PCR results, confirming the presence of MTC bacteria. In addition to IS6110-PCR, drug susceptibility molecular tests and PCR-based molecular typing were performed in the Biomedical Research and Study Centre. The results of PCR-based methods for MTC detection, molecular drug susceptibility testing and molecular typing of several specimens are summarized in Table 2. In three cases (CSF-3024, CSF-3025 and L-202), our results were in good agreement with the clinical findings and were used for elaboration of therapeutic strategy. In one case (L-204), the PCR methods showed a weak amplification signal and an atypical genetic pattern in Mixed Linker PCR. A suspicion of a false positive result was confirmed by the diagnosis of atypical pneumonia. Unfortunately, Abbott LCx assay was not applied to these specimens, and laboratory investigations, except as smear negative bacterioscopy, were not available. Anti-TB chemotherapy was begun for these drug-susceptible persons.

Interestingly, only two of these methods, IS6110-PCR and Mixed-Linker PCR, were initially designed for a low amount of DNA present in clinical material. The described molecular methods for drug susceptibility testing (Inno-LiPA kit, *rpoB* and

katG gene fragment sequencing) are usually applied for larger amounts of DNA isolated from bacterial culture. In most cases, a low content of DNA is an obstacle for further application of molecular methods in clinical material. In our study, these methods were shown to be sensitive enough to give correct results for clinical material when DNA was concentrated using the Boom method.

Discussion

Nucleic acid amplification gives the best chance of detecting MTC in smear negative samples. IS6110-PCR is rapid and relatively simple method; PCR results are available in 1 or 2 days instead of several weeks needed to growth mycobacteria in the BACTEC system. IS6110-PCR was shown to be able to detect a mycobacterial genome in variable clinical material – sputum, bronchial lavage, CSF etc. The results were comparable with other studies in which PCR was used for diagnosis of (mainly pulmonary) TB, where the sensitivity and specificity ranged between 70-100 % when a culture of MTC was taken as golden standard (Forbes 1997). However, in practice, many problems occur due to inhibition or due to cross-contamination with the products from previous PCRs (Nordhoek et al. 1994). We separated rooms and instruments used at each PCR step: PCR mixture preparation, sample addition and PCR product analysis. Also, specimens with negative PCR results were controlled for the presence of inhibitors. For this aim, PCR-negative specimens were repeatedly tested with a parallel addition of *M. bovis* DNA. Absence or weak amplification of *M. bovis* BCG DNA indicated the presence of inhibiting factor.

As PCR is able to detect both dead and live MTC bacteria, some patients may remain PCR-positive for mycobacterial DNA several months after treatment. Therefore, 44 discrepant PCR-positive and BACTEC-negative samples were evaluated in the context of the clinical findings. Clinical data was available for 38 patients. Fifteen (39.5 %) of them had active TB, confirmed clinically and by X-ray examination. Eight patients had already received a drug therapy course for a period from three days to several months and seven patients have not started treatment. Interestingly, only four of those patients were detected as MTC-positive by LCx assay. Eight (21 %) patients had inactive TB with after-effects, thus confirming that PCR can detect dead or dormant mycobacteria. In general, the clinical findings were in agreement with the shown presence of the MTC genome among 66 % of the false positive samples. Seven (18 %) specimens were, possibly, contaminated during the bronchoscopy procedure. Extrapolation of the PCR results for the remaining six (16 %) patients was problematic.

This study showed the advantages of PCR-based TB detection prior to mycobacteria culturing and admitting of chemotherapy. It is likely that, in the cases of smear positive results, there is no additional need in IS6110-PCR, and it is useful only to distinguish MTC from the other acid-fast bacteria. Therefore, IS6110-PCR can be recommended in cases of smear- or BACTEC-negative results. Invention of PCR-based detection methods may increase a number of recovered TB cases and may dramatically shorten the time required for TB diagnostics.

Acknowledgements

The present study was supported by a grant from the Science Council of Latvia (grant # 02.0011.15.1). We thank Prof. Olgerts Marga (Rīga Stradiņš University, Latvia) for his encouragement and help to initiate and to coordinate this study.

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Uz inserciju secības IS6110 balstīta polimerāzes ķēdes reakcija: lietojums patogēno mikobaktēriju noteikšanai

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Kopsavilkums

Insercijas sekvence IS6110 ir *Mycobacterium tuberculosis* kompleksam (MTC) raksturīgs mobilais ģenētiskais elements. Saimnieku loks šim insercijas elementam ir šaurs, un tādēļ to var lietot mikobaktēriju ātrai noteikšanai. Darba mērķis bija salīdzināt šā ģenētiskā elementa diagnostisko vērtību ar pazīstamām molekulārām un mikrobioloģiskām metodēm. Dažādus klīniskos paraugus no 170 tuberkulozes slimniekiem pārbaudīja uz IS6110 klātbūtni ar polimerāzes ķēdes reakciju (PCR). MTC noteikšana ar IS6110-PCR deva visaugstāko pozitīvo atbildi (40,6 %), salīdzinot ar LCx testu (27,6 %) un tādām noteikšanas metodēm kā baktēriju kultivēšana BACTEC sistēmā (20,6 %) vai uz Levenšteina-Jensena barotnes (15,3 %). Parādīts, ka IS6110-PCR ir ātra un specifiska MTC identifikācijas metode. Tai ir īpaša vērtība tajos gadījumos, kad ir nepieciešams apstiprināt mikobaktēriju klātbūtni, ja mikroskopiskā izmeklēšana un kultivēšana bijušas nesekmīgas.

APPENDIX 3

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Prevalence of Beijing genotype in Latvian multidrug-resistant *Mycobacterium tuberculosis* isolates

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SUMMARY

SETTING: Predominant genotypes of *Mycobacterium tuberculosis* include the Beijing family, which has caused large tuberculosis outbreaks and has been associated with increased virulence and multidrug resistance (MDR).

OBJECTIVE: To search for the Beijing genotype among Latvian MDR patients to characterize their DNA isolates at the molecular level.

DESIGN: 109 MDR isolates were spoligotyped and tested for gene mutations by automatic nucleotide sequencing.

RESULTS: Of the 109 isolates examined, 95 were located in six clusters of 2 to 63 isolates each. The 63 isolates in the largest cluster had an identical pattern corresponding to the Beijing genotype. The remaining isolates were of a non-Beijing genotype and formed another large group whose similarity ranged from 72% to 100%. Mutations in the *rpoB* and *katG* genes were compared in the Beijing and non-Beijing strains. In both groups, the *rpoB* gene mutations predominated in codons S531L (52.2%) and D516V (14.7%). Double mutations in the *rpoB* gene were observed in 8.2% of the isolates, most of them located among Beijing-type isolates. The *katG* gene mutation S315T (98.4%) was prevalent among all isolates.

CONCLUSION: Molecular analysis of MDR isolates of *M. tuberculosis* demonstrates that the Beijing genotype, most likely due to recent transmission, is prevalent in Latvia among MDR patients and that this genotype can be associated with double mutations.

KEY WORDS: tuberculosis; drug resistance, multiple; genotype; mutation; DNA fingerprinting

STRAINS OF the Beijing genotype of *Mycobacterium tuberculosis* have been responsible for considerable morbidity and mortality worldwide.[1] This genetically highly conserved family of strains has caused large outbreaks of tuberculosis (TB) predominantly affecting young people. The Beijing genotype strains are often resistant to isoniazid (INH) and streptomycin (SM) and are associated with multi-drug resistance (MDR).[1,2] The Beijing genotype is also reported to have a selective advantage over the other *M. tuberculosis* genotypes[3] and induce a febrile response to treatment.[4] The Beijing genotype predominates in China and some geographic areas of Asia and Eastern Europe,[3,5] and now seems to be spreading to other areas in the world.

Latvia is one of the countries with the highest rate of MDR-TB in the world.[6] Between 1999 and 2001, primary MDR-TB reached 9%, and since 1991 the MDR incidence has increased 2.4 times. In a response to these alarming numbers the DOTS (directly observed treatment, short course) strategy was implemented in 1995, and DOTS-Plus in 1997.[7]

The aim of the present study was to examine the previously undetermined prevalence of the Beijing genotype of *M. tuberculosis* in Latvian MDR isolates. We also analyzed mutations in the *katG* and *rpoB* genes of *M. tuberculosis* which are known in part to determine INH[8] and rifampin (RMP)[9] resistance. We compared the mutations in isolates of the Beijing genotype with those in the other isolates to determine whether specific predominant gene mutations can be used as genetic markers for MDR in countries with a high incidence of MDR-TB.

MATERIALS AND METHODS

Spacer oligonucleotide typing (spoligotyping) can be regarded as the gold standard for the identification of the Beijing genotype in strains of *M. tuberculosis*.[2] Hybridization to spacers 35 to 43 in the polymorphic DR region of *M. tuberculosis* DNA is 100% specific for the Beijing genotype. In this study, 109 cultures were randomly selected from typical Latvian MDR TB patients (28 female and 81 male) with primary (36%) and relapsed (64%) pulmonary TB cases. The

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Article submitted 22 November 2002. Final version accepted 12 May 2003.

random selection of our samples reflects the rate of primary (33%) and relapsed (61,5%). MDR-TB in general population. *M. tuberculosis* infection between 1999 and 2001. Patients ranged in age from 21 to 71 years, with a mean age of 44 years. Each patient was resistant at least to RMP and INH and contributed only one isolate of *M. tuberculosis*. Selected cultures represented about 25% of all MDR cultures obtained annually by the State Tuberculosis and Lung Disease Centre, where all Latvian TB patients are monitored. Cultures were grown on Löwenstein-Jensen 'LJ) medium slants for 4–6 weeks. Drug susceptibility was determined by using both the BACTEC system (Becton Dickinson, Sparks, MD) and the absolute concentration method on slants.[10,11] Critical concentrations for INH were 0.2 μg/ml and 2.0 μg/ml in LJ medium and 0.1 μg/ml in the BACTEC system, while for RMP they were 40.0 μg/ml in LJ medium and 2.0 μg/ml in the BACTEC system. Genomic DNA was isolated from cultivated bacteria by an internationally standardized procedure[12] and dissolved in TE buffer.

