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ABSTRACT BOOK

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I. STATE-OF-THE-ART LECTURES
2008: CENTENARY OF THE BCG VACCINE

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In December, 2008, we will mark the 100 years since *Albert Calmette* (1863-1933) and *Camille Guérin* (1872-1961) first presented their discovery –"bacille bilié"– to *Academie des Sciences*. 1908 is the year when the two French scientists started working with a virulent strain of Mycobacterium bovis ("lait Nocard") that had been isolated by Edmond Nocard (1850-1903) from a cow with tuberculous mastitis (Calmette,A.,1936). In 1921, after 230 successive passages on 5% glycerinated beef-bile potato medium over the course of 13 years , they obtained a resulting culture ("une race de bacilles-vaccins don't les caractères d'atténuation sont héréditairement fixés" – *Frappier, A*. and *M. Panisset*, 1957).

There is evidence for an ongoing evolution of BCG strains since their original derivation (*Behr, M. et al.,* 1999, *Honda,I*., 2006, *Brosch et al.*, 2007).

The problems associated with the standardization and quality control of BCG vaccines are caused by the fact that the various substrains used in different laboratories have differing biological characteristics, there are differences in the production methods and the assay procedures, as well a lack of reliable protection test for evaluation of the immunity in animals and humans (*Rodopska,S*.,1960,1971,1983, *Smith,D.W.* et al.,1971, *Osborn,T.W*., 1983, *Georghiu,M*. et al.,1988, *Behr,M.A.* et al.,2002).

The Bulgarian production strain

In 1948, Dr. *Srebra Rodopska* (1913-2006) brought the BCG strain from Paris after having been acquainted with the production of liquid BCG vaccine in the Pasteur BCG Laboratory founded by

Albert Calmette in Paris. As in about 1% of the newborns vaccinated orally suppurative cervical lymphadenitis had appeared, the Paris strain was substituted with the *Moscow strain* BCGI. After the new strain was introduced for production in Bulgaria, cervical lymphadenitis became an extremely rare complication.

Freeze-dried BCG vaccine

Freeze-dried BCG vaccine is one of the most beneficial achievements of medical science in Bulgaria (the regular production started in 1963). The purpose of BCG vaccination is to provide resistance to tuberculous infection in the form of cellular immunity; this has turned out to be induced most effectively by using live BCG, but not dead bacilli, in humans.

The properties of the freeze-dried BCG vaccine have been investigated in extensive studies through the following tests: viability, heat stability, oxygen consumption, local reactions (1, 3 and 12 months after immunization) and tuberculin sensitivity (3 and 12 months after immunization) in children. The variations found in the number of culturable particles (NCP) between the individual lots were within the acceptable limit ($1.5 – 6.0 \times 10^6$ /ml) and were not associated with any significant differences when investigated in the field. The examinations of one of the most important characteristics of the product exported mostly to tropical regions have shown good and consistent heat stability. The product possesses acceptable residual virulence and induces adequate tuberculin sensitivity in the children immunized by one and the same qualified team.

Since 1991 when the Laboratory became UNICEF and PAHO supplier, the product has been exported to more than 120 countries, being on the WHO list of UN Pre-qualified BCG vaccines (WHO, 2008).

Seed Lot System and the way of minimizing changes within BCG strains

The importance of minimizing the phenotypic changes which might occur very readily in BCG strains during the course of vaccine production was emphasized during the Meeting "WHO Consultation

on the Characterization of BCG Strains" in London (Corbel,M.J. et al., 2003). The reduction in emergence of variants could be achieved by minimizing the number of passages from the Master seed. Seed cultures should be established from single colonies and kept under stable conditions, e.g. freeze-dried.

Quality control

The key to effective quality control is rigorous testing and validation applied to the starting materials, to the different stages of the production process and to the final product.

The necessity for improvement of the quality control of BCG vaccines has been identified as a WHO priority (WHO, 2005). WHO has recognized the need to improve both characterization of BCG vaccine and the assays used for its quality control, taking into account recent advances in *genetics and molecular biology*.

Techniques of the molecular biology

- To detect DNA specific for virulent mycobacteria ; possible use in detecting virulent mycobacteria in BCG vaccine;
- To determine the capacity of a given product to protect against virulent challenge in animal models;
- To reveal the causes, the mechanism and the relevance of the phenotypic variations;
- To characterize BCG substrains;
- Possibility of monitoring consistency of the production of BCG vaccines;
- To distinguish between Mycobacterium tuberculosis and M.bovis and the BCG strain, to subdivide the BCG substrains, to check for variations arising during the production.

Genetic properties of the Bulgarian BCG vaccine

Genotyping assay was performed on:

- Master Seed Lot SL222
- Working Seed Lot 7-93
- Commercial vaccine batches

Main characteristics of BCG Sofia

- Presence of RD2 Region (typical for the early BCG strains)
- DU2-type Duplication (from Rv3299 to Rv3316)
- Novel 1.6-kb deletion that affects the Rv3697c and Rv3698 (related to membrane protein in the cell wall structure)

Mycobacterium bovis BCG Sofia

- SL 222 Sofia reveals the genetic characteristics which pertain only to the closest to the original Calmette and Guérin strains;
- The genetic identity, the provenance and the genetic stability of the Bulgarian BCG vaccine for a period longer than 30 years have been proved.

Conclusion

BCG vaccine is still the only currently available means for immunization against tuberculosis infection. The message of the researchers involved in the search for new tuberculosis vaccines (Vienna – Second International Conference on TB Vaccines for the World, 2006, Varadero, Cuba – TBVaccines07) was that an effective TB vaccine by the year 2015 is an ambitious goal. They also considered it to be a realistic goal. Over the last 6 years the candidate vaccines were moved from the laboratory into clinical development programmes. The first generation of new TB vaccines is currently in phase I or II of clinical trials while the next generation of TB candidate vaccines is in various stages of development, in preclinical testing or is scheduled for clinical testing.

A vaccine to both prevent TB and reduce its transmission is urgently needed but BCG vaccine will remain in use in the foreseeable future. Due to the fact that the use of BCG vaccines will continue,

either as stand alone or as a prime vaccine in prime-boost immunization strategies in conjunction with new vaccines against tuberculosis, the necessity for further work in better characterization of the BCG vaccine and of the production strain has been identified (WHO, 2004, 2005). The BCG vaccine is still subject to regulatory practices devised many years ago. Since then, the defining of new concepts has begun.Collaborative studies are needed for many of the molecular methods and applications, and investment in training, equipment and facilities is needed in order to promote and enhance these developments.

BCG ADJUVANCY AND NEW STRATEGIES FOR VACCINES IN DEVELOPING COUNTRIES

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Summary

Due to the epidemiology of tuberculosis in Africa, African children are routinely vaccinated with the Mycobacterium bovis BCG within the first week of their life. Thousand of BCG vaccinations performed in children infected with HIV at the birth indicate that this kind of vaccination is safe in HIV-infected host and dissemination of BCG is a rare event.

The design of a protective vaccine against infections such as HIV or hepathisis B, based on synthetic/recombinant peptides, is limited by their poor immunogenicity. However, it has been observed that the synthetic V3 loop peptides of HIV when covalently bound to PPD (purified protein derivative of Mycobacterium tuberculosis) as carrier protein are capable to exhibit over time a uniform increase in neutralizing antibodies when injected intradermally in PPD skin test-positive subjects.

BCG (live Calmett-Guerin strain of Mycobacterium bovis) has been shown to possess a strong systemic and mucosal adjuvant activity, which can induce both humoral and cell-mediated immune response. In particular, viable BCG has been described that induced the signaling via both Toll-like receptors (TLR2 and TLR4) causing the maturation of immature DC into fully activated mature DC. Moreover, infection of DC with BCG facilitates secretion of pro-inflammatory cytokines (IL-1, IL-12, TNF, IFN, etc.) and up regulates CD40, CD80, CD83, CD86, and MHC class I molecules. Moreover, DC exhibit potent antigen presenting ability through uptake of BCG, and this activity can be used on soluble antigens other than BCG itself. Furthermore, conditioned DC can be a temporal bridge between CD4 TG helper and CD8 cytotoxic T cells.

BCG has been also described to induce a potent B cell response with production of IgG and IgA. As one of the possible risk of HIV transmission in new-born occurs at the oropharyngeal mucosa level through breast feeding, the possibility to increase mucosal immune responses, particularly IgA-mediated, could represent an important mechanisms of prevention from HIV transmission.

Finally, we have recently demonstrated that BCG induce differentiation and maturation of monocytes/macrophages into activated DCs determining up-regulation of MHC Class I, Class II, CD80, CD86 and CD40 in cells.

The approach of developing recombinant BCG constructs expressing other pathogens proteins, such has the simian immunodeficiency virus (SIV) Gag, Pol, Env, and Nef proteins, it has been shown to be successful in inducing protection in mice and rhesus macaques, also when administered intranasally in Th1 and Th2 deficient condi-

tions. However, the safety aspects linked to the use of live recombinant bacteria limit and delay the usage of this kind of vaccine in the next years.

A more reliable approach in using the strong adjuvancy power of the BCG, is represented by the co-administration/co-formulation of BCG with recombinant vaccine products. In this context, it has been recently observed that the co-administration at the childbirth of the Hepathisis B together with the BCG is capable to induce an antibody response against the Hepathisis B vaccine much stronger compared when the two vaccines are given separately, making this vaccine strategies particularly effective in developing countries.

II. GUEST SPEAKERS` PRESENTATIONS (GS)

GS–01
MYCOBACTERIUM TUBERCULOSIS COMPLEX MOLECULAR EVOLUTION METHODS. RESULTS FROM A SOUTH-EASTERN EUROPE PERSPECTIVE

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Summary

The study of the population molecular structure of the *Mycobacterium tuberculosis* complex (MTC) is the focus of active research both for public health and evolutionary genetics goals. It is now admitted that MTC is a made up of a limited number of founding genotypes, which were shown to be spatially and temporarily structured, however there are still many blackholes in our knowledge of the MTC genetics landscape and especially on the time-frame of its evolutionary history. Indeed, the population structure of MTC is a never-ending dynamical process which results from the superimposition of numerous clonal complexes which are under the control of ecological, anthropological and epidemiological parameters. Combined to this layer of genetic diversity is the genetic diversity of hu-

man hosts with their own actions on the pathocenosis, i.e. the action-reaction dynamic process of infection, escape, control in individual patients. Little is known about the population molecular genetics of MTC in the Balkan region, a mosaic though strategic region of Europe with a moderate prevalence of tuberculosis.