Spoligotyping was performed on the 109 Latvian MDR isolates in the International Reference Laboratory of Mycobacteriology, Statens Serum Institute, Copenhagen, Denmark. For spoligotyping, a 20-fold dilution of *M. tuberculosis* DNA was used for polymerase chain reaction (PCR) and subsequent hybridization reactions as previously described.[13] The H37Rv strain was used as a positive control. Autoradiograms were scanned and analyzed using the GelCompar software version 4.0 (Applied Maths, Kortritjk, Belgium), and similarity analysis were based on the Jaccard correlation coefficient (maximum tolerance 2.0%) and the UPGMA clustering method. All spoligopatterns identified by the GelCompar software as more than 80% identical were controlled visually. A cluster was defined as two or more isolates having spoligopatterns that were 100% identical.

Automatic nucleotide sequencing. The 109 *M. tuberculosis* isolates were analyzed for mutations carrying the RMP and INH resistance by nucleotide sequencing of the *rpoB* and *katG* gene fragments. A 257 bp fragment of the *rpoB* gene, that determines RMP resistance in 96% of the cases,[9] was amplified using primers with the following sequences[14]: 71.B–5′-GGTCGGCATGTCGCGGATGG-3′ and 72.B–5′-GCACGTCGCGGACCTCCAGC-3′. A 704 bp fragment of the *katG* gene was amplified using the direct primer 74–5′-CGGGATCCGCTGGAGCAGATGG GC-3′, targeting to the 52 end nucleotides before the codon 315 and the reverse primer 75–5′-CGGAAT TCCAGGGTGCGAATGACCT-3′, positioned 158 nucleotides downstream from codon 463.[15] DNA from *M. bovis* BCG-Connaught strain was used as the drug susceptible control in both reactions. The purified PCR products were applied for automatic nucleotide sequencing of both chains using the ABI PRISM Big-Dye™ Terminator Cycle Sequencing Reaction Kit, and the results were read by the ABI PRISM 3100 DNA Analyser (Applied Biosystems, USA).

RESULTS

Spoligotyping

Twenty variants of spoligopatterns were distinguished on 109 MDR isolates of *M. tuberculosis* tested by spoligotyping (Figure 1). Of the 109 isolates, 95 were located in six different clusters of 2 to 63 isolates each, of which the two largest clusters accounted for 63 and 19 isolates each (Figure 2). The remaining 14 isolates did not show clustering. As indicated in the dendrogramme (Figure 2), the 109 isolates could be subdivided into two main groups, one containing 63 and the other 46 isolates. The largest one, called the 'Beijing genotype' group, was formed by the 63 (58%) isolates, all showing Beijing-characteristic nine-spacer spoligopatterns (spacers 35–43) and lacking spacers 1–34 (Figure 1, SP1). The Beijing genotype group was the largest cluster of spoligopatterns. Another main group, called 'non-Beijing', was formed by the 46 isolates with 19 variants of spoligopatterns (Figure 1, SP2–SP20) and is shown at the top of the dendrogramme (Figure 2). In this group is the second largest cluster after Beijing, consisting of 19 isolates (Figure 1, SP12). All except one non-Beijing isolate shared >72% pattern similarity within the group. The one isolate had a spoligopattern (Figure 1, SP2), different from those in both groups; its similarity coefficient with the non-Beijing group was less than 40%. This isolate was included in the non-Beijing group, however, as it did not share the spoligopattern with the Beijing group and had Arginine (Arg) at codon 463 of the *katG* gene (see below).

Automatic nucleotide sequencing

The *rpoB* gene 257 bp fragment sequencing results for the 109 MDR *M. tuberculosis* isolates are shown in the Table. Altogether, 16 variants in single and double codon mutations were found in 102 (93.6%) of 109 isolates, including the isolate with the combination of codon deletion and two codon mutations at positions 524–527. In both groups of Beijing and non-Beijing genotype, the following *rpoB* gene mutations predominate: S531L in 52.2% and D516V in 14.7% of the 109 isolates. Altogether, nine double mutations were detected, six of which were located at different pairs of codons and eight of those were found in isolates comprising Beijing genotype. Finally, the 63 Beijing isolates showed a higher percentage of double mutations (12.7%) in comparison to the group of 46 non-Beijing isolates (2.2%) ($P < 0.05$). Several isolates (6%) did not contain mutations in the region examined.

The *katG* gene fragment sequencing showed a single nucleotide change at codon 315 in 107 (98.4%) of the 108 isolates for which sequencing results were

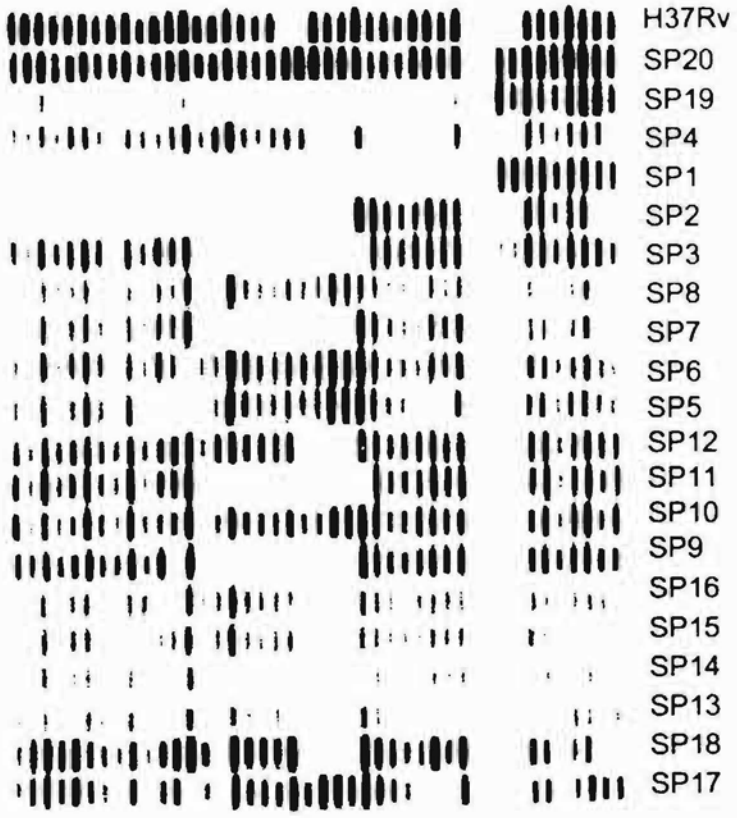

Figure 1 Computer-generated, normalised image showing 20 representative variants of spoligopatterns. H37Rv is the standard reference strain and the rest, SP1 to SP20, are variants of spoligopatterns.

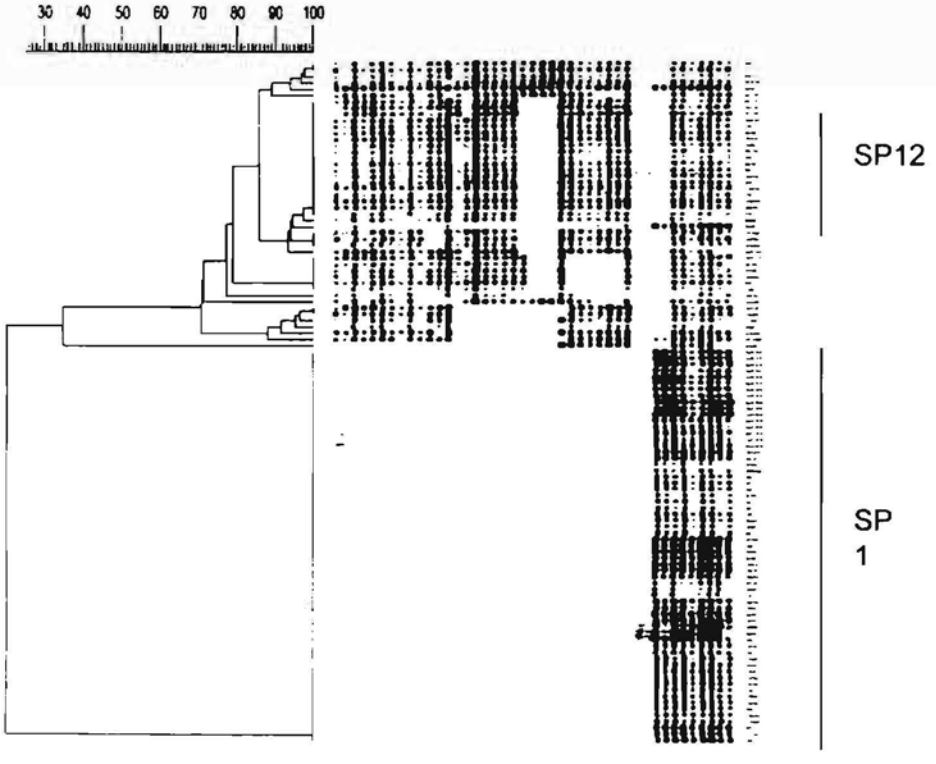

Figure 2 Results of spoligotyping of 109 MDR *M. tuberculosis* isolates and the corresponding dendrogram. SP1 and SP12 are spoligopatterns of the isolates located in the two largest clusters

Table Mutations in 257 bp fragment of the *rpoB* gene found in 109 MDR isolates of *M. tuberculosis*

Mutation localization	Number of mutations in 63 isolates with Beijing genotype	Number of mutations in 46 isolates of non-Beijing genotype	Mutations in all 109 *M. tuberculosis* MDR isolates
S531L	34 (54%)	23 (50%)	57 (52.2)
D516V	8 (12.6%)	8 (17%)	16 (14.7)
D516Y+P353S	5	—	5 (4.6)
S531F	4	—	4 (3.7)
H526D	—	5	5 (4.6)
H526Y	1	2	3 (2.8)
D516Y	2	—	2 (1.8)
H526L	—	1	1 (0.9)
H526R	1	—	1 (0.9)
S531W	—	1	1 (0.9)
L533P	2	—	2 (1.8)
Q510H+H526Y	—	1	1 (0.9)
D516V+P535L	1	—	1 (0.9)
D516V+K519T	1	—	1 (0.9)
R529P+S531L	1	—	1 (0.9)
Deletion+mut. (524–527)	1	—	1 (0.9)
Wild type	2	5	7 (6.4)

available. In 103 (94.7%) isolates it was change AGC→ ACC, and in four (3.7%) it was a mixture of ACC and ACA. The 4 isolates with ACC and ACA mixture position 315 had different IS*6110*-RFLP patterns (data not shown). In all 107 strains, the nucleotide change from AGC to ACC or ACA resulted in substitution of Serine (Ser) for Threonine (Thr). In one (0.9%) of the 108 isolates the 704bp long *katG* gene fragment did not contain a mutation. All isolates with the Beijing genotype had the same substitution: S315T.

In addition, a genetic polymorphism at codon 463 of the *katG* gene was detected by automatic DNA sequencing. All 63 isolates comprising in the Beijing group had Leucine (Leu) (CTG) at position 463 of the 704 bp fragment of the *katG* gene. Whereas 45 of the 46 isolates from the non-Beijing group showed Arg (CGG) at position 463 including the isolate sharing less than 40% similarity with other members of this group (Figure 1, SP2). The remaining one isolate (Figure 1, SP20) of 46 within the non-Beijing group showed a mixture of Leu and Arg at position 463 indicating the presence of two strains in this sample. Indeed, RFLP analysis showed a high number (n = 25) of IS*6110* bands for this sample (data not shown). No further investigation of this sample was possible since the corresponding culture was not preserved.

DISCUSSION

Spoligotyping of the 109 MDR *M. tuberculosis* isolates revealed a high number of Beijing genotype strains among MDR patients in Latvia. The specific reason for the high prevalence of this genotype is unknown, but explanations include: more recent transmission of Beijing MDR strains, higher virulence of Beijing MDR strains, higher evolutionary development of strains with the Beijing genotype and development of MDR strains as a result of prolonged inadequate chemotherapy.[2] It is also unknown, whether the Beijing genotype has a higher probability of acquiring drug resistance than the other *M. tuberculosis* genotypes. A less likely explanation for the situation in Latvia could be the evolution of bacteria.

The high prevalence of the Beijing genotype among MDR-TB patients in Latvia from 1999 to 20001 can be partly explained by recent transmission. It was shown that the sample of 63 MDR isolates of the Beijing group described above have 80–100% identical IS*6110*-RFLP patterns (data not shown). It is also known, however, that Beijing strains are genetically closely related[1] and do not necessarily reflect ongoing transmission. We don't know for how long time the Beijing genotype has been present in Latvia, as collecting and storage of *M. tuberculosis* strains was started only in 2000. We have also identified the Beijing genotype by spoligotyping and IS*6110*-RFLP in 12 drug-susceptible *M. tuberculosis* isolates obtained from patients during 1999–2001, but, in general, our observations suggest that Beijing is not particularly common among drug-susceptible isolates in Latvia (data not shown). These results are in agreement with reports showing the spread of Beijing genotype over recent years in neighbouring countries Estonia and Russia, where it was also associated with recent transmission of drug-resistant strains.[19,20]

In this study, the Beijing genotype of *M. tuberculosis* was not associated with younger age of patients, as was previously reported.[5] The mean age of Latvian patients hosting the Beijing genotype was 46 years; most (35.2%) of these patients were aged between 41 and 50 years. In contrary, the younger patients, those between 21–30 years of age, constituted only 11% of the Beijing patients. A similar age distribution was observed in non-Beijing group: the mean age was 42 years, 36% of patients were 41–50 and 18% were 21–30 years old. It is obvious that the distribution of age groups for the Beijing and non-Beijing genotypes follows the general trend of TB in Latvia, where the highest incidence is found among the population 44–54 years of age (160 per 100 000 in 2000). This could indicate that transmission of TB in Latvia occurs within a specific age group. This observation is important for diagnosis of TB in the cases where bacterial cultures are unavailable but transmission is obvious. For example, in 2000, 34.7% of the new cases among Latvian children were detected only during contact investigation.

Instead of specific age, there is known to be a higher incidence of TB and a corresponding higher incidence of MDR-TB among the males in Latvia. The ratio of females to males in our study in both Beijing and non-Beijing groups was 1:3, which reflects the incidence of TB in females and males in the general population.

Clinical information was available for 34 patients from Beijing group who received DOTS therapy at State Tuberculosis and Lung Disease Centre in 1999. Of these patients, 15 (43%) completed treatment and one has been cured, whereas seven patients continue to produce culture-positive sputum and 11 (31%) have died. This information allows us to predict the general trends resulting from TB treatment and to develop adequate TB monitoring methods in clinical practice.

The most common RMP-resistance determining mutations detected in this study, S531L and D516V, are in complete agreement with previous studies.[9,14] In both of these studies, it was shown that the same mutations dominate in both Beijing and non-Beijing strains as well as among other genotypes. The higher occurrence of double mutations (12.7% against 2.2%) in the *rpoB* gene was the most obvious difference between Beijing and non-Beijing groups in our study. It is likely that the two mutations in different codons result from a prolonged exposure of bacteria to RMP. Perhaps, double mutations induce change in protein folding that give the MDR strain a survival advantage in presence of RMP. In our study, 93.7% of the RMP resistant isolates contained mutations occurring in the highly variable 69 bp region of the *rpoB* gene. This observation is also in agreement with the previous studies.[9,14] The presence of this highly variable locus should stimulate the search for a new generation of antibiotics that are analogous to RMP.

A high percentage of mutations at position 315 (98.4%) in the *katG* gene among INH resistant strains had also been observed in this study. Similar results were obtained in St-Petersburg area of Russia,[21] where Ser315Thr substitutions were predominant (92%) among MDR-TB isolates. However, a wider mutation spectrum was observed in USA and in western European countries.[22,23] Van Soolingen et al. have discussed codon 315 mutations being associated with the high level resistance to INH as a result of inappropriate treatment.[24] Our results suggest that Serine substitution at codon 315 of the *katG* gene is characteristic of INH-resistant strains and can therefore serve as a genetic marker for INH resistance in our region. Surprisingly, substitution at codon 315 was absent only in one INH-resistant strain. This can be explained by the observation that there are more genes which can be responsible for IHN resistance—*inhA*, *ahp*C and kasA,[25,26] as well as the recently described *ndh* gene,[27] but mutations in these genes are of lower frequency than those in the *katG* gene. According to Sreevatsan et al., all strains of *M. tuberculosis* can be assigned to three phylogenetic groups based on functionally neutral polymorphisms present at codon 463 of the *katG* gene and codon 95 of the *gyrA* (a subunit of DNA gyrase) gene.[16] Therefore, all our isolates of the Beijing genotype with Leu at codon 463 could be assigned to the phylogenetic Group 1. This correlates with results from other investigations confirming that the Beijing group shares a common ancestor with *M. bovis*.[17,18] In this study, automatic sequencing of the *katG* gene fragment of the *M. bovis* BCG strain showed Ser (wild type) at codon 315 and Leu at codon 463; the latter indicating its relationship to phylogenetic Group 1. The remaining strains that constituted the non-Beijing group with Arg at codon 463 could be assigned either to Group 2 or to Group 3 on the basis of polymorphisms at codon 95 of the *gyrA* gene. This could be the aim of further investigations.

This study demonstrates a high frequency of the Beijing genotype among MDR TB patients in Latvia. The double mutations in the *rpoB* gene among strains may show that MDR Beijing strains were exposed to inappropriate RMP treatment for a long time, thereby underlying a historical lack of health policy measures. In Latvia, the highest incidence of TB, reaching 74 cases per 100 000 population, was observed in 1998.[28] Since then, the application of the DOTS strategy and individual treatment regimens have proven effective for MDR-TB patients. As a result, the incidence of MDR-TB in Latvia has stabilised, and the cure rate for new TB cases has increased from 70.0% to 77.9% in the last 3 years.[28] In addition, the creation of a MDR-TB registry in 2000, in combination with the implementation of molecular typing methods, will provide a strong basis for improved monitoring of TB in Latvia.

Acknowledgements

We thank Vibeke Ø Thomson and Eskild Petersen from Statens Serum Institut for essential assistance in this study, and Vitauts Kalnins (University of Toronto, Canada) for help in preparing this manuscript.