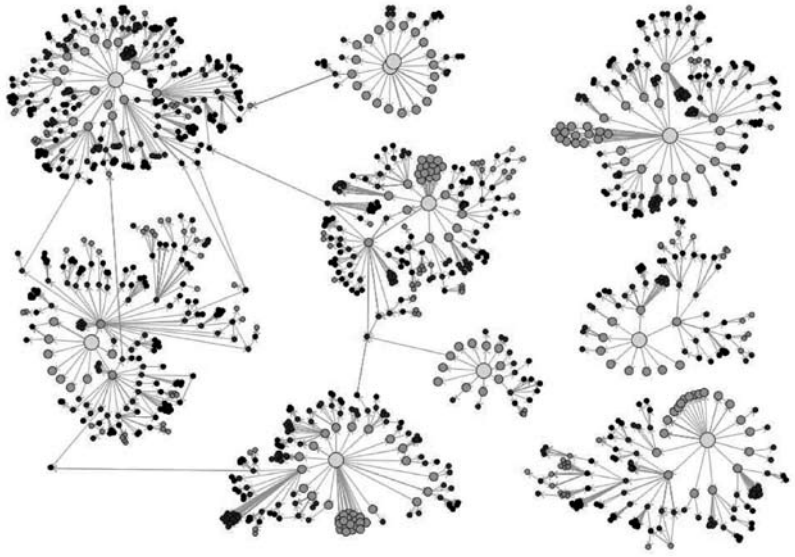
The Balkans is the historical and geographical name used to describe a region of South-eastern Europe. The region has a combined area of 550.000 square kilometers and an approximate 55 millions people. The region takes its name from the Balkans mountains. Although it is a region of moderate TB incidence, it is a crossroad of various cultures, hence a region of great interest to study the origin, the genetic diversity and the population structure of *Mycobacterium tuberculosis* complex (MTC); the Balkans are also a region of great strategic importance and some countries in this region including the smaller ones built on the former Yougoslavia go through much difficulties, lesser access to health services, hence more efforts should be put to improve health infrastructures and economy in these countries which are not yet part of the 27 countries in the United Europe.

After an update on the perspectives offered by recent development in (i) genotyping methods (ii) new publicly available databases for bacterial genotyping (iii) new algorithms and bioinformatical methods that all contribute to an expanded knowledge of MTC population molecular strucutre, and a thorough discussion in terms of cost/benefit, we will try to build a snapshot of the current MTC genetic diversity which is observed in the Balkan region, based on both published and unpublished results. In particular, we will put a specific emphasis on recent results obtained in Bulgaria and Albania, and will compare the structure observed with previously studied neighboring countries. Results obtained using spoligotyping, classical 5 and 12 VNTR[1] or even 24 loci typing schemes will be reported.

We will also discuss the limitations of current molecular epidemiological methods and will suggest new possibilities to improve MTC molecular population research. Such perspectives would be interesting both for the sustainable development of this part of Europe and for

[1] Variable Number of Tandem Repeats

the building of a common european identity which will respect both genetic diversity and people rights. The possibility to use recent results obtained using the 3R genes (DNA repair, replication, recombination) to achieve a better knowledge of MTC will also be discussed.



GS–02
ANTIBIOTIC RESISTANCE AND DRUG SUSCEPTIBILITY METHODS

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Summary

Tuberculosis (TB) represents a major public health problem, especially in low-resource countries where the burden of the disease is higher. Multidrug-resistant and extensively drug resistant TB constitute a serious threat for the effective control of the disease stressing the need for the rapid detection of resistance to first- and second-line drugs. Conventional methods to detect drug resistance in *Mycobacterium tuberculosis* have traditionally relied on slow and cumbersome procedures requiring a minimum of 3–4 weeks to produce results. The proportion method, the resistance ratio method and the absolute concentration method, all performed on solid culture media became the reference standard for this purpose. Later on the radiometric BACTEC TB-460 system using an enriched liquid-medium was used for drug susceptibility testing especially to first-line drugs, decreasing the turnaround time to obtain results. The dependence of the system on the use of radioactive materials was an inconvenience for a wider application especially in low-resource countries.

Several new approaches have been proposed and tested in the last years for the rapid and timely detection of drug resistance of *M. tuberculosis*. Liquid culture-based methods including the Mycobacterial Growth Indicator Tube (MGIT), the BacT/Alert 3D system, and the Versa TREK system, all commercially available, rely on the fact that faster growth is usually obtained in liquid medium. The MGIT system has been the most widely used and evaluated; both in its manual and automated version the BACTEC MGIT960 sys-

tem. Studies that evaluated the MGIT manual system for susceptibility testing to first-line drugs reported good results and accuracy for detecting resistance, especially for isoniazid and rifampicin. Very few published studies, however, exist evaluating the manual MGIT for susceptibility testing to second-line drugs or directly in sputum samples. The automated version, BACTEC MGIT960, has also been thoroughly evaluated for susceptibility testing to first-line drugs with good results. A recent meta-analysis of the many published studies found high accuracy and high predictive values associated with the use of BACTEC MGIT960 (Piersimoni et al., 2006). Also, a multicentre study has been performed to test susceptibility to several second-line drugs giving accurate results when compared to the BACTEC TB-460 radiometric method. The investment required to obtain the automated equipment and its large capacity of workload may limit, however, its adoption by small- and medium-sized diagnostic laboratories in low-resource countries.

Colorimetric methods have also been recently proposed and evaluated for the rapid detection of drug resistance in *M. tuberculosis*. In this category two types of tests are considered, those based on the use of redox indicators to assess the growth of *M. tuberculosis* in the presence or absence of an antibiotic and those based on the nitrate reductase assay. A coloured oxidation-reduction indicator added to the culture medium after *M. tuberculosis* has grown in the presence or absence of antibiotics is the basis of the first test. Reduction of the indicator produces a change in colour of the medium that is easily interpreted visually with results available in 7-8 days. (Palomino et al. 2007). The nitrate reductase assay is based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite which is then easily detected in a coloured reaction. Reduction of nitrate after growth in the presence or absence of antibiotics is also the basis of this test. Both colorimetric tests have been thoroughly evaluated in different settings to detect resistance to first-line drugs and some second-line antibiotics with very good results. Recent meta-analyses of colorimetric methods using redox indicators or the nitratase test for susceptibility testing of *M. tuberculosis* have shown evidence of the high accuracy of these methods (Martin et al., 2007; Martin et al.,

2008). An additional application of the nitrate reductase assay is that it can be used directly on sputum samples.

Another category of methods proposed for the rapid detection of drug resistance are those based on the detection of micro-colonies of *M. tuberculosis*. The microscopy observation drug susceptibility (MODS) and thin-layer agar (TLA) methods belong to this category. Both methods are based on the early detection of the characteristic growth of *M. tuberculosis* forming cords, which are detected microscopically. MODS is performed in liquid medium while TLA is performed on agar. Results are available within a week (Moore et al.

Other techniques like those based on phage technology (*FastPlaque*), the E-test, slide-culture technique and TK medium have also been proposed and tested. From these only the *FastPlaque* has been evaluated in several settings giving variable results for the detection of rifampicin resistance.

Molecular methods have also been proposed for the early detection of drug resistance, although they are mainly aimed at the detection of resistance to rifampicin and isoniazid; among these the INNO-LiPA Rif.TB and the GenoType MTBDRplus, both commercially available have been described and evaluated. DNA sequencing to look for gene mutations associated with drug resistance remains a reference standard.

The continuous emergence of drug resistance calls for improved methods for its timely detection, which will contribute for a better control of the disease and help contain the dissemination of drug resistant TB.

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GS–03
ADVANCES IN THE MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS AND ANTI-TUBERCULOSIS DRUG RESISTANCE IN EUROPE

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Purpose

To highlight findings and ongoing research in the molecular epidemiology of tuberculosis with emphasis on anti-TB drug resistance.

Methods

DNA fingerprinting.

Results

The population structure of *Mycobacterium tuberculosis* differs significantly by area. Generally, the circulating genotypes are characteristic of a given area and the clonality among isolates is significantly higher in high prevalence settings. Recent research pointed out that the average proportion of Beijing genotype strains in 10 provinces of China amounts 73%, but varies significantly by province within this country. The highest density was observed in the Beijing province; 93%.

In an earlier study in Ho Chi Minh City, Vietnam, the Beijing genotype was found significantly correlated with low age, which probably reflects an association with active and recent transmission and, hence, with emergence. In the last two years a study was conducted to examine the situation in the rural Mekong Delta of Vietnam.

Of 2097 isolates with complete genotyping data (90% of all registered patients), 681 (32%) were of the Beijing genotype; 1063 (51%) of the Vietnam genotype (EAI) and 353 (17%) of other genotypes. This distribution of genotypes was found markedly different from that found earlier in Ho Chi Minh City, where 50% of the isolates were of the Beijing genotype. This suggests that a high density of Beijing strains may be related to crowding of the human population.

The overall case notification rate in Vietnam declined significantly with 5.1% per year (95% CI -5.9%~-4.4%). The overall annual decrease in case notification was 5.9% (95% CI -11.3%~+2.1%) for the Beijing, versus 4.4% (95% CI -9.6%~-1.2%) for the Vietnam genotype. However, in the age group 15-24 years, the case notification rate increased by 5.2%, reflecting an increase for both the Beijing (9.2% per year) and the Vietnam genotype (2.0%). This finding may indicate that also in rural Vietnam the Beijing strains will be increasingly found in the future. As also in the Mekong Delta a highly significant correlation was observed between the Beijing genotype and multi-drug resistance (MDR) among new cases, this may partially explain the lack of anticipated decrease in the TB rates in the lowest age category.

In an earlier population-based study in Vietnam a correlation was observed between presence of a BCG vaccination scar and the Beijing genotype. However, this correlation was not significant. Recently, two evolutionary lineages of the Beijing strains were distinguished on the basis of IS*6110* RFLP profiling and insertion of IS*6110* in the NTF region. Atypical Beijing strains more closely resemble the common ancestor of all Beijing strains. Typical Beijing strains have a variety of mutations in putative mutator genes which may provide them with a selective advantage over other *M. tuberculosis* strains. In Vietnam, 25% of the Beijing strains represent the atypical and 75% the 'typical' lineage. Re-analysis of the previous study learned that atypical Beijing strains are inversely correlated with BCG vaccination. This may reflect that atypical Beijing strains are less capable of circumventing the BCG-induced immunity than typical Beijing strains. Within the framework of the European Union project 'TBadapt', a consortium of 11 international research partners is trying to disclose the evolutionary adaptation of *M. tuberculosis* in response to vaccination and exposure to anti-tuberculosis drugs.

In Europe, IS*6110* RFLP profiles of MDR-TB isolates are being collected to trace international transmission and we analyzed the data from the period of 2003-2007. Twenty-four European countries, including 20 from the European Union, reported 2,494 MDR-TB cases, of which 65% were reported from Estonia, Latvia and Lithuania. A total of 672 (40%) IS*6110* RFLP profiles were available from 19

countries. Of these 672 cases, 288 (43%) belonged to 18 European clusters associated with MDR-TB cases in 16 countries. The size of the clusters ranged from 2 to 174 cases. Beijing strains caused the cases in 7 clusters, representing 84% of the clustered MDR-TB cases and including the largest cluster, of which 148 cases (85%) were reported in Estonia. As in general only 6-7% of the TB cases in Europe are caused by Beijing strains this clearly indicates the significant role of Beijing strains in transmission of MDR-TB in this region.

Extensively drug resistant (XDR) TB strains have been reported all over the world and we searched our data on resistance profiles from *M. tuberculosis* isolates in Europe for the prevalence of XDR-TB. Ten percent of 1,537 MDR-TB cases subjected to second-line drug resistance testing in 19 countries appeared to represent XDR-TB and this was significantly correlated to the Beijing genotype. The main determinant of XDR-TB was origin of the case from countries of the Former Soviet Union.