The project was funded by the FEBS short-term fellowship, INCO-Copernicus ERB IC15-CT98-0328 project and the Council of Sciences of Latvia (program Nr 01.0012.9.1). Troels Lillebaek was supported by the Danish Lung Association and European Community Grant 2000-0630.

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RÉSUMÉ

CONTEXTE : Les génotypes prédominants de *Mycobacterium tuberculosis* décrits de par le monde incluent la famille Beijing qui a provoqué de grandes épidémies de tuberculose et est associée à une virulence aiguë et à une multirésistance (MDR) dans cette maladie.

OBJECTIF : Rechercher le génotype Beijing parmi les patients MDR de Lettonie afin de caractériser au niveau moléculaire leurs isolats de DNA.

SCHÉMA : On a pratiqué le spoligotypage sur 103 isolats MDR et l'on a recherché les mutations géniques par un séquençage automatique de nucléotides.

RÉSULTATS : Sur les 109 isolats examinés, 95 appartenaient à six grappes comportant de 2 à 63 isolats au sein de chaque grappe. Les 63 isolats de la plus grande de ces grappes avaient un type similaire correspondant au génotype Beijing. Les isolats restants étaient d'un génotype non-Beijing et formaient un autre grand groupe dont la similarité s'étalait entre 72% et 100%. Les mutations dans les gènes *rpo*B et *kat*G ont été comparées entre les souches appartenant au génotype Beijing et à un génotype non-Beijing. Dans les deux groupes, les mutations géniques *rpo*B ont prédominé dans les codons S531L (52,2%) et D516V (14,7%). Des mutations doubles dans le gène *rpo*B ont été observées dans 8,2% des isolats, la plupart d'entre elles étant localisées parmi les isolats du type Beijing. La mutation S315T du gène *kat*G (98,4%) était prévalente dans tous les isolats.

CONCLUSION : L'analyse moléculaire des isolats MDR de *M. tuberculosis* démontre que le génotype Beijing, le plus probablement dû à une transmission récente, est prévalent en Lettonie parmi les patients MDR et que ce génotype peut aller de pair avec des mutations doubles.

RESUMEN

CONTEXTO : Los genotipos predominantes de *Mycobacterium tuberculosis* descritos en todo el mundo incluyen la familia Beijing, que ha causado grandes brotes epidémicos de tuberculosis y que ha sido asociado con el aumento de la virulencia y con la multirresistencia a los medicamentos (MDR) de esta enfermedad

OBJETIVO : Buscar el genotipo Beijing en los pacientes MDR en Letonia, para caracterizar sus aislados de ADN a nivel molecular.

DISEÑO : En 109 aislados MDR se realizó una espoligotipificación y tests de mutación génica por secuenciación automática de nucleótidos.

RESULTADOS : De los 109 aislados examinados, 95 pertenecían a 6 conglomerados, con 2–63 aislados en cada conglomerado. Los 63 aislados del conglomerado más grande tenían, en un 100%, un tipo similar correspondiente al genotipo Beijing. Los aislados restantes eran de un genotipo no-Beijing y formaban otro gran grupo, cuya similaridad iba de 72 a 100%. Se compararon las mutaciones en los genes *rpoB* y *katG* entre las cepas con genotipo Beijing y aquéllas con genotipo no-Beijing. En ambos grupos, las mutaciones del gen *rpoB* predominaban en los codones S531L (52,2%) y D516V(14,7%). La doble mutación en el gen *rpoB* se observó en el 8,2% de los aislados, la mayoría de ellas habiendo sido localizadas en los aislados de tipo Beijing. La mutación S315T del gen *katG* (98,4%) era prevalente en todos los aislados.

CONCLUSIÓN : El análisis molecular de los aislados MDR de *M. tuberculosis* demuestra que el genotipo Beijing, el más probablemente debido a una infección reciente es prevalente en Letonia en los pacientes MDR y que este genotipo puede estar asociado con mutaciones dobles.

APPENDIX 4

IDENTIFICATION OF METHICILLIN RESISTANCE IN *STAPHYLOCOCCI*

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Grampozitīvie koki, tai skaitā meticilīnrezistentie stafilokoki – S. aureus un koagulāzes negatīvā S. epidermidis grupa ir kļuvuši par nozīmīgiem hospitālo infekciju ierosinātājiem.

Tā kā šiem mikroorganismiem ir raksturīga polirezistence, to precīza diagnostika klīnikā ir ļoti nozīmīga. Darbā izmantotas sekojošas meticilīnrezistences noteikšanas metodes: disku difūzijas metode, mecA gēna noteikšana ar polimerāzes ķēdes reakciju, E-tests. Rezultātu apstiprināšanai izmantotas 2 automātiskās sistēmas – Sceptor un Mini Api. Rezultāti parāda, ka disku difūzijas metode ir absolūti precīza, novērota 100% rezultātu sakritība ar "standartu"-mecA gēna noteikšanu. E-testa rezultāti četros gadijumos atšķīrās un neuzrādija rezistenci, kas bija pierādīta ar pārējām 4 metodēm. Visas izdalītās meticilīnrezistentās kultūras bija polirezistentas – nejutīgas pret klīnikā biežāk lietojamiem antibakteriālajiem preparātiem.

Introduction

Antimicrobial resistance continues to threaten the clinical use of antibiotics. Among the organisms currently causing the greatest problems are a whole range of gram-positive cocci such as *Staphylococcus* spp., *Streptococcus pneumoniae*, *Enterococcus* spp. etc. These are the organisms that are most frequently isolated from clinical specimens in surgical hospitals (Bax et al., 2001; Gravenitz, 2001; Zilevica et al., 2001).

Staphylococci are pathogens that can cause a wide variety of diseases ranging from localized wound infections to life-threatening systemic diseases. In the last decades the multitude of resistant strains of *S. aureus* have developed and spread globally. Multiresistance is commonly associated with the occurrence of methicillin resistance, an intrinsic resistance to virtually all beta-lactams (Deplano et al., 2000; Oliveira et al., 2002).

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were first detected in the UK in 1960, shortly after the introduction of penicillinase-resistant penicillins into the clinical practice. Since then MRSA has become one of the leading causes of nosocomial infections worldwide (Witte et al., 1997).

The incidence of MRSA among the clinical isolates of *S. aureus* varies from country to country. Some countries such as Denmark and Iceland (> 0.2%) as well as Switzerland (1.8%) have managed to keep MRSA at a controllable level. However, in some countries, the MRSA incidence level is high - in France, Spain and Italy, more than 30% of hospital isolates of *S. aureus* are resistant to methicillin (Murchan et al., 1988; Blanck et al., 2002; Pascual, 2002).

Coagulase negative *Staphylococci* (CoNS), particularly the *S. epidermidis* group, have emerged as significant pathogens in nosocomial infections since the 1980s.

One of the factors that contributed to this phenomenon was increased use of indwelling devices and prosthetic implants as well as poor catheterization techniques, followed by other reasons such as more aggressive chemotherapy, advances in the cardiac and implant surgery, and lastly, the awareness and improvement in microbiological techniques, which has made the identification of these pathogens easier and more precise.

The proportion of methicillin-resistant *S. epidermidis* (MRSE) among hospital CoNS has increased from 8 to 30% in the 1980s to 38% and even 90% nowadays (Mehtar, 1994; Bouza, 2002, Carratala, 2002). MRSE strains are considered reservoirs of antimicrobial resistance genes that can be transferred to other *Staphylococci*, so contributing to the development of methicillin resistance among microorganisms (Kragsbjerg et al., 2000).

To control the spread of resistance among *Staphylococci*, appropriate infection control practices should be applied in hospitals, including precise microbiological diagnosis and detection of antimicrobial susceptibility of isolated agents (Leclercq, 2000).

This study was undertaken for evaluation and comparison of several methicillin resistance identification methods in *Staphylococci* and detection of antimicrobial susceptibility of isolated MRSA and MRSE to a panel of antimicrobials.

Materials and methods

Bacterial strains

A total of 117 methicillin resistant *Staphylococci* strains isolated from clinical specimens in a surgical hospital and collected within 1998-2002 were included in this study. Among them, there were 25 coagulase-positive MRSA and 92 coagulase-negative methicillin-resistant *Staphylococci*, including 63 strains of *S. epidermidis* (*sensu stricto*), 17 strains of *S. haemolyticus*, 9 strains of *S. hominis*, 2 strains of *S. warneri*.

Most of these isolates were from indwelling artificial devices, blood, abscesses, etc. All isolates were gram-positive clustering cocci. They were identified to a species level by conventional tests such as coagulase test-tube reaction, phosphatase activity, haemolysis, susceptibility to novobiocin, etc. and the automated BBL Crystal system (Becton-Dickinson).

22 methicillin-sensitive strains were included as control strains.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities for all isolates were tested by the disk diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines using Mueller-Hinton agar (MHA, Oxoid, UK) against the following panel of antibiotics: penicillin, gentamicin, cefazolin, erythromycin, clindamycin, vancomycin, ciprofloxacin, trimethoprim-sulfamethoxazole.

Methicillin resistance was identified by the standard agar diffusion technique with a commercial oxacillin disk (potency 1 μg of oxacillin). Because of the difficulties in detecting cross-resistance among penicillinase-resistant penicillins (methicillin, oxacillin), the oxacillin disk is now the recommended choice for detecting methicillin-resistant *Staphylococci* (Koneman et al., 1997).

Plates were incubated at 35°C for 24 h when zones of incubation were measured. Plates with CoNS cultures were incubated for 48 h.

S. aureus ATCC 29213 was used as a methicillin-susceptible control strain, NCTC 8325 as a methicillin-resistant control strain.

To confirm the results of the disk diffusion method, detection of the *mec*A gene by the polymerase chain reaction (PCR) and E-test were used.

Detection of the *mec*A gene

At present, the detection of the *mec*A gene, which is responsible for methicillin resistance in practically all clinical methicillin-resistant Staphylococcal strains, is considered the oxacillin resistance reference test. This is introduced in practice as an alternative to another "gold standard" – the oxacillin agar screen plate test used in our and many other clinical laboratories worldwide. PCR detection of *mec*A was started in Latvia in January, 2001.

66 clinical isolates of *Staphylococci* were included in this study. Both coagulase-positive and coagulase-negative *Staphylococci* (*S. aureus* – 24 strains, *S. epidermidis* – 31 strains, *S. haemolyticus* – 8 strains, *S. warneri* – 1 strain) and *S. hominis* – 2 strains were used for detection of the *mec*A gene by PCR amplification. 54 strains from them were preliminary methicillin-resistant, 12 strains – methicillin sensitive.

DNA isolation

Chromosomal DNA was extracted by the lysostaphin-CTAB method as described by Jones, with modifications (Jones et al., 1953; Hookey et al., 1998). The cell cultures were separated from Mueller-Hinton medium by centrifugation at 7000 rpm for 2 min, suspended in 1 ml of TE-glucose (25mM Tris-HCl [pH 8.0], 1.0% [wt/vol] D-glucose), and centrifuged at 7500 rpm for 5 min. The cells were resuspended in 100 µl of lysostaphin (1 mg/ml in TE-glucose; Sigma) – 50 µl lysozyme (50 mg/ml in TE-glucose; Sigma) and incubated at 37°C for 1 h. 80 µl of NaCl-cetyltrimethylammonium bromide (CTAB) solution (0.7M NaCl, 10% [wt/vol] CTAB; Sigma) was added with mixing and incubated at 65°C for 10 min. Sodium chloride (100 µl of a 5M stock solution, SDS (30 µl of 10% [wt/vol] SDS; Sigma), and proteinase K (4 mg of proteinase K; Sigma) were added with mixing and incubated at 55°C for 30 min. The lysate was extracted with equal volumes of phenol-chloroform, and the DNA was precipitated from the aqueous phase with one volume of isopropanol and diluted in 100 µl of sterile distilled water.

The DNA concentration was determined by UV spectrophotometry at A_{260}, and the extract was stored at 4°C. Approximately 50 to 100 ng of DNA was taken for PCR amplification. Extraction took place from 1 to 2 days.

PCR amplification of the *mecA* gene

PCR was performed with the following primers, previously designed by Geha et al. (Geha ET al., 1994): *mec*A1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and *mec*A2 (5'- CCA ATT CCA CAT TGT TTC GGT CTA A). The PCR reagent mixture consisted of 200 µM concentrations of dNTPs, 10mM Tris (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, a 0.25 µM concentration of each primer, and 1.25U of *Taq* polymerase (*Fermentas*, Lithuenia). DNA amplification thermal cycling profile ("Progene", *Techne*, UK): initial denaturation at 94°C for 5 min, followed by 30 cycles of amplification (denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, extension at 72°C for 30 sec), ending with the final extension at 72°C for 2 min. A positive result was indicated by the presence of the 310-bp amplified DNA fragment revealed by electrophoresis on a 1.5% agarose gel. Results were obtained within 4 h. Each PCR included methicillin- resistant strain as a positive control and water as a negative control.

E-test

E-test (AB Biodisk) was used as a quantitative technique for determination of antimicrobial susceptibility and minimal inhibitory concentration (MIC) (Bolmstrom et al.

1988). Mueller-Hinton medium, supplemented with 2% NaCL was used for inoculation. Incubation time with E-test strips was 24 h and 48 h for *S. aureus* and CoNS, respectively.

In cases when incomparable results were obtained using the disk diffusion technique, PCR and E-test, two automated systems were used: the automated Sceptor system and Mini-Api system.

Results

The collection of methicillin-resistant *Staphylococci* (MRS) strains was carried out on the basis of the results of the oxacillin disk method. 66 of them – 24 *S. aureus* (from them 18 MRSA, 6 MSSA) and 42 CoNS strains (36 MRS, 6 MSS) were analysed for the presence of the *mec*A gene. According to PCR results, all 18 strains of *S. aureus* and 36 strains of coagulase-negative *Staphylococci*, phenotypically resistant to methicillin, showed the presence of the 310-bp fragment of the *mec*A gene, thereby confirming methicillin resistance (the method's sensitivity = 100%) (Fig. 1).

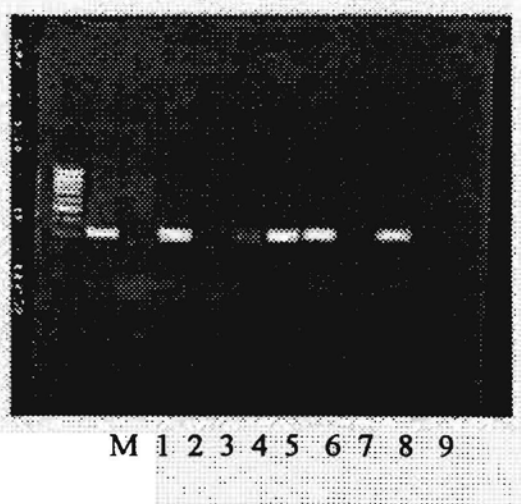

Figure 1. 2% agarose gel electrophoresis analysis of 310-bp fragments of the mecA gene from DNA isolates of Staphylococcus sp.
M – molecular mass standard. "100bp Ladder Plus", slots No. 1-7 – strains from SIT, slot No. 8 – PCR negative control, slot No. 9 – PCR positive control (methicillin-resistant strain). PCR results show that strains No. 1, 3, 5, 6 and 7 are mecA-positive, strains No. 2 and 4 – mecA negative.

E-test, used in parallel with PCR, demonstrated different results: 4 from methicillin-resistant Staphylococcal strains, which exhibited methicillin resistance in both basic methods, i.e. the disk method and PCR, showed a methicillin-sensitive pattern.

These four cultures were tested additionally for methicillin resistance in automated Sceptor and Mini Api systems (Table 1). As can be seen from Table 1, all 4 strains proved to be methicillin-resistant.

Table 1

Methicillin resistance in *Staphylococci* according to different methods of detection

No	Patent	Micro-organisms	Method for detection of methicillin resistance						
			Oxacillin disk	E-test	*mec*-gene	Sceptor		Mini Api	
							MIC mcg/ml		break point mcg/ml
1	C.	*S. epidermidis*	R	S	R	R	>4	R	>0.25
2	S-s	*S.epidermidis*	R	S	R	R	4	R	>0.25
3	A.	*S. epidermidis*	R	S	R	R	4	R	>0.25
4	S-a	*S.warneri*	R	S	R	R	>4	R	>0.25

For 11 of the 12 control strains, the PCR response, phenotypically sensitive to methicillin, was negative – the *mec*A gene was absent. One strain, exhibiting oxacillin MIC 1 μg/ml, proved to possess the *mec*A gene and should be recognized as methicillin-resistant. One of the possible explanations for this fact can be a higher sensitivity of the PCR method in comparison to standard methods, as PCR allows "to touch" bacteria at the genetical level. Another explanation could be the heteroresistance of bacteria.

Methicillin-resistant *Staphylococci* are invariably regarded as resistant to all other beta-lactam antibiotics. In addition, many clinical isolates demonstrate multi-resistant patterns. In our studies of antimicrobial susceptibility of methicillin-resistant *Staphylococci* strains, a high-level resistance to many commonly used antibiotics was registered (Table 2). 100% of *S. aureus*, *S. haemolyticus*, *S hominis* were resistant to erythromycin, 100% of *S. hominis* were resistant to gentamicin. 35-75% of isolated strains were resistant to Trimethoprim / Sulphamethoxazole, 68-95% to clindamycin. No vancomycin resistance was documented.

Table 2

Susceptibility of MRS to antimicrobials

Micro-organism	No. of cul-tures	Antimicrobials				
		Cipro-floxacin	Clinda-mycin	Erytro-mycin	Genta-micin	Trimetho-prim/Sulpha-methoxazol
S.aureus	25	S – 88% R – 12%	S – 32% R – 68%	S – 0 R – 100%	S – 76% R – 24%	S – 65% R – 35%
S.epidermidis	63	S – 73% R – 27%	S – 4.8% R – 95.2%	S – 11% R – 89%	S – 60.3% R – 39.7%	S – 29.8% R – 70.2%
S.haemolyticus	17	S – 62.5% R – 37.5%	S – 0 R – 100%	S – O R – 100%	S – 62.5% R – 37.5%	S – 25% R – 75%
S.hominis	9	S – 89% R – 11%	S – 22% R – 78%	S – 0 R – 100%	S –0 R – 100%	S – 45% R – 55%
CoNS total	93	S – 73% R – 26.4%	S – 5.5% R – 94.5%	S – 8.8% R – 91.2%	S – 63.7% R – 36.3%	S – 30.5% R – 69.5%

Discussion and conclusions

Antimicrobial susceptibility tests measure the ability of an antimicrobial agent to inhibit bacterial growth *in vitro*.