Conclusion

Since 1993, IS*6110* restriction fragment length polymorphism (RFLP) typing has gained recognition as the gold standard in the molecular epidemiology of tuberculosis. However, although the introduction of this methodology caused a revolution in the epidemiology of tuberculosis, it is technically demanding and time consuming. Therefore, the widespread introduction of the PCR-based variable number of tandem repeat (VNTR) typing seems an important step forward. A part of the collection of nearly 15,000 *M. tuberculosis* isolates in The Netherlands is currently being subjected to 24 loci VNTR typing. The overall concordance between IS*6110* RFLP and VNTR typing is high and the detailed results will be discussed.

GS–04
CLONAL EXPANSION AND MICROEVOLUTION OF MULTI-DRUG RESISTANT M.TUBERCULOSIS

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Summary

Drug resistant *Mycobacterium tuberculosis* complex (MTBC) strains have emerged as a serious threat to tuberculosis (TB) control worldwide[1]. Rates of multidrug resistant (MDR-TB, resistance at least to isoniazid and rifampicin) strains have reached levels of up to 14% among patients never treated and up to 40 % among previously treated patients in several MDR TB "hot spots" such as the Henan province in China or Karakalpakstan (Uzbekistan) and Kazakhstan in Eastern Europe[1-3]. MDR TB is associated with much poorer treatment outcomes compared to drug-susceptible TB and a considerably increased risk of developing further resistances[2,4,5]. Moreover, prolonged periods of infectivity result in enhanced transmission of drug resistant strains further accelerating rates of drug resistance[6].

Even more worrisome is the emergence of a nearly untreatable form of TB – namely extensively drug-resistant (XDR) TB – which is defined as MDR with an additional resistance to any fluoroquinolone and at least one of three injectable drugs (i.e., amikacin, kanamycin, or capreomycin). A recent survey confirmed the worldwide presence of XDR strains with levels of up to 15% of MDR TB cases[7]. Considering the difficulties and problems associated with treatment of resistant TB, high levels of MDR and XDR TB have the clear potential to jeopardize TB control on a local or national level.

In addition to various measures for strengthening TB control such as rapid case detection, proper treatment and rapid detection

of drug resistance, the long term effect of the emergence of drug resistant strains on the worldwide TB epidemic also depends on the relative fitness of MDR and XDR compared to susceptible strains[6]. While initial experiments indicated a lower fitness of resistant MTB strains, recent results using laboratory-derived mutants demonstrated that the fitness of resistant strains depends on the kind of mutations (codon and position) as well as on the genetic background of the strain (i.e. strain lineage). Adverse effects on bacterial fitness can be reversed by compensatory mutations occurring during long term infection and ongoing transmission. Indeed, MDR variants with enhanced fitness compared to susceptible progenitor strains have been described[8]. Using this data in models for prediction of the MDR TB epidemic, it turned out that even in the case of a well-functioning TB control system, small subpopulations of comparatively fit MDR clones might out compete susceptible and less fit resistant strains and become the dominant clones in future with dramatic consequences for TB treatment and control[9].

The most striking association between mycobacterial genetic background and drug resistance documented so far has been described for strains of the so called Beijing genotype. These strains have been found to be involved in outbreaks and transmission of MDR TB in several areas of the world[10]. In Eastern Europe, several studies report a clear association between Beijing genotype infection and drug resistance[10]. In our own work, we confirmed that Beijing genotype strains are a major cause of TB, are strongly associated with any form of drug resistance (patients with Beijing genotype infection had a 4 times higher risk to have an MDR TB when compared with patients infected with a non-Beijing strain) and strongly contribute to the transmission of MDR TB the Aral Sea region in Karakalpakstan (Uzbekistan) and Turkmenistan [11]. In particular, two large clusters of rapidly spreading clones have been determined which also represent the dominant strains in an ongoing survey of MDR TB in Karakalpakstan. Similar observations have been recently reported from South Africa where a rapidly spreading highly resistant clone represents nearly half of all cases in the George subdistrict[12]. The combination of experimental and epidemiological observations pro-

vide strong evidence pointing to a microevolution in resistant MTBC strains that selects for mutations compensating for resistance phenotypes and resulting in a particularly virulent, and thus highly successful pathogen. As a consequence, these rapidly spreading, highly resistant and "hypervirulent" *M. tuberculosis* strains may become dominant in the future in some regions of the world.

To date, the nature and consequences of microevolution in resistant *M. tuberculosis* complex outbreak strains have not been defined yet. Molecular mechanisms involved in enhanced capacities to acquire resistance, enhanced virulence, and in the adaptive evolution of successful spreading MDR clones are entirely unclear.

To shed some light on this point, we have applied a massively parallel sequencing approach (Illumina Genome Analyzer System) to compare the genome sequences of a susceptible and a MDR variant of a outbreak strain from a region with the highest rate of MDR TB observed worldwide (Karakalpakstan)[1,3]. First analyses revealed a striking genomic diversity that will be explored further.

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GS–05
GENOMIC ANALYSES OF BCG VACCINE STRAINS

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Summary

The elucidation of the genome sequence of *Mycobacterium bovis* BCG Pasteur provided us with the blueprint of the most widely used vaccine in the world [1]. The sequence revealed a number of surprises, not least of which was the extensive gene duplication events that had taken place during the derivation of BCG. This was the first

example of genome rearrangements in a member of the *M. tuberculosis* complex, and most likely reflects the in vitro culture pressures that led to the attenuation of the *M. bovis* progenitor. The first of these duplications, DU1, is only found in BCG Pasteur and encompasses the origin of replication; hence BCG Pasteur would appear to have two functional copies of *oriC*. The second event, DU2, exists in 4 forms across the BCG daughter strains with a core region that contains *Rv3300c-glpD2* conserved across all four configurations. The distribution of DU2 allows a duplication genealogy to be defined that is concordant with of Behr and colleagues [2] which was constructed using deletion events (Figure 1).

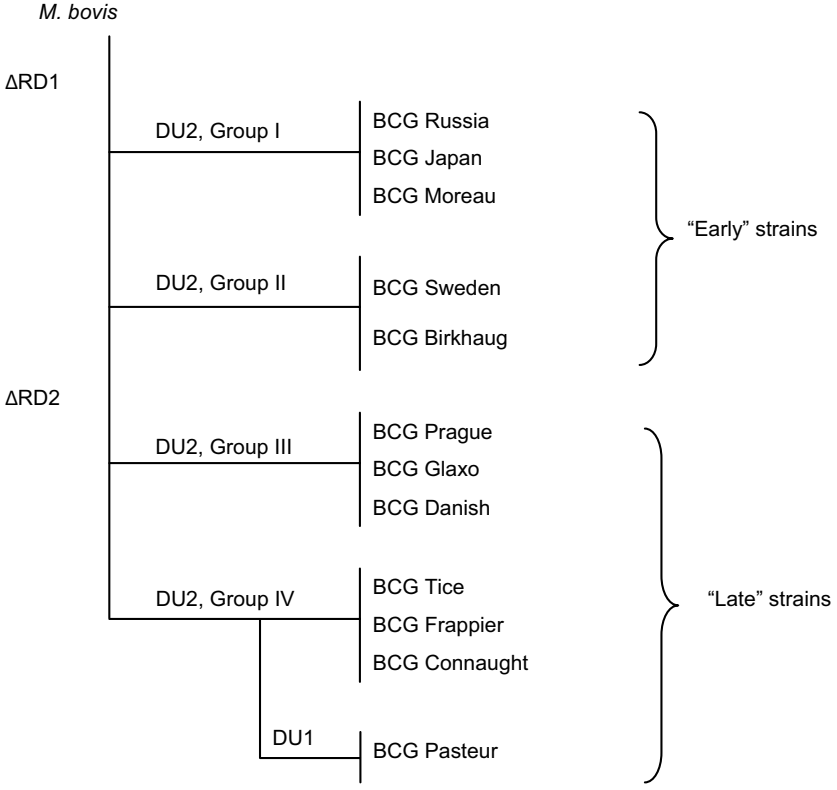

Figure 1: Duplication Phylogeny of BCG strains.

The DU2 duplication exists in 4 configurations across BCG strains that can be mapped back to the deletion-based phylogeny. The DU1 duplication in BCG Pasteur is a unique event restricted to the latter strain.

The key role for the RD1 deletion in the attenuation of the *M. bovis* progenitor has been clearly shown. However, the role of these duplication events, if any, in the attenuation process is still undefined.

BCG was derived by the in vitro serial passage of a French strain of *M. bovis*. Comparison of the genome sequences of *M. bovis* 2122 (a UK strain) and *M. bovis* BCG Pasteur revealed 736 single nucleotide polymorphisms (SNPs), with 440 non-synonymous SNPs (that cause an amino acid change in the encoded protein), and 204 synonymous SNPs (that do not translate into an amino acid change). To determine the role of these SNPs in both the attenuation of BCG and the genetic basis of phenotypic variation across BCG substrains, we screened the distribution of these SNPs against a panel of BCG and *M. bovis* strains. We selected *M. bovis* strains from both the UK, as these are closely related to the sequenced strain *M. bovis* 2122, and French *M. bovis* strains that were predicted to be closely related to the BCG vaccine strains since they had the same spoligotype as the BCG strains. In this way we hoped to determine the distribution of these SNPs in UK and French *M. bovis* strains, allowing us to determine which differences between the 2122 strain and BCG Pasteur were simply differences between French and UK *M. bovis* strains, and which were specific to the BCG lineage and hence implicated in attenuation. In total we examined 13 French strains, 7 UK strains, and 13 BCG daughter strains.

Genotyping was performed with IPLEX chemistry, on the SEQUENOM genotyping platform (Sequenom Inc.) [3]. Only 701 SNPs gave reliable data, and this could then be used to construct a phylogenetic tree. From this we could see that the UK, French and BCG strains clustered together, but that the French strains were more closely related to the BCG strains as we expected. We were also able to validate and extend the phylogenies of BCG that had previously been proposed using deletion or duplication events. Furthermore, the analysis allowed us to define a "maximum" SNP distance between virulent *M. bovis* and BCG, reducing our previous 736 SNPs to 185

SNPs. Hence, these SNPs may have played a role in the attenuation of BCG, and we are functionally characterising some candidates.

Acknowledgments

This work is a collaborative project between UCD, VLA (Weybridge), Institute Pasteur (Paris), the Wellcome Trust Sanger Institute, and the Global Health Institute (Lausanne), and I acknowledge all my collaborators across these centres. Funding for this work was provided by the Wellcome Trust and Defra (UK).

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GS–06
IMMUNE RESPONSES TO TUBERCULOSIS

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Summary

Tuberculosis (TB) is a chronic bacterial infection that still causes more deaths worldwide than any other infectious disease. It is re-

sponsible for 3 million deaths for years and one-third of humanity is infected by *Mycobacterium tuberculosis* (Mtb). Key reasons for this are the unavailability of an efficacious vaccine for adult disease; the rise of multidrug-resistant strains, and the dreadful association of TB with human immunodeficiency virus infection. Moreover, TB is a disease in which traditional prophylactic approaches have been largely unsuccessful.

On the first encountering Mtb, the majority of individuals produce an effective immune response leading to the control of the infection. Consequently, most of these individuals experience a mild disease (primary TB), often without clinical relevance. T cells specific for Mtb antigens are, in fact, expanded and limit Mtb growth either directly by killing intracellular Mtb or indirectly by secreting cytokines such as IFN-γ and TNF-α, which promote killing by macrophages. However, the immune response that causes such a clinical cure does not clear the bacilli from infected individuals and after primary TB, Mtb persists causing a latent infection.

The notorious success of Mtb as a highly adapted human pathogen rests on its ability to evade the immune response and, in turn, to persist in an immunocompetent host. The persistence of Mtb in macrophages contrasts with the observed induction of a vigorous specific immune response, which is, however, rarely effective in eradicating the infection and suggests the ability of Mtb to induce some form of immune-regulation. In immunocompetent adults TB typically occurs as a reactivation of pre-existing *foci*. This suggests the existence of a dynamic balance between the host immune system and Mtb. In most cases, the host response is sufficient to forestall activation of the disease for a lifetime. However, occasionally the immune response fails in some way and the infection reactivates to cause active disease. The immune factors contributing to the establishment of latent infection and the immunological components that are required to maintain such an infection and prevent reactivation are still unknown. Regarding the adaptive response of mycobacteria to host's antimicrobial defenses, it is unclear how the bacilli survive in the face of a strong immune response, whereas it is now clear that they profoundly change several metabolic activities leading to a non replicating status (dormancy).

In any case, the capacity of immune system to limit primary infections and to be unable to control mycobacterial growth in the presence of a vigorous memory immune response represents a paradox in the immune response to Mtb. The inability of the immune response to clear reactivation TB is a failure of the memory immune response rarely observed in other infectious diseases. Since there is no variation in the antigenic array on resuscitated mycobacteria that cause post primary disease other factors must be responsible for the observed failure in controlling postprimary TB. It will be discussed the possibility for an excessive regulation and of the capacity of Mtb to interfere with innate immunity as possible causes concurring to the inability of the immune response to control reactivation TB.

GS–07
HOST – PATHOGEN INTERACTIONS IN TUBERCULOSIS

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Summary

My presentation will focus on host-cell pathogen interactions in tuberculosis. I will start with a brief introduction to the TB disease. It is important to remember that merely an estimated 5-10% of individuals exposed or infected with *Mycobacterium tuberculosis* bacteria actually develop TB. In other words, the human population, as a whole, is extremely resistant to the disease. Despite this resistance, approximately 1.5 million people die of tuberculosis each year. This figure can be explained by fact that an estimated two billion people are infected with latent TB. Thus, although the activation rates of the

disease are rather low, this huge reservoir leads to high numbers of diseased and deaths. Talk will be divided in the following parts:

I.Factors Influencing the Development of the TB Disease

Multiple factors explain why certain individuals are more likely to develop the disease than others, including nutrition and hygiene, HIV co-infection, sex and age, as well as host genetic factors and the genotype of the infecting strain. This will be discussed in more details.

II. Public Health Measures

We currently possess two main tools to combat *Mycobacterium tuberculosis*. The first is the BCG vaccine. Unfortunately, BCG is probably not sufficiently efficient to prevent TB, at least in adults. The second tool consists of four first-line antibiotics. It is noteworthy that since the 1960's, no new first-line antibiotics has been developed. Thus, The WHO projects that by 2020, there would be 1 billion newly infected people, 200 million sick, and 35 million deaths from tuberculosis. If we wish to prevent this scenario, and live in a TB-free world, we must develop a new vaccine and new drugs to fight the disease.

III. Host-Pathogen Interactions

When an individual is infected with *Mycobacterium tuberculosis*, the bacilli enter his or her lungs. In order to become potentially infectious, the bacilli must first enter the alveoli. Inside the alveoli, they interact with cells, mainly macrophages and dendritic cells, through different receptors.

I will focus on the interactions between the bacillus and the first cells it encounters in the lungs during the infection. In spite of the immune response, one of the major virulence mechanisms in TB is the ability of the bacillus to parasitize the macrophages; that is, to persist and multiply within the cells. On of the fundamental questions of tuberculosis basic research is how the bacillus can persist and multiply within these cells.

In the remainder of my talk, I will like to focus on three aspects. The first is the entry of the bacillus into the cell, and the receptors involved in this phagocytosis process. The second is the cell's reaction to the infection. Finally, I will review the strategies used by the bacilli to persist and survive inside the cell.

1. Cell Entry

Many receptors are involved in the phagocytosis of *Mycobacterium tuberculosis*, which will be discussed extensively, with a particular focus on DC-SIGN, a receptor on which we have worked in the past years [1,2].

2. HOST CELL RESPONSE TO THE INFECTION

I will then discuss the host cell response to infection and the consequences, in terms of intracellular signalling and cytokine secretion, of the mycobacteria-host cell interactions through the receptors discussed above, notably DC-SIGN and Dectin.

3 INTRACELLULAR SURVIVAL STRATEGIES OF THE BACILLUS

I will then focus on the strategies used by the bacillus to survive inside the cell. *Mycobacterium tuberculosis* can survive inside macrophages and this is not typical in bacteria [3]. I believe that if we manage to understand how the bacillus can parasitize the host cell, and to identify the mycobacterial genes involved in this process, we might be able to design new antibiotics, which are specifically directed against these genes. We might also be able to generate new mutants and attenuated strains of TB, in order to develop new vaccines.

a. Understanding the TB Genome

The publication of the TB genome in 1998 was a major milestone in the history of TB [4]. We can now wonder how to convert this mass of information into a useful understanding. Indeed, the TB genome is extremely frustrating, since over 40% of its genes do not reveal any information.

b. Functional Genomics

Functional genomics can help understanding the TB genome, which will be discussed, through mainly the example of Signature tagged Transposon Mutagenesis (STM) used at various levels, from the whole organism to the subcellular level [5].

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III. ORAL PRESENTATIONS (OP)

OP–01

STANDARDIZED PCR-BASED GENOTYPING OF *M.TUBERCULOSIS* ISOLATES FROM HIGH TUBERCULOSIS BURDEN REGIONS: LESSONS FROM BRAZIL AND COUNTRIES WITH PREVALENT BEIJING LINEAGE

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Study objectives

Genotyping of Mycobacterium tuberculosis isolates is a powerful tool for epidemiological control of tuberculosis (TB). Its use in high TB burden countries has been restricted hitherto by constrained resources,

technical limitations of standard IS6110 fingerprinting, and, in general, by dominance of geographically specific and genetically homogeneous strain lineages. A recently internationally standardized PCR-based genotyping system based on amplification of 15 to 24 mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) loci, combined with spoligotyping, has been shown to be proficient for molecular-guided evaluation of TB transmission in three independent European population-based studies. However, the applicability of this system has not so far been tested in high TB burden countries.

Methods

We tested the applicability of this standardized genotyping system for molecular epidemiological analysis of 369 M. tuberculosis isolates from 3 regions of Brazil. Deligotyping, targeting multiple large sequence polymorphisms (LSPs), and MIRU-VNTRplus identification database were additionally used to confirm phylogenetic identification.

Results

The high congruence between the different typing results showed the country-wide supremacy of the Latin-American-Mediterranean (LAM) lineage, comprised of three main branches. Nevertheless, by distinguishing 321 genotypes among the 369 isolates, combined MIRU-VNTR typing and spoligotyping demonstrated the presence of multiple distinct clones. Noteworthy, 27 of the 32 clusters identified were exclusively composed of patient isolates from a same city, consistent with expected patterns of local TB transmission.

Conclusion

Taking into account recent economic development, high-capacity mycobacterial genotyping may now become an usable tool to guide TB control efforts, at least on sentinel sites or targeted risk-populations, in Brazil and likely in other countries with high TB burden. Settings with prevalent M. tuberculosis Beijing lineage might

however constitute a special case. The relevance of introducing a few supplementary hypervariable MIRU-VNTR loci for epidemiological analysis in such settings will be discussed.

OP–02
ORIGIN OF PATIENTS INFLUENCES THE EPIDEMIOLOGY OF TUBERCULOSIS IN WESTERN SWEDEN

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Summary

Considering the large population of immigrants in Sweden, this study focused on tuberculosis epidemiology among foreign-born patients versus Swedish born patients. We further wanted to investigate if the highly diverse origins of the patients influence the epidemiological picture of the disease, and whether a close relationship exists between the geographical origin and the characteristics of the isolate harbored (e.g., fingerprint, ancestral versus evolutionary-recent lineages, etc.).

Single *Mycobacterium tuberculosis* clinical isolates from 357 patients collected over 2001 to 2005 were genotyped using two methods: spoligotyping and the 12 loci MIRUs (Mycobacterial Interspersed Repetitive Units).

The patients originated from 46 countries, often from areas with high TB incidence. The study sample comprised a high proportion (75%) of foreign-born patients; only 25% were born in Sweden, while 27% were born in Africa (21% Somalia), 17% in Europe (10% former Yugoslavia), 14% in Asia (5% Viet Nam), 13% in the Middle East (6%

Iraq), and 2% in South America. The male to female ratio was 0.9 for the foreign born group versus 1.6 for the Swedish born. The foreign born were also younger (38 years) than the Swedish born (66 years; $p<0.001$) and drug-resistance was more common (any resistance, 13% versus 2%; $p<0.01$). Phylogeographical analysis based on combined spoligotyping and MIRU-typing showed that genotypic lineages found among foreign-born patients were significantly different than for Swedish born patients (detailed results presented in an accompanying poster presentation). Most of the ancestral or older lineages were found among the foreign born patients, while the evolutionary recent lineages were more common in Swedish born patients.

This presentation will attempt to underline some striking differences in epidemiological, demographical or clinical aspects of tuberculosis among Swedish versus foreign-born patients.

OP–03
PEDIATRIC TUBERCULOSIS IN NORTHERN BUENOS AIRES AREA DURING THE PERIOD 2001-2007

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Introduction

Pediatric tuberculosis (PTB) worldwide varies significantly regarding to the burden of the disease in different countries. Children with TB may represent 10%-20% of TB, approximately 3.1million children fewer than 15years of age.

Purpose of the study: To describe the PTB incidence in the Northern Buenos Aires (NBA) and to compare the proportions of both children and adults infected by multidrug-resistant Mycobacterium tuberculosis (MDR) strains.

Methods

It was an observational, retrospective study. Data were collected from 2001to 2007 from medical and laboratory records of the TB Control Program (TBCP).

Results

During this period 7,220 TB cases were reported being 2,128 (29.5%) confirmed by culture. A total of 431 (6.0%) out of 7,220 cases occurred in children. From 136 (31.6%) PTB cases the isolate was obtained by culture, the median age in children was 12 years (95%CI: 12.0-14.0; range: 0.2-15.0) and 120 (88.2%) presented a respiratory localization of the disease. Nine (6.6%) children were infected by a MDR isolate: 6.7% (6/89) had not had a previous treatment history (NTP) but in 15.4% (4/26) it was possible to identify the infectious contact (CON), while 14.3% (3/21) of the cases occurred in children with previous treatment history (PTP). In adults these figures were: NTP, 4.0% (56/1383); CON, 12.2% (6/49) and PTP, 12.5% (70/561) respectively. There were no significant differences between proportions of MDR in children and adults: NTP (0.3359), CON (0.9752), PTP (0.8766), besides children were at higher risk to develop a drug-resistant TB (odds ratio: 17.55, 95%CI: 6.41-48.04). Household TB contacts were simultaneously identified and related to 13 (9.6%) PTB cases by molecular epidemiology studies.