The disk diffusion method (the modified Kirby-Bauer method), originally described in 1966, is recommended for clinical and surveillance purposes in view of its technical simplicity and reproducibility. The method is well standardized, has been widely evaluated and is suitable as a general method for all rapidly growing pathogens (Acar, 1980; Hasselman et al., 2000).

Methicillin resistance in *Staphylococci* can be detected by using an oxacillin disk, and two gold standard methods are recommended by the European Antimicrobial Resistance Surveillance System (EARSS), namely, oxacillin screen plate and detection of the *mec*A gene by PCR (Goettsch et al., 2000).

The precise diagnosis of methicillin resistance is technically different because of the hetero-resistant nature of MRS. However, it is of greatest importance – MRS are resistant to all other beta-lactams regardless of the *in vitro* results obtained. These beta-lactams include all penicillins, cephalosporins, amoxicillin-clavulanic acid, ampicillin-sulbactam, imipenem.

We have used 3 basic methods for identification of methicillin resistance in *Staphylococci*, namely, the standard agar diffusion (disk) test, detection of *mec*A gene, E-test.

It is well known that the most reliable is the detection of the *mec*A gene, a genotypic marker of resistance by PCR – the high level of resistance to penicillinase-resistant penicillin requires the presence of the *mec*A gene that encodes the penicillin-binding protein PBP 2a (Frebourg et al., 1998; Lowy, 1998; Goettsch et al., 2000).

According to our results, the disk diffusion method has a high specificity – only one of the cultures evaluated as methicillin-sensitive due to the disk method proved to have a resistant pattern in the PCR test. This could be explained by the hetero-resistant nature of microorganisms (Berger-Bachi, 1997; Zilevica, Vingre, 2001).

The E-test proved to be less reliable. However, its value is in the quantitative determination of resistance (MIC).

Undoubtedly, at least 2 methods are necessary for detection of methicillin resistance. We recommend the use of PCR for detection of the presence of the *mec*A gene in methicillin-sensitive strains.

Acknowledgement

This study was supported by the Grant 02. 0011. 07 of the Latvian Council of Science.

Summary

Gram-positive cocci, particularly the methicillin-resistant *Staphylococcus aureus* and coagulase-negative *Staphylococcus epidermidis* group, have emerged as major agents of nosocomial infections. Owing to the multi-resistance of these agents, precise diagnosis of the methicillin resistance of *Staphylococci* is of greatest clinical importance. In our studies, the following methicillin resistance identification methods were used: the disk diffusion method, detection of the *mec*A gene by PCR, E-test. The results were confirmed using the automated Sceptor and Mini Api systems.

Our findings indicate that the results of the disk diffusion method were identical to "standard" PCR results. In 4 cases, the E-test showed different results and did not detect the resistance confirmed by the other four methods. All the MRS strains were multiresistant to most commonly used antibacterials.

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APPENDIX 5

Resubmitted to *Research in Microbiology*

Characterization of *rpsL*, *rrs* and *embB* mutations associated with streptomycin and ethambutol resistance in *Mycobacterium tuberculosis*

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To characterize molecular mechanisms of first-line drug resistance in *Mycobacterium tuberculosis* and to evaluate the use of the molecular markers of resistance (gene point mutations), we have analyzed 66 multi-drug resistant (MDR) isolates from Latvian tuberculosis patients. They were all resistant to rifampin (RIF), isoniazid (INH) and streptomycin (SM), 33 were resistant to ethambutol (EMB). Enzymatic digestion by *MboII* and nucleotide sequencing of the *rpsL* gene fragment revealed single nucleotide substitution K43R in 40 (61%) of the 66 SM resistant *M.tuberculosis* isolates. Of the other 26 SM resistant isolates, 16 (24%) had mutations at positions 513A→C and 516C→T of *rrs* gene and 10 (15%) had the wild type sequence. The single-stranded DNA conformation polymorphism (SSCP) method was applied to the *embB* gene to detect EMB resistance. Using 103bp fragment SSCP analysis substitutions in *embB* gene were found in 15 (45%) and by sequencing in 17 (52%) of the 33 EMB resistant isolates. Surprisingly, SSCP revealed nucleotide mutation at codon M306 in five (15%) of 33 *in vitro* EMB susceptible MDR isolates.

Key words: Mycobacterium tuberculosis, drug resistance, gene mutations.

1. Introduction

For approximately 50 years chemotherapy was effectively used against tuberculosis (TB) caused by the *Mycobacterium tuberculosis* complex [2]. Unfortunately, inappropriate treatment regimens compounded by non-compliance of patients in completing the prescribed course of treatment have lead to the appearance of drug-resistant strains. Today a high proportion of TB patients in Latvia harbour multi-drug resistant (MDR) strains, i.e., strains resistant at least to rifampin (RIF) and isoniazid (INH). In a survey of 1751 pulmonary TB patients in 2000, 9.3% of the patients had primary MDR and 27.1% had acquired MDR *M.tuberculosis* bacilli [7]. Many of the culture isolates from MDR *M.tuberculosis* patients show resistance not only to RIF and INH, but also to other first-line antituberculous agents: streptomycin (SM), ethambutol (EMB) and, less frequently, to pyrazinamide [7].

The mode of action of anti-TB drugs and the molecular mechanisms of drug resistance have been extensively studied. In most cases gene mutations in *M.tuberculosis* were found to be responsible for resistance to first – and to second –line drugs [16,20]. We have previously reported results from the study of MDR *M.tuberculosis* isolates from Latvia showing that mutations in the *rpoB* and *katG* genes, associated with RIF and INH resistance, were found in 93.6% and 98.4% cases, respectively [23,24]. Therefore, mechanisms of mycobacterial resistance to SM and EMB should be also investigated.

SM which was shown to be an effective anti-TB drug in 1944 [2], acts by binding to the 30S ribosomal subunit, thereby interfering with polypeptide synthesis by inhibiting translation [26]. Two genes, *rpsL* and *rrs*, encoding the ribosomal protein S12 and the 16S rRNA are known to be responsible for the SM resistance [6,8]. EMB is another effective and highly specific agent used in chemotherapy in combination with other drugs [3]. It is believed that EMB is targeting arabinosyltransferases that catalyse the incorporation of arabinan into cell wall polymers arabinogalactan and lipoarabinomannan [5, 27]. Mutations associated with resistance to EMB have been found in the *embB* (arabinosyltransferase) gene, especially, at codon M306 [22].

It would be interesting to know the molecular mechanisms of drug resistance to *M.tuberculosis* in a region with a high level of MDR such as Latvia and to compare it with that in other parts of the world. Therefore, we examined *rpsL, rrs* and *embB* genes known to be associated with resistance to SM and EMB, respectively, in *M.tuberculosis* isolates from Latvian MDR patients. Knowledge of frequently occurring mutations at specific codons that correlate with the genetic relatedness of MDR strains should contribute to our understanding of resistance to first-line anti-TB drugs both at regional and at international levels. This information might be further used in order to develop and adopt molecular methods for testing drug susceptibility directly in clinical specimens.

2. Materials and Methods

2.1. Cultures and susceptibility testing. For this study, 66 cultures of *M.tuberculosis* from patients with a primary and relapsed pulmonary MDR-TB were randomly selected. They were typical TB patients from different regions of Latvia admitted to the State Centre of Tuberculosis and Lung Diseases between 2001 and 2002. Cultures of the *Mycobacterium tuberculosis* complex were grown on Löwenstein-Jensen medium for 4-6 weeks. Drug susceptibility was determined both by the absolute concentration method on slants with the H37Rv strain as the positive control and by the BACTEC radiometric method (Becton Dickinson, Sparks, Md.) [9,19]. In the absolute concentration method, resistance was defined as growth on solid media containing graded concentrations of drugs greater than a 20CFU at a specific drug concentration. The minimal inhibiting concentrations on Löwenstein-Jensen medium were 0.2μg/ml and 2.0μg/ml for INH, 40.0μg/ml for RIF, 4.0μg/ml for SM and 2.0μg/ml for EMB. All 66 cultures were resistant to RIF, INH and SM, 33 were resistant to EMB.

2.2. DNA isolation and PCR. Native DNA was isolated from the mycobacterial cultures by lysozyme/proteinase K, CTAB procedure and precipitated with isopropanol [25]. Purified DNA was dissolved in 20-50μl of TE buffer (10mM TrisHCl, 1mM EDTA [pH 8]). DNA isolated from MT14323 and *Mycobacterium bovis* BCG strains was used for controls.

The 306bp fragment of *M.tuberculosis rpsL* gene (GenBank accession number L08011) was amplified using primers SM1-CCAACCATCCAGCAGCTGGT and SM2-ATCCAGCGAACCGCGGATGA. Modified primers SM3-GATGACGGCCTTCGGGTTGT and SM4-TCTAGCTGCCCGTATCGCC and primers SM5-GTAGTCCACGCCGTAAACGG and SM6-AGGCCACAAGGAACGCCTA were used for analysis of the 530 loop (238bp fragment) and region 912 (240bp fragment) of the *rrs* gene [11, 12]. The 103bp fragment of the *embB* gene (GenBank accession number Z80343) was amplified using primers adopted in part from Rinder et al. [18]: 46-CTGCTCTGGCATGTCAT and 47-AGCGGAAATAGTTGGAC. The 10-20ng of DNA diluted in TE buffer was added to the PCR mix containing PCR buffer, $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 200μM each of dNTP and 1U Taq DNA Polymerase (*Fermentas*, Lithuania) to a final volume 50μl. Ten pmols of each primer were used for one reaction mix. The optimized cycling protocol was used for each set of primers in the "Progene" thermal cycler (*Techne*, England). PCR products were analysed in 1.5% agarose gels. In each set of reactions one negative control and one positive control (*M. bovis* BCG-Connaught strain DNA) were included.