Conclusions

Culture and drug-susceptibility testing to determine the resistance pattern of the isolates should be recommended to optimize therapeutic efforts addressed for PTB cases, which is a useful parameter for assessing the impact of TBCP activities.

OP–04

UTILITY OF NEW 24-LOCUS VNTR FORMAT FOR DISCRIMINATING *M.TUBERCULOSIS* ISOLATES IN BULGARIA

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Purpose

The objective was to assess novel versus traditional molecular markers for epidemiological studies of M. tuberculosis in Bulgaria. The general interest of this study was to evaluate new 24-VNTR-locus format (i) in the relatively heterogeneous M. tuberculosis population (ii) circulating in the setting of a single country (iii) devoid of the significant influx of the foreign-born population.

Methods

A study panel included 133 M. tuberculosis isolates from newly diagnosed, epidemiologically unlinked, pulmonary TB patients from different regions of Bulgaria. IS6110-RFLP, spoligotyping, and 24-loci MIRU-VNTR typing was used for strain typing.

Results

The 133 strains were subdivided into 36 spoligotypes: 20 singletons and 16 clusters (2 to 33 isolates/cluster). The population

structure of M. tuberculosis in Bulgaria was dominated by two spoligotypes ST125 (S family) and ST53 (T family) with strikingly different distribution patterns. Spoligotype ST53 was found in similar and high proportion in the neighboring Greece and Turkey and almost equally distributed across different regions of Bulgaria. Contrarily, ST125 was specific for Bulgaria, especially the southern part of the country. High-resolution typing methods were further applied to a selection of 73 strains. Iinterestingly, IS6110-RFLP was less variable marker than even classical 12-loci MIRU scheme, even in high IS6110-copy number isolates. The 73 strains were subdivided into 69 types by 24-loci MIRU-VNTR scheme, 59 types by classical 12-loci MIRU-VNTR scheme, and 51 types by IS6110-RFLP typing. A combination of the five most polymorphic loci (MIRU40, Mtub04, Mtub21, QUB-11b, and QUB-26) was shown to achieve a high discrimination (HGI=0.988).

Conclusions

15- and 24-loci MIRU-VNTR schemes excellently differentiated strains in our study. A reduced 5-loci MIRU-VNTR set is preliminarily suggested for the first-line screening of M. tuberculosis clinical isolates in Bulgaria. At the same time, a comprehensive secondary subtyping of the clustered isolates should probably target all the 15 discriminatory loci.

Acknowledgments

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OP–05

NONTUBERCULOUS MYCOBACTERIA ISOLATED FROM A TB – PREVALENCE SURVEY CONDUCTED IN ZAMBIA: CONFUSION WITHIN IDENTIFICATION OR CLINICALLY RELEVANT?

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Purpose of the study

To investigate the distribution of nontuberculous mycobacterial species (NTM) in our TB prevalence survey conducted in 2005-2006 in two communities in Zambia.

Methods

Sputum was collected and cultured from 8044 individuals door to door survey. All ZN positive cultures were subjected to the Genotype Mycobacterium CM assay (CM). When no conclusive result was obtained, the 16S rDNA gene was sequenced. Sequences obtained were analysed using SeqMan (Lasergene) and identified by matching with BLAST (GeneBank) and RIDOM database.

Results

NTM were cultured from 324 (4.0 %) individuals. More NTM's were isolated in the rural area (6,3%) then the semi-rural area (1,7 %). Using CM Assay, 175 out of 324 (54%) could be identified as M. intracellulare followed by other common mycobacteria like M. gordonae (2.5%). M. fortuitum (3.7%), M. kansasii (0.6 %), M. abscessus (0.3 %) and M. malmoense (0.3 %). In 3 isolates, M. avium was found. In total, 81 isolates were analysed by 16S rDNA sequencing. The species that could be identified were M. terrae (9.9%), M. asiaticum (8.6%), M. nonchromogenicum (3.7%) M. mucogenicum (2.5%), M. triplex (6.2%), M. szulgai (1.2%), M. parafinicum (1.2%), M. goodii (2.5%) and M. murale (1.2%). Finally 33 (41%) of the isolates were classified as Unknown Mycobacteria Species Zambia (UMSZ type 1 to 12) because a 100% match was not found in BLAST or RIDOM. Two isolates were identified as Nocardia while 16 were classified as unknown as they need further analysis.

Conclusion

Overall we isolated 4 times more NTM's then MTB complex in our TB prevalence survey. The ratio NTM/TB was much higher in the rural area compared to the semi-urban area (9.1 and 1.3 respectively). This study illustrates that a reliable identification system is vital in performing TB culture prevalence surveys.

OP–06

EVALUATION AND USER STRATEGY OF MIRU-VNTRPLUS, A MULTIFUNCTIONAL DATABASE FOR ON-LINE ANALYSIS OF GENOTYPING DATA AND PHYLOGENETIC IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ISOLATES

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Purpose of the study

MIRU-VNTRplus, an online multifunctional database for phylogenetic analysis based on standardized MIRU-VNTR typing, spoligotyping, single nucleotide polymorphism and regions of difference, is now freely accessible at http://www.MIRU-VNTRplus.org. A collection of 186 well-characterized reference strains representing the primary MTBC lineages was used to build up the database and to allow easy user's strain identification. Here, we evaluated the database consistency both by testing the reference collection against itself and by using an external population-based dataset.

Methods

A population-based collection from Brussels, comprising 807 isolates from patients originating from 61 countries, was used as

external dataset. Genetic lineages were pre-identified based on 24-MIRU-VNTR-spoligotyping analysis using the Bionumerics package. The consistency of strain lineage identification was evaluated by testing MIRU-VNTRplus best matches of isolate genotypes and by testing groupings based on tree analyses. The sensitivity and specificity for lineage detection were defined as the proportion of correct best matches identified among the test samples, and the proportion of correct best-matches found among the total best-matches identified, respectively.

Results

Best-matched analysis, based on 24-locus MIRU-VNTR, of the reference collection against itself or against pre-identified lineage of the external dataset gave a specificity of 100% and 99.3% respectively at a stringent cut-off (0.17), and 98.9% and 96.8% respectively at a relaxed cut-off (0.3).In tree-based evaluations, groupings of the reference strains were fully consistent in the internal evaluation, while in the external tree-based tests with strains of pre-identified lineages, groupings within expected reference lineages were obtained for 99.3 % test genotypes.

Conclusion

Based on this evaluation, a user-strategy was defined, including best match analysis followed, if necessary, by tree-based analysis. The MIRU-VNTRplus database is a powerful tool for high-resolution clonal identification, which has little equivalent in terms of functionalities among the bacterial genotyping databases available so far.

OP–07

ANALYSES OF MUTATIONS CONFERRING RESISTANCE TO SECOND-LINE DRUGS IN MYCOBACTERIUM TUBERCULOSIS STRAINS FROM A HIGH-INCIDENCE REGION

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Background

Resistance to second-line drugs (fluoroquinolones and injectable aminoglycosides) has become a serious problem for the treatment of *Mycobacterium tuberculosis* infections. Resulting XDR-tuberculosis implies an enormous threat for tuberculosis control worldwide. It is therefore of great importance to analyze the genetic basis of clinical resistance.

Methods

M. tuberculosis strains from a high-incidence region in Uzbekistan (Karakalpakstan) which displayed resistance to ofloxacin (n = 56) and to capreomycin and/or amikacin (n = 111), respectively, were sequenced concerning the resistance determining regions (GYRA, GYRB, RRS**,** TLYA). 25 of the strains showed resistance to both second-line drugs and were therefore XDR-TB.

Results

The most common mutation seen among ofloxacin resistant strains is the substitution of aspartic acid to glycine at codon 94 (32.1%) followed by the substitution of alanine to valine at codon 90 (30.4%) in the GYRA region. 17.9% of the ofloxacin resistant strains display substitutions of aspartic acid other than glycine at codon 94 and changes of serine for proline at codon 91 in GYRA, respectively. Two ofloxacin resistant strains show SNPs in GYRB at codon485(CGT →TGT) and at codon543(GCG →ACG), respectively, that have not been reported previously.

Concerning resistance to amikacin and capreomycin the most common mutation seen is the substitution of nucleotide adenine to guanine at position 1401 (67.6%) in RRS followed by a change of cytosine to thymine at position 1402 (2.7%).

Conclusion:

This is the first study analyzing *M.tuberculosis* strains from a high-incidence region for fluoroquinolone and aminoglycoside resistance determining mutations. In addition to the previously described mutations, new SNPs in GYRB and TLYA were detected, whose impact needs to be further elucidated. Sequence analysis in general is of great importance for the rapid detection of XDR strains.

OP–08

VARIATION OF DISTRIBUTION OF MUTATIONS BETWEEN PHYLOGENIC LINEAGES OF M.TUBERCULOSIS

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Introduction

The detection of mutations in the rifampicin resistance determining region (RRDR) of M. tuberculosis rpoB is routinely used to predict phenotypic resistance to rifampicin giving high levels of specificity and sensitivity. DNA sequencing is used to identify all mutations in the RRDR. Commercially available PCR-reverse hybridization systems are available which detect the presence of mutations at the RRDR and identify four commonly seen mutations (D516V, H526Y, H526D and S531L) in conjunction with DNA sequencing all mutations can be identified.

Purpose of the study

Data sets from a variety of institutions around the world have shown differing distributions of mutations at the RRDR although invariably reporting the most common four. As phylogenic lineages are not uniformly distributed across the globe this begs the question as to whether these mutational distributions are related to phylogenic lineage.

Methods

It has been shown that VNTR typing can be used to indicate the four phylogenic lineages of M. tuberculosis, all of which are seen in London. In this study the rpoB genotypes of the RRDR have been determined in 233 rifampicin isolates from individual patients using a combination of PCR-reverse hybridization and sequencing. The phylogenic lineage was determined for each by interpreting a 15 loci VNTR (12MIRU+3ETR) profile. Where >1 VNTR profiles with the same rpoB genotype were seen only a single isolate was included for analysis. The distribution of rpoB mutations was determined for each of the 4 lineages.

Results

The distribution for all isolates was 5% D516V, 8% H526Y, 8% H526D, 54% S531L and 25% other including 4% wild type. 11% of lin-

eage I isolates had a genotype other D516V, H526Y, H526D or S531L, this was 20%,27% and 19% in lineages II, III and IV respectively.

Conclusion

In conclusion it appears that distribution of mutations at the RRDR is dependent upon phylogenic lineage.