2.3. Analysis of the rpsL and rrs genes. 306bp fragments of the *rpsL* gene from each of the 66 SM resistant DNA isolates were digested with *MboII* endonuclease (*Fermentas*, Lithuania) for 15 min at 37°C and separated on 6% PAAG. Fragments of the *rrs* gene encoding the 16S rRNA 530 loop and 912 region were analyzed by automatic nucleotide sequencing.

2.4. Analysis of the embB gene. The 33 EMB resistant and 33 EMB susceptible MDR *M.tuberculosis* isolates were analyzed for presence of mutations in the 103bp fragment of *embB* gene by the single-stranded DNA conformation polymorphism method (SSCP). After heating for 5 min at 96°C with an equal volume of loading buffer (0.05% bromphenol blue, 0.05% xylene cyanol and 95% formamide) the 103bp fragments were snap-cooled and immediately loaded onto the gel. The SSCP analysis of the DNA fragments was performed in a 6% polyacrylamide gel containing 3.5% glycerin at 200V on a 14×16×0.2cm gel plate cooled to 10°C (*BioRad*, USA). After 3h of electrophoresis the separated DNA bands were visualized by ethidium bromide and by silver staining.

2.5. Nucleotide sequencing. Automatic nucleotide sequencing was used to confirm the results of SSCP and *MboII* digestion methods. Both DNA chains of the purified PCR products were sequenced using the ABI PRISM BigDye™ Terminator Cycle Sequencing Reaction Kit, and the results were read using the ABI PRISM 3100 DNA Analyser (Applied Biosystems, Inc., Foster City, Calif.). The data were assembled using Applied Biosystems software and nucleotide sequences were compared to the published sequences of the genes.

3. Results

3.1. SM resistance. The 306bp fragments of *rpsL* gene from the 66 SM resistant DNA isolates were analysed both by digestion with *MboII* endonuclease and by automatic sequencing. The *MboII* digestion

patterns are shown in Fig. 1. Cleavage of the 306bp fragment, observed in 26 (39%) isolates, indicates the presence of the wild type codon K43. In opposite, absence of cleavage in 40 (61%) of the isolates indicated nucleotide change at codon K43. Nucleotide sequencing of the 40 isolates confirmed change from AAG to AGG (K→R) at position 43 (K43R). Digestion with *MboII* was, therefore, confirming wild type nucleotide sequence at codon K43. The 26 isolates with the wild type *rpsL* sequence were further examined for mutations in two regions of the *rrs* gene encoding for the 16S rRNA. Analysis of the 912 region showed the wild type sequence in all the isolates tested. In contrast, 16 or 24% of all tested 26 isolates showed substitutions in the 530 loop region at nucleotide positions 513A→C (n=11) and 516C→T (n=5). The remaining 10 of these isolates or 15% of the all isolates tested showed the wild type sequence in these regions of the *rpsL* and *rrs* genes. In summary, 56 (85%) of the tested 66 SM resistant isolates, had nucleotide substitutions either at amino acid position K43 of *rpsL* gene or at nucleotide positions 513A or 516C of the *rrs* gene.

3.2 EMB resistance. SSCP analysis was applied to 103bp fragments of the *embB* gene from 33 EMB resistant MDR isolates. The analyzed fragments included codon M306 known to undergo nucleotide changes. Results of the SSCP analysis were then compared to results from nucleotide sequencing of the same fragments. The SSCP analysis showed patterns in 15 (45%) of the 33 EMB resistant isolates that were different from that of the EMB susceptible isolates (Fig. 2). The remaining 18 (55%) of the EMB resistant isolates showed SSCP patterns indistinguishable from those of the EMB susceptible isolates. Automatic sequencing of the 15 EMB resistant isolates that differed in SSCP patterns from the susceptible ones showed a nucleotide point mutation at codon 306 ATG→GTG or ATG→ATC resulting in the substitution of amino acid Methionine for Valine or Isoleucine. Nucleotide sequencing of the 18 EMB resistant isolates that could not be distinguished from the EMB susceptible isolates by SSCP analysis, showed the wild type sequence in 16 (48%) of the isolates and mutations in 2 (6%) isolates: ATG→ATA (M306I) in one and GGC→GAG (Gly314Glu) in the other. Obviously, these nucleotide substitutions don't change conformation of the 103bp single-stranded DNA fragments under the used electrophoresis conditions of the SSCP analysis. In summary, single nucleotide substitutions were detected by SSCP in 15 (45%) and by sequencing in 17 (52%) of the 33 EMB resistant isolates. The SSCP method revealed 15 (88%) of the 17 isolates that had mutations in the studied fragment of the *embB* gene showing good correlation with sequencing results.

SSCP method was also used to examine the 33 EMB susceptible MDR isolates. Interestingly, 6 (18%) of the 33 isolates showed SSCP patterns different from patterns given by a typical wild type sequence. Subsequent nucleotide sequencing revealed mutations in all 6 isolates: the mutation ATG→GTG (M306V) in four isolates, mutation ATG→ATC (M306I) in one isolate and the silent mutation GGC→GGT (Gly294) in another.

4. Discussion

The high MDR-TB level in Latvia can be explained by mono drug treatment regimens in the past century compounded by present social conditions today such as inadequate housing, poor nutrition and, generally, low socio-economic levels conductive to increase of MDR-TB cases. How could these factors influence the mechanism by which drug resistance in *M.tuberculosis* develops? Therefore, the alterations in mycobacterial genome should be studied and the correlation with the *in vitro* susceptibility testing results should be evaluated. The PCR-based methods are very useful for that aim since are able to detect specific nucleotide changes in sequences present in a low copy numbers. At the same time, the correct information on drug susceptibility is crucial for the clinicians and should be available for slowly growing mycobacteria in a short period of time. We have confirmed the suitability of the different methods for detection of changes in the already known genes in order to apply those further to clinical specimens of the *M.tuberculosis*. The results of our previous studies on prevalent mutations in the *rpoB* and *katG* genes in MDR isolates showed possibility to apply molecular methods directly to clinical specimens as it was later introduced in State Centre of Tuberculosis and Lung Diseases [23].

We have studied two genes, *rpsL* and *rrs*, known to be partially responsible for SM resistance. A percentage of the *rpsL* (61%) and the *rrs* (24%) gene mutations in part correlates with the previous reports, where alterations in the *rpsL* gene have been detected in 52.0 to 56.8% and mutations in the *rrs* gene were detected in 8.0-15.6% of the analysed SM-resistant isolates [10,15,21]. A slightly higher prevalence of K43R mutations may be explained by the high prevalence of Beijing genotype among Latvian MDR *M.tuberculosis* strains [24]. It is also based on observation that in our study that strains with the Beijing genotype (confirmed by RFLP analysis and spoligotyping) had the K43R mutation, whereas isolates with non-Beijing genotype showed the wild type sequence in the analyzed fragment of the *rpsL* gene (data not shown). Interestingly, there was one exception, when isolate with non-Beijing type, confirmed by the previously mentioned methods, had mutation K43R. This case is in contrary to previous founding [1] and should be additionally studied. Mutations in the *rrs* gene could therefore, confer SM resistance in the isolates lacking *rpsL* gene mutations. Mutations have been very rarely found in the both genes simultaneously; possible interplay of

mutations was proposed in several such examples [13,21]. By R.Cooksey [4], [13] a high level of phenotypic resistance to SM *in vitro* (>500μg/ml) is associated with mutations in the *rpsL* gene, whereas mutations in the *rrs* gene occur only in isolates with the low-level resistance to SM (MICs, 10μg/ml). Interestingly, none of our isolates showed mutation neither at *rpsL* codon K88 nor at *rrs* gene 912 region, both of those have been widely described by the other investigators [12,16]. Again, obtained results can be explained by the prevalence of particular genotypes in Latvian strains. At least, 10 (15%) remaining isolates may have alternative low-level resistance mechanisms mediated by drug-modifying enzymes or by reductions in drug uptake.

Many of the EMB resistant isolates could be rapidly identified by the analysis of the *embB* gene encompassing codon 306. We have evaluated SSCP method for analysis of the *embB* gene short fragment. Compared to nucleotide sequencing, SSCP is sensitive enough to reveal mutations M306V and M306I but not the change ATG306ATA, shown in one isolate and also resulting in Ile. Not surprisingly, that only 52% of the analysed isolates showed nucleotide alterations in the *embB* fragment. Therefore, analysis of alterations in the *embB* gene in order to detect EMB susceptibility is of limited value and should not be recommended for fast screening of clinical specimens. Ramaswamy [17] have analysed sequences of 12 genes that could be involved in EMB resistance and had showed that 68% of the isolates had mutations in the *embB* gene. At the same time, 51% of the isolates had mutations associated with EMB resistance in the only 1 of the 12 genes indicating multiple molecular pathways leading to the EMB resistant phenotype.

Surprisingly, in our study SSCP analysis revealed mutations at codon M306 in the *embB* gene of the five *in vitro* EMB susceptible MDR isolates. Similar results have been reported from Northwestern Russia, where mutations at codon M306 were found in 14 (48.3%) of the 29 EMB-resistant strains and in 48 (31.2%) of the 154 EMB susceptible strains [14]. Like in our study, the discrepancy between the results of phenotypic and genotypic EMB resistance tests was found in MDR strains only. Authors hypothesize that there could be a drug target in tubercle bacilli, other than one encoded by the *embB*; it could be affected by the EMB during the treatment with the other first-line anti-TB drugs.