OP–09

RAPID DETECTION OF RESISTANCE TO SECOND-LINE DRUGS OF *MYCOBACTERIUM TUBERCULOSIS* USING THE *MYCOBACTERIUM GROWTH INDICATOR TUBE* (MGIT) MANUAL SYSTEM

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Summary

The emergence of multidrug-resistant tuberculosis (MDR-TB) and, more recently, of extensively drug-resistant (XDR)-TB is a real threat for TB control programmes. Especially in low-income high-burden countries there is a great need for rapid, reliable, and economical methods for testing the susceptibility of *M. tuberculosis* to antibiotics. Using standardized 7H11 agar conventional methods, it takes a minimum of 3 weeks to identify resistant strains on solid media. Fully automated commercial systems such as the BACTEC

MGIT 960 have shown its reliability for the rapid detection of resistance to second-line drugs, however, it requires heavy and costly equipment. The MGIT manual system has not been standardized until now for susceptibility testing to second-line drugs. The objective of this study was to establish critical concentrations for the major second-line anti-TB drugs, ofloxacin (OFL), kanamycin (KAN), ethionamide (ETH), and capreomycin (CAP) with clinical isolates of *M. tuberculosis* and compare the results with those obtained with the standard proportion method on 7H11 media. High concordance was found between the manual MGIT and the conventional method except for ETH. The great advantage of the manual MGIT is that it obviates the need for heavy and expensive equipment. Tubes can be read under an UV lamp or using a simple semi-automated tube reader (BD). The MGIT manual system is a good alternative for low-income countries or in settings where resources are limited.

OP–10

ETHAMBUTOL RESISTANCE IN-VITRO AND IN-VIVO CONFERRED BY TRANSFER OF AN EMBB306 POINT MUTATION IN *MYCOBACTERIUM TUBERCULOSIS*

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Summary

Ethambutol (EMB) is a first-line drug used for the treatment of tuberculosis. The role of EMBB306 mutations for the development of EMB resistance in *Mycobacterium tuberculosis (M. tuberculosis)* was controversially discussed. Mutations in the codon 306 of the EMBB gene, which were found in up to 68% of EMB resistant clinical isolates, were suggested as molecular marker for EMB resistance. However, in recent studies EMBB306 mutations were detected in EMB susceptible strains and thus questioning their importance for the EMB resistance mechanism.

To answer this question, we used allelic-exchange techniques generating EMBB306 mutants of *M. tuberculosis* H37Rv. The wild-type EMBB306 ATG codon was replaced by codons EMBB306 ATC, ATA or GTG, respectively. The level of EMB resistance conferred by particular mutations was measured in vitro (minimal inhibitory concentration [MIC]) and in vivo following oral EMB therapy in a mouse model of tuberculosis.

All obtained EMBB306 mutants showed a clear increase in EMB MIC's compared to the wild-type H37Rv. While H37 growth was significantly affected by EMB treatment of infected mice, the growth of one selected EMBB306 GTG mutant strain was not inhibited under EMB treatment when compared with untreated controls.

These experiments for the first time provide direct experimental evidence that EMBB306 mutations confer a clinically relevant degree of EMB resistance in *M. tuberculosis.*

OP–11

RECONSTRUCTING THE EVOLUTIONARY ITINERARY OF A MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* OUTBREAK STRAIN BACK FROM ITS DRUG SUSCEPTIBLE PROGENITORS

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Background

Undoubtedly, comparative analyses of a multidrug-resistant (MDR) *Mycobacterium tuberculosis* outbreak strain with its closest non epidemic drug sensitive progenitor(s) is crucial to understand the natural molecular mechanisms leading to the emergence of MDR tuberculosis (MDR-TB) outbreaks. However, the main obstacle to carry out such comparative analyses consists in the difficulty to identify and/or to make available these progenitor strains.

Methods

Because TB progression from infection to active disease greatly varies between patients, we hypothesized that the progenitors of an outbreak strain could still be recovered from newly identified TB cases soon after the onset of the outbreak. We also reasoned that these parental strains would share several IS6110 transposition sites with their derived outbreak strain. To confirm theses suppositions, we first determined, to the base pair, the IS6110 insertion sites in the genome of an *M. tuberculosis* strain involved in a severe MDR-TB outbreak in Tunisia. Using these data, we next developed an IS6110

site-specific PCR-based assay and searched for the outbreak-specific IS6110 genomic locations in a representative pool of isolates recovered from the same area after the onset of the outbreak.

Results

By analysing the transposition itinerary of IS6110, we were able to reconstruct the evolutionary history of the MDR outbreak strain and to identify its closest parental strains in the post-outbreak drug sensitive pool.

Conclusion

Future widespread application of such a strategy, combined to comparative and functional genomics, will result in better understanding of the natural sequential molecular events leading to the emergence of TB outbreaks.

OP–12

PERFORMANCE OF HAIN LIFESCIENCE MTBDR+ ASSAY IN THE DIAGNOSIS OF TUBERCULOSIS AND DRUG RESISTANCE IN SAMARA, RUSSIAN FEDERATION

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Background

Samara is a region in Russian Federation with a high incidence and prevalence of multi-drug resistant tuberculosis (MDRTB). Although of critical importance for patients' management and prevention of MDRTB transmission, no rapid diagnosis and drug susceptibility testing (DST) on clinical specimens is available in Russia at this moment.

Methods

A total of 168 smear-positive individual sputum samples were tested for rifampicin (RIF) and isoniazid (INH) resistance using HAIN MTBDR+ assay according to the manufacturer's guidelines. DST on cultures were performed using liquid media automated system (Becton Dickinson BACTEC MGIT960).

Results

Readable MTBDR+ assay results were obtained for 151 DNA extracts (89.9%); valid phenotypical DST results were obtained for 161 (95.8%) initial sputum specimens. The sensitivity of the assay depended on the concentration of *Mycobacteria* in the sputum samples (82.8% readability in samples graded 1+ compared to 100.0% readability in specimens graded 3+). Comparative analysis demonstrated good agreement between molecular and phenotypic DST results: molecular and phenotypic results were identical in 141 (94.6%) and 140 (94.0%) specimens for RIF and INH susceptibility respectively. The specificity and sensitivity of the MTBDR+ assay was 90.7%; 96.2%, and 83.3%; 97.3% for the detection of RIF and INH resistance respectively. MTBDR+ assay tends to overestimate drug resistance with "mutant" RPOB and "mutant" KATG and/or INHA patterns registered respectively in four and six DNA samples, from which phenotypically sensitive strains were derived. This is probably due to initial sputum samples containing mixtures of resistant and sensitive bacilli.

Conclusion

Good agreement between phenotypical and molecular DST results and short turnaround times (1 working day) allow to recommend the assay for implementation in high and medium TB burden regions with high prevalence of MDRTB strains. Further studies are needed to address issues of assay sensitivity and correct recognition of sensitive and resistant bacilli in mixed specimens.

OP–13

RAPID DETECTION OF RIFAMPICIN RESISTANCE USING THE DIRECT MGIT METHOD IN AFRICA

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Purpose of the study

Great vigilance is imperative when new drugs are used in the treatment of tuberculosis (TB). Due to amplification of resistance, these new drugs could rapidly become ineffective if administered in combination with first-line drugs to partially resistant or multidrug-resistant TB (MDR-TB) patients. The use of a rapid test is therefore indispensable to exclude such patients from the trial. The performance and practicability of the rapid test used in the frame of the "Gatifloxacin for TB project (OFLOTUB)" are presented.

Methods

Rapid detection of MDR-TB was performed on two smear-positive sputa from 1067 patients by the manual MGIT method (Becton Dickinson, USA) in Benin, Guinea, Kenya and Senegal and by the MGIT 960 method in South Africa. Decontaminated sputum specimens were directly inoculated into one control MGIT and one rifampin (R) containing MGIT tube. Indirect drug susceptibility testing (DST) was also performed on isolates using the proportion method. Quality control of DST was performed at the ITM.

Results

A total of 992 (93.0%) R sensitive (Rs) patients were included in the trial, while 75 patients (7.0%) were excluded due to R resistance (Rr) (1.8%), invalid (4.4%) or contaminated results (0.8%). Comparing direct MGIT and indirect DST results, 3 discordant results (0.3%) were obtained. Three patients were Rs by MGIT and Rr by DST. The median turn-around time for reporting Rs or Rr was 8-10 days (3-15) with the manual MGIT and 4 days (2-14) with the MGIT 960 system.

Conclusion

The direct MGIT test gave excellent results within 2 weeks. No false Rr results were observed and only 0.3% false Rs results were detected. Moreover, the manual MGIT method is convenient for performing direct DST in low resource settings. Supply of MGIT tests is, however, critical and easy access to consumables should be available in those countries.

OP–14
NON-TUBERCULOUS MYCOBACTERIA ISOLATED IN CLINICAL LABORATORIES: ANY CHANGE OVER THE YEARS?

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Summary

State-of-the-art presentation on the (clinical) significance of atypical mycobacteria isolated in a routine diagnostic laboratory, observed trends in low- and high-TB-incidence countries, and implications of introducing culture as a diagnostic tool in populations highly exposed to environmental mycobacteria.

Soon after the discovery of the tubercle bacillus by Robert Koch in 1882, various non-tuberculous mycobacteria (NTM) were described. Their clinical relevance was only accepted after some decades of occasional and systematic isolation from well-documented patients.

Worldwide, the most commonly encountered pathogenic NTM are *M. avium*, *M. intracellulare*, *M. kansasii* and *M. ulcer-*

ans. Less frequent are slow growers like *M. marinum*, *M. xenopi* and *M. simiae* and rapid growers like *M. chelonae* and *M. fortuitum*. Although human-to-human transmission has not been documented, micro-epidemics due to some of these opportunistic pathogens have been described as a result of a common environmental source of infection. It is clear, however, that not all NTM isolated in a clinical laboratory are truly the cause of disease, but rather the result of colonization in the patient or contamination of the specimen during the sampling, transporting or processing.

Until the early 1980's, identification of NTM was mainly based on phenotypic characteristics such as growth rate, pigmentation, ability to produce niacin etc. Nowadays, genotypic identification has taken over in many laboratories using probe hybridization assays, sequencing of the 16SrRNA gene or the 16S-23S spacer region, PRA of the hsp65 etc. This resulted in a huge increase in the number of valid NTM species.

Populations in countries with a high TB and/or HIV incidence are often also highly exposed to environmental mycobacteria. It is not clear to what extent this exposure results in infection and disease, and whether the number of diagnosed NTM diseases will increase with the introduction of culture as a diagnostic tool. Also, the introduction of liquid media that do not allow visual inspection of the macroscopic aspects, require a specific approach for identification. Furthermore, some NTM might have a selective advantage in liquid media, possibly overgrowing *M. tuberculosis*-complex present in the same specimen. Finally, the diagnostic criteria to determine the clinical relevance of NTM as formulated by the American Thoracic Society might need revision in view of this.

OP–15

RESISTANCE TO SECOND-LINE DRUGS IN 153 MULTI-DRUG RESISTANT TUBERCULOSIS CASES IN THE NETHERLANDS, DERIVED FROM VARIOUS GEOGRAPHIC ORIGIN

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Introduction

Multi-drug resistant tuberculosis (MDR-TB) is a threat to TB control efforts worldwide. Purpose of the Study: To investigate the frequency of resistance to second-line drugs among MDR-TB cases in the Netherlands and its correlation with the patient's geographic origin.

Methods

We performed a retrospective laboratory database study of multi-drug resistant Mycobacterium tuberculosis complex strains, isolated in the Netherlands between January 1993 and October 2007, collecting all available data on second line drug susceptibility. The 25-wells Middlebrook 7H10 agar dilution method was applied for second line drug susceptibility testing. The following drugs and breakpoint concentrations were used: para-aminosalicylic acid (breakpoint 1 μg/ml), amikacin (5 μg/ml), cycloserine (50 μg /ml), prothionamide (5 μg /ml), ciprofloxacin (2 μg/ml), clofazimine (2 μg/ml) and rifabu-

tin (2 μg/ml). Patients were sorted by region of origin using World Health Organisation administrative regions.