In general, our data confirms the findings of the previous studies made on genes associated with resistance to first-line anti-TB drugs in a majority of *M.tuberculosis* isolates. More studies should be done to identify additional mechanisms of drug resistance in *M.tuberculosis* bacilli. Combined with the studies of *M.tuberculosis* genome (insertion sites, repetitive units, direct repeat region etc.) it should contribute our understanding of TB spread in population and of evolution of mycobacteria.

Acknowledgements:

This work was supported by the Latvian Council of Sciences (grant Nr 02.0011.15). We thank Vitauts Kalnins (University of Toronto, Canada) for his essential help in preparing this manuscript.

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Figures, Tables

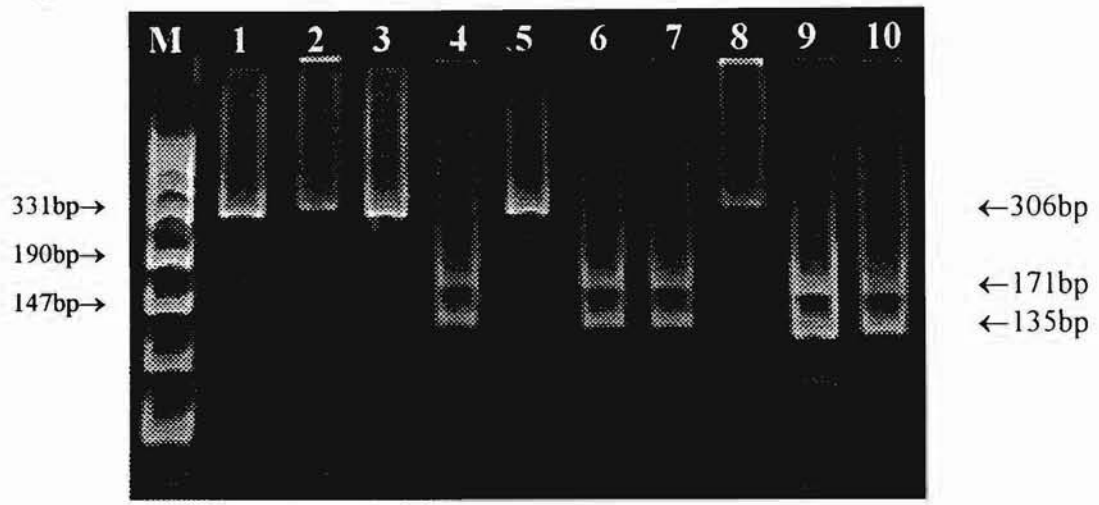

Figure 1. Digestion of the 306bp fragment of the *rpsL* gene with endonuclease *MboII*. Lane M, DNA molecular weight marker "pUC19 DNA/*MspI* (HpaII)"; lanes 1 – 10, digested *rpsL* gene fragments of SM resistant *M.tuberculosis* DNA isolates. Lanes 1, 2, 3, 5 and 8 contain undigested 306bp PCR products indicating a mutation at codon L43. Lanes 4, 6, 7, 9 and 10 contain digested PCR product (171bp and 135bp fragments) indicating the wild type codon L43 in this position.

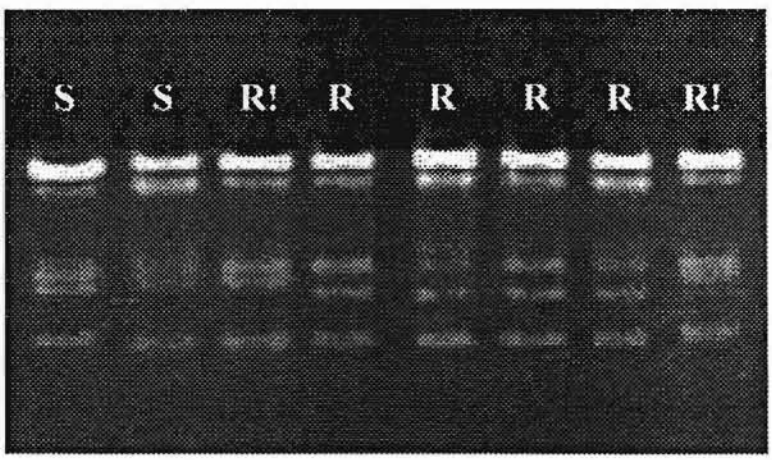

Figure 2. PCR-SSCP analysis of a 103bp fragment of the *embB* gene in 6% PAAG. S – 103bp fragment of the *embB* gene from EMB susceptible *M.tuberculosis* isolate; R – 103bp fragment of the *embB* gene from EMB resistant isolate; R! – 103bp fragment of the *embB* gene form EMB resistant isolate with a SSCP pattern undistinguishable from S.

APPENDIX 6

Resubmitted to ANTIMICROBIAL AGENTS AND CHEMOTHERAPY
New-data Letter

Spectrum of *pncA* mutations in multi-drug resistant *Mycobacterium tuberculosis* from Latvia

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Pyrazinamide (PZA) is a very effective anti-tuberculous agent particularly when used in combination with rifampin (RIF) and isoniazid (INH) (2). PZA becames active when bacterial pyrazinamidase converts it to toxic pyrazonoic acid (29). PZA resistance in *Mycobacerium tuberculosis* strains is associated with the loss of pyrazinamidase activity mainly due to mutations in the 561bp long *pncA* gene gene coding region or in gene expression regulating part (13, 26).

The molecular basis of resistance to PZA in *M.tuberculosis* have been extensively studied in last years. Nevertheless, an additional studies should be done in order to evaluate molecular methods for drug susceptibility testing directly in clinical specimens, since PZA susceptibility testing *in vitro* is complicated (Scorpio 97). In order to understand the molecular basis of PZA resistance, we examined *pncA* gene in 28 PZA resistant and 10 PZA susceptible cultures of MDR (multi-drug resistant) *M.tuberculosis* isolates from 38 Latvian MDR patients. In last decades, situation in Latvia can be characterised by a high TB morbidity and MDR-TB level (Surveill). Cultures were collected in State Centre of Tuberculosis and Lung Diseases between 2001 and 2002. No PZA monoresistant cultures were observed during that period. Cultures of the *M.tuberculosis* were grown on Löwenstein-Jensen medium for 4-6 weeks. Drug susceptibility was determined both by the absolute concentration method on slants and by the BACTEC method (Becton Dickinson, Sparks, Md.) (9,24) All 38 cultures were resistant to RIF, INH and SM, and 28 of 38 were resistant to PZA. Native DNA was isolated as previously described (28). We used primer set P1/P6 (27) to amplify the 720-bp fragment of the 558bp *pncA* gene and the surrounding DNA regions. PCR products were further analysed by automatic sequencing of both DNA chains using the ABI PRISM 3100 DNA Analyser (Applied Biosystems, Inc., Foster City, Calif.).

Results from automatic sequencing of the 28 PZA resistant isolates are shown in Table 1. Altogether, point mutations were found in 23 (82%) of the 28 PZA resistant isolates and were located in ten different codons within open reading frame of the *pncA* gene leading to an amino acid change. In addition, one mutation also resulted in premature synthesis termination. Codons T76 and Y103 were most frequently affected (43%). One isolate showed a mixture of the wild type and a mutant sequence, with mutations at two codons, C14 and Y103. We propose that the two mutations in this strain arose independently, because RFLP and spoligotyping analysis showed a single strain pattern (data not shown). Five of the isolates showed the wild type *pncA* gene sequence indicating alternative mechanism for PZA resistance. No mutations were detected in ten of the PZA susceptible MDR *M.tuberculosis* isolates.

In comparison to previous reports, we have detected mutations at three novel codons: C14Y, D63G and V180F; the remaining seven mutated codons have been previously described (3,14,15,17,19,27,31). In our study, most frequently mutations occurred in codons T76 and Y103, in spite of the R140, L85 and T47 codons, reported previously (3,14,15,17,19,27,31). Seven of our mutated codons (Q10, C14, P62, D63, C72, T76 and C138) were located in *pncA* gene three hot regions, as suggested by Scorpio (27), partially confirming his hypothesis.

To conclude, nucleotide sequencing remains the most appropriate tool for the analysis of the *pncA* gene where mutations are randomly dispersed. Taking in account a high percentage (82%) of mutations found in the *pncA* gene in Latvian isolates, we propose a direct sequencing on clinical specimens a screening method for molecular testing of PZA susceptibility.

This work was supported by the Latvian Council of Sciences (grant Nr 02.0011.15). We thank Vitauts Kalnins (University of Toronto, Canada) for his essential help in preparing this manuscript.

TABLE 1. Mutations detected in *pncA* gene of PZA resistant *M.tuberculosis* isolates

Number of isolates	Change(s) in :	
	Nucleotide sequence	Amino Acid
5 (18 %)	A→C at position 226	T76→P
5 (18%)	T→C at position 307	Y103→H
2	A→G at position 188	D63→G
2	C→D at position 216	C72→W
2	T→G at position 254	L85→R
1	C→T at position 28	Q10 →Ter*
1	G→A at position 41	C14→Y
1	C→A at position 184	P62→T
1	A→T at position 308	Y103→S
1	G→A at position 413	C138→Y
1	G→T at position 537	V180→F
1	WT / C→ G at position 42 + T→C at position 307	WT / C14→W + Y103→H
5 (18%)	WT	WT

Ter* - chain synthesis terminating codon
WT - wild type

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