Results

We found 153 patients with MDR-TB; eighteen were native Dutch (12%). Complete second-line drug susceptibility testing was performed for 131 patients. Resistance to second-line drugs was noted in primary samples of 28 patients (21%). Resistance to a single second-line drug was most frequent (9 prothionamide, 6 para-aminosalicylic acid, 4 amikacin, 4 ciprofloxacin, 1 cycloserine; 24/28=86%). Four patients had strains resistant to multiple second line drugs; two were extensively drug resistant M. bovis. In patients of European and Central Asian origin, resistance to second line drugs was most frequent and involved the widest range of drugs. Prothionamide resistance was frequent among African and American patients; Amikacin resistance was frequent among South-East Asians.

Conclusion

Resistance to second line drugs is infrequent among MDR-TB patients in the Netherlands. Most second line drug resistance is recorded among immigrants, with substantial differences in second line drug resistance in patients originating from different geographical areas. A patient's origin can be a factor in determining the appropriate second-line drug treatment.

OP–16

FREQUENCY OF MUTATIONS AMONG MDR-AND-XDR M.TUBERCULOSIS STRAINS IN DIFFERENT GEOGRAPHICAL SETTINGS

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Purpose of the study

To assess the frequency of mutations among MDR and XDR strains, in order to: (i) develop genetic methods to identify resistant strains; (ii) to evaluate the usefulness of existing molecular assays in different geographical settings.

Methods

DST was performed by proportion method on Löwenstein-Jensen according to WHO guidelines. Direct sequencing of PCR products was carried out with an ABI Prism 3730 DNA analyzer (Applied Biosystems).

Results

Rifampin (RIF)– and isoniazid (INH)-resistant isolates collected in Italy (I, n 153 and 202), Mozambique (MOC, n 73 and 73), Burkina Faso (BF, n 15 and 17) and Bulgaria (BG, n 11 and 10) were analyzed by sequencing rpoB hotspot region for RIF-R and katG plus inhA promoter regions for INH-R.

RIF-R

Country	TOT	rpoB hotspot					No Mut
		D516V	H526D	H526Y	S531L	Other	
	N	%	%	%	%	%	%
I	148	7,2	2,6	7,2	62,1	15,0	5,9
MOC	73	2,7	8,2	8,2	63,0	15,1	2,7
BF	15	66,7	13,3	13,3	6,7	0,0	0,0
BG	10	0,0	0,0	9,1	72,7	9,1	9,1

INH-R

Country	TOT	katG 315	inhA promoter	No Mut
	N	%	% tot (katG-associated)	%
I	202	68,3	12,9 (7,0)	25,7
MOC	73	94,5	11 (9,6)	4,1
BF	17	88,2	50 (50,0)	11,8
BG	10	40,0	60 (0,0)	0,0

Conclusion

Sequence analysis data from resistant strains show geographic differences in the prevalence of mutation conferring MDR phenotype. This may explain the difference in sensitivity of existing molecular diagnostic tools especially in Western low-burden countries. The analysis of the frequency of mutations for fluoroquinolones and aminoglycosides resistances will contribute to the development of new tools for fast detection of the XDR phenotype.

OP–17
AN OUTBREAK OF MULTI-DRUG RESISTANT TUBERCULOSIS IN REFUGEES IN AUSTRIA IN 2005/2006

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Summary

In 2005/ 2006 the Austrian reference laboratory for tuberculosis identified MDR isolates from 4 cases of TB showing genotypes indistinguishable from each other. An epidemiological investigation was conducted to clarify the chain of transmission of this MDR-TB strain. Case series investigation by reviewing tuberculosis notification reports and hospital discharge letters. The 38 year old primary case of the MDR-TB-cluster had initially been identified as a case of non-MDR pulmonary TB in June 2004, 7 months after being detained for illegal immigration. In March 2005 he got lost for follow-up for 4 months. In June 2005 he presented himself with pulmonary and laryngeal TB due to MDR-TB. After discharge the case again got lost for follow upuntil April 2006, when he was readmitted with recurrent MDR-TB. A 3-case-cluster of pulmonary MDR-TB sharing the identical strain with the primary case was detected in April 2006: His 5 month old daughter and a 25 year old friend with his 6 month old son. As MDR-TB has originated in the HIV-seronegative community in Austria, there is a clear need for implementing national guidelines for the management of drug resistant tuberculosis in Austria.

OP–18
IN-VITRO DRUG SUSCEPTIBILITY OF 2275 CLINICAL NON-TUBERCULOUS MYCOBACTERIUM ISOLATES OF 48 SPECIES IN THE NETHERLANDS. RECOMMENDATIONS FOR TREATMENT

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Introduction

Treatment of disease due to nontuberculous mycobacteria is complicated. For many species the relation between in vitro susceptibility and in vivo outcome of treatment is unclear or there is a lack of in vitro susceptibility data. Purpose of the Study: To provide insight in in vitro activity of antimycobacterial drugs to clinical isolates in the Netherlands, we compiled identification and drug susceptibility testing results from our national reference laboratory.

Methods

We subjected 2275 strains of 49 different species of nontuberculous mycobacteria, isolated from 2072 patients in the Netherlands between 2000 and 2007, to drug susceptibility testing with the 25 wells Middlebrook 7H10 agar dilution method. Isoniazid, rifampicin, rifabutin, ethambutol, clarithromycine, ciprofloxacin, cycloserine, prothionamide, amikacin, clofazimine and streptomycin were included in the test panel.

Results

The pattern of drug susceptibilities and minimum inhibitory concentrations was found conserved within species and differed significantly between species. Most nontuberculous mycobacteria were susceptible to clarithromycin. Susceptibility to ciprofloxacin and amikacin was less frequent and limited to Mycobacterium kansasii, M. xenopi, M. fortuitum and species phylogenetically related to these three species. Susceptibility to first-line anti-tuberculosis drugs was rare, except for M. kansasii and phylogenetically related species. Slowly growing nontuberculous mycobacteria were susceptible to second-line anti-tuberculosis drugs such as rifabutin, cycloserine, clofazimine and prothionamide; the latter also had activity against M. fortuitum and related rapid growers.

Conclusion

Clarithromycin and rifabutin are most active against nontuberculous mycobacteria. The activity of other second-line anti-tuberculosis drugs is not supported by clinical data. To improve its utility, the selection of drugs in DST should be changed to more drugs with proven clinical efficacy, correlating with in vitro susceptibility. The DST results have additional taxonomical value. They may reconfirm previous identification results and disclose phylogenetic relationships between species.

OP–19
OTITIS MEDIA CAUSED BY NON-TUBERCULOUS MYCOBACTERIA, AN EMERGING INFECTION IN THE NETHERLANDS?

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Introduction

Recently, a rise in incidence of ear infections due to nontuberculous mycobacteria (NTM) was noticed in the Netherlands. Purpose of the study: We investigated the risk factors, clinical features, therapy and outcome. Methods: We examined medical records of all patients in the Netherlands from whom NTM were isolated from samples derived from the middle ear or mastoid between January 1993 and June 2007.

Results

We found thirteen patients. Ten had otomastoiditis caused by Mycobacterium abscessus and three by M. avium complex (MAC). Most M. abscessus cases emerged since 2005. Patients with MAC were younger than patients with M. abscessus. Previous middle ear infections, tympanotomy tubes and prior use of otic drops containing steroids with aminoglycosides or quinolones were the main risk factors. Clinical presentation was non-specific, with chronic otorrhea, mastoiditis and hearing loss. The diagnosis derived from ac-

id-fast bacilli microscopy and mycobacterial culture from biopsy materials or otorrhea pus. In ten patients cleaning surgery was performed. For M. abscessus drug treatment usually consisted of clarithromycin monotherapy or clarithromycin and a fluoroquinolone. Patients with MAC otomastoiditis were treated with clarithromycin combined with anti-tuberculosis drugs. Drug therapy and surgery initially led to eight relapses; after multiple rounds of surgery and drug treatment, ten patients were cured, two still receive treatment and one died. For six patients, NTM otomastoiditis resulted in permanent hearing loss.

Conclusion

NTM otomastoiditis is rare but emerging in the Netherlands and mostly affects young children with tympanostomy tubes, a history of ear infection and topical treatment with steroids and aminoglycosides or fluoroquinolones. Adherence to American Thoracic Society treatment recommendations for M. abscessus soft tissue and bone infection, advocating clarithromycin and parenteral antibiotics, was very limited. Most patients only attained cure after multiple rounds of surgery and drug treatment. For MAC, treatment guidelines were better adhered to.

OP–20

ARE RODENTS AND INSECTIVORES A RESERVOIR FOR *BURULI ULCER* IN BENIN?

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Introduction

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is a debilitating skin disease. Very little is known about the epidemiology of the disease. The reservoir and mode(s) of transmission of this disease are not yet determined, although *M. ulcerans* specific DNA (IS2404) has been identified in water related organisms.

Mammals are until now the only naturally infected creatures found from which *M. ulcerans* could be cultivated. Several rodents can be experimentally infected by *M. ulcerans* confirming that they are susceptible to *M. ulcerans*. Therefore we suggest a rodent or insectivore species as part of the reservoir of BU.

Methods

To test this hypothesis, rodents and insectivores were trapped in Benin. Trapping occurred in high and low endemic villages. Organs from rodents and insectivores were analyzed by culture and PCR methods for the detection of *M. ulcerans*.

In total, 575 animals were collected: 336 in high endemic villages, 225 in low endemic villages, and 14 grasscutters bought in Cotonou.

Results

None of the samples tested positive for IS2404 and no *M. ulcerans* could be cultured. From 49 animals (8.5%) mycobacteria other than *M. ulcerans* were cultured. More mycobacteria were cultured from insectivores than from rodents (23.3% vs. 2.1%, $p<0.001$) and from animals trapped in the fields as compared to houses (12.9% vs. 4.5%, $p=0.004$). In the wet season more mycobacteria were isolated than in the dry season (18.0% vs. 3.9%, $p<0.001$). A slightly higher isolation rate of mycobacteria was found in high endemic villages as

compared to low endemic villages, this difference was however not significant (11.3% vs. 7.1%, p=0.156).

Conclusion

Different arguments are available for the role of rodents and insectivores as a reservoir for BU, but until now the natural occurrence of *M. ulcerans* in these animals has not been confirmed. Further studies on African mammals as reservoir for BU are needed.

OP–21

THE ROLE OF PROTOZOA IN THE ENVIRONMENTAL RESERVOIR OF M.ULCERANS

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Purpose

Mycobacterium ulcerans disease (Buruli ulcer) occurs most commonly in defined areas related to rivers, swampy terrain or lacustrine systems. Since *M. ulcerans* and free-living amoebae share this habitat a study on a possible link between *M. ulcerans* and free-living amoebae was carried out.

Methods

The isolation of free-living amoebae as well as mycobacteria was attempted from water and biofilm specimens taken from protected and unprotected sources of water in villages with high and low endemicity of Buruli ulcer in Benin. We also investigated experimentally the interaction of *M. ulcerans* with *Acanthamoeba polyphaga*, a phagocytic protozoon.

Results

Water bodies in highly endemic areas contained significantly more amoebae than those in low endemic areas. After experimental infection of *A. polyphaga* with *M. ulcerans* both light and transmission electron microscopy demonstrated acid fast bacilli within amoebae. Phagocytosis occurred rapidly with 35% of amoebae showing intracellular acid fast bacilli after 3 hours. Moreover *M. ulcerans* persisted and survived with *A. polyphaga* for up to 14 days without influencing the growth curve of the amoebae independent of toxin production by the *M. ulcerans* strains. We observed that during the experiment the bacilli rather got expelled and rephagocytised by the amoebae and multiplied during this process than multiplying within the amoebae. *M. ulcerans* strains with restored virulence persisted in higher numbers in *A. polyphaga* than strains adapted to the laboratory.

Conclusions

These results have implications on our understanding of the epidemiology and ecology of *M. ulcerans*. If free-living amoebae would

truly be one of the environmental hosts of *M. ulcerans* this would be very important since amoebae are common inhabitants of natural aquatic environments and since they have been found to be resistant to extreme parameters while encysted.

OP–22
A NOVEL PORTABLE SCREENING DEVICE FOR INFECTIOUS PULMONARY TUBERCULOSIS

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Introduction

The highest burden of tuberculosis is found in the less developed countries of Africa and Asia where the current practice of passive case finding by smear microscopy is failing to control the disease. A more effective intervention is required to detect and treat infectious cases earlier in the transmission chain. Purpose of study: We describe a novel device to screen for M. tuberculosis in breath. The RBS Breathalyser system (Rapid Biosensor Systems Ltd) is a fully portable device comprising a disposable plastic collection tube into which the patient coughs. The tube is then inserted into a battery powered instrument which provides a digital readout in less than 5 min. The device utilizes novel immunosensor and bio-optical technology to detect TB antigen.

Methods

The device was subjected to independent testing in a small pilot study in an outpatient clinic in Ethiopia (N=58). Known TB patents were excluded. Patients were instructed to only cough into the device and not to expectorate sputum from their lungs. Gentle nebulisation was provided to enable patients to cough freely into the device. All patients tolerated the process well and the whole testing process took less than 10 min. Chest radiography and sputum smear microscopy were undertaken. Although not routinely undertaken is this setting samples were sent for culture on Lowenstein Jensen (results pending).

Results

The instrument proved simple to use and required little training. 25 patients were found negative by the breath test and smear microscopy. 12 were found positive in both tests. 15 were RBS Breathalyser positive but smear negative, 8 of which was diagnosed pulmonary tuberculosis on the basis of chest radiography and/or clinical evidence. 6 patients were RBS Breathalyser negative but smear positive.

Conclusion

These preliminary findings suggest that this novel screening device may assist early identification of infectious TB in poor resource settings. Further evaluation should be undertaken.

OP–23
DIRECT CONCENTRATION OF TB FROM SPUTUM AND ENHANCEMENT OF THE SENSITIVITY OF SMEAR MICROSCOPY

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Introduction – direct smear is insensitive. Indirect smear after concentration by centrifugation is more sensitive but cannot be performed by sites without access to a centrifuge. TB-Beads provides an alternativesimple method to concentrate TB from sputum and achieve ahighly sensitive smear microscopy.

Purpose of the study

Evaluation of microscopy results after magnetic separation of TB from sputum using TB-Beads

Methods

81 frozen unprocessed samples from the WHO sputum bank were investigated. 0.5-1.0 ml sputum was thinned using an equal volume of 2% NaOH, 0.5% NALC . After 15 min, one tenth volume of T-Bead Capture Buffer was added and mixed thoroughly.50µl of TB-Beads were added and incubated for 10 min. The beads were then collected using a magnet, the supernatant removed and the beads smeared onto astandard glass slide. After drying, the slide was stained using a standard auramine method. The slide was observed using a fluorescent microscope and x20 objective.

Results

The results of our TB-Bead concentration and microscopy were compared to the microscopy results following centrifugal concentration (the latter results were supplied with the samples). 40 samples

were smear negative by both approaches, 38 samples were positive by both approaches. An additional 3 samples were positive by only the TB-Bead concentration approach. All microscopy positive samples, including those positive after TB-Bead concentration, were confirmed by culture and final clinical diagnosis.

Conclusion

Microscopic analysis of the samples extracted by the TB-Beads was 100% specific and more sensitive compared to microscopic analysis of the samples prepared by centrifugal concentration. The advantages of the TB-Beads approach include a reduced risk of infection, higher and more rapid throughput and the possibility for implementation at the peripheral laboratory level. In addition, TB-Beads are stable at ambient temperature and resistant to proteases, acids and alkali.

OP–24
IMPROVING DETECTION OF MYCOBACTERIA BY IMMUNOCAPTURE-QPCR IN WATER SAMPLES

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Summary

Detection of mycobacteria by traditional methods is often necessary but cumbersome. With the aim to improve the diagnostic in

mycobacteriology, we developed a rapid and sensitive test-procedure to estimate the mycobacterial charge by immunocapture-qPCR, which we optimized in water-samples. The validation of the test on tap water-samples was performed using culture-based methods as the gold standard for comparison.

THE PROCEDURE IS COMPOSED OF FOUR STEPS:

1) Non-specific concentration of the total bacteria content through filtration of water samples and their detachment into buffer.
2) Specific immunomagnetic concentration of Mycobacteria,

The specificity and efficiency was ensued by raising polyclonal antisera against outer cell-wall preparations of the environmental Mycobacteria most frequently found in samples of tap-water (M.kansasii, M.abscessus, M.xenopi, M.avium and M.gordonae). Over 80 % of Mycobacteria present in spiked samples were captured with IgG-fractions fixed on magnetic beads, independently of the starting concentration.

3) Genomic -DNA preparation of bead-captured Mycobacteria by physical disruption
4) Quantification of the mycobacterial charge by Mycobacterial genus-specific qPCR, a home-developed assay targeting a mycobacterial housekeeping gene.

In our hands, the complete test-procedure has a detection-limit of 50 mycobacterial genomes per 1000ml of water sample, and can be fully performed in one day. The test-procedure is ready to use in tap-water, which constitutes a potential source of iatrogenic and environmental mycobacterial infections mainly in aged and immune-compromised persons, and it could be incorporated to routine analysis of drinking water.

We are currently working on the rapid evaluation of the real pathogenic potential of the samples through both molecular estimation of bacterial viability and species identification by high resolution melting of PCR amplicons. The applicability of the test-procedure to clinical samples will be discussed.

Keywords

Immunocapture, quantification, diagnostic, environmental Mycobacteria, drink water

OP–25
QUANTIFERON-TB GOLD ASSAY IN TUBERCULOSIS DIAGNOSIS

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Introduction

Purpose of the study: To evaluate QuantiFERON TB Gold assay (QF), in respect to agreement with Tuberculin Skin Test (TST), BCG vaccination, immunosuppresion and exposure to M.tuberculosis (MTB)

Methods

1047 whole blood samples were tested. Clinical data was partially available in many instances. Sputum cultured were 137 patients.

Results

- In 1047 cases studied, QF(+) were 321(30,7%), QF(–) 681(65%) and indeterminated 45(4,3%)
- TST data was available in 905 cases: among TST(+) individuals, QF(+) were 246(40,5%), QF(–) 352(58%) and indeterminated 9(1,5%). In TST(–), QF(+) were 47(15,8%) QF(–) 228(76,5%) and indeterminated 23(7,7%) individuals.
- BCG vaccination, TST data was available in 414 individuals: 245(59,2%) were TST(+) QF(–), 85 (20,5%) were TST(+) QF(+), 79 (19,1%) were TST(–) QF(–) and 5 (1,2%) were TST(–) QF(+)
- In 775 cases, there was sufficient data for immunologic status: 117 were immunosuppressed and 658 immunocompetent. In im-

munosuppressed, TST(+) were 33(28,2%) and TST(–) 84(71,8%), while QF(+) were 32(27,4%), QF(–) 68(58,1%) and indeterminated 17(14,5%). In immunocompetente, indeterminate results were much lower (2,3%)

- MTB exposure, BCG vaccination, TST data was available in 218 cases. Among them, in 86 BCG non vaccinated-MTB exposure individuals, QF(+) was 25(29,1%), while TST(+) 46(53,5%)
- Sputum cultured, QF tested were 137: In 40 positive cultures, 36 MTB and 4 NonTB were isolated. All MTB(+) cases were QF(+)

Conclusions

The QF assay is especially usefully in diagnosis of latent TB, particulary in cases of BCG vaccinated, as well as in immunosuppressed individuals.

OP–26

CHALLENGES OF QUANTIFERON-TB GOLD IN-TUBE IMPLEMENTATION IN A RESOURCE LIMITED SETTING

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Purpose of the study

Interferon gamma release assays (IGRAs) are replacing the tuberculin skin test as a diagnostic tool for Mycobacterium tuberculosis

infection in developed countries. However, data on test performance in high HIV-TB burden settings is limited. The aim of this study is explore the challenges of the QuantiFERON-TB Gold In-Tube (QFT-G) in a resource limited setting with a high prevalence of HIV.

Methods

112 smear positive TB patients were recruited from clinics in Lusaka. From each patient three sets of tubes of QFT-G blood were taken. Two sets were handled exactly the same according to the manufacturer's protocol (sets A and B), whilst for the third set (set C), incubation was delayed for 24 hrs. We subsequently investigated other factors that may causes discrepancies between samples including incubation time on the Elisa plate and the effect of pipetting errors to learn more about the reliability and the reproducibility of the test.

Results

There was excellent agreement between sets A and B of 91.7% (κ=0.8). However, there was fair agreement between sets A and C (77%; κ =0.54) as well as sets B and C (78%; κ = 0.57). Pipette errors of 10 µl of either the sample or conjugate did not affect of the results. However, the position of the sample on the Elisa plate (implying differences in incubation time) influenced the optical density.

Conclusion

QFT-G tests were reproducible with a fair level of agreement. The position of the sample on the Elisa plate influenced the final results but QFT-G seemed less affected by variations of pipette volume. It remains a challenge to routinely apply QFT-G in such a resource limited setting with power outages, shortage of trained laboratory staff, high HIV prevalence and various logistical issues in sample collection and processing.

OP–27

A QUANTITATIVE REAL-TIME PCR (QPCR) ASSAY TO DETERMINE *MYCOBACTERIUM AVIUM* SUBSPECIES PARATUBERCULOSIS LOAD IN DAIRY PRODUCTS

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Introduction

Mycobacterium avium subsp paratuberculosis (Map) has concerned the dairy industry by reports of its possible association with Crohn's disease in humans. While the 'gold standard' for Map detection remains isolation in culture, the purpose of this study was to develop and assess the potential of real time PCR assays coupled with a standardized Map DNA extraction method, to quantify Map in a range of dairy products.

Methods

Dairy products (milk, milk powder, yogurt and a range of cheeses) were artificially contaminated with various levels of Map. Map DNA was extracted from homogenized cheese and milk samples using the *Adiapure*® kit (*Adiagene*, France) subjected to two independent real time PCR assays targeting sequences from the IS900 insertion element and a region of the f57 sequence. Both assays utilized *TaqMan*® probe chemistry with FAM labelled probes. Each assay was duplexed with a VIC-labelled internal positive control. Specificity and sensitivity of the assays were assessed and validated using cheeses prepared from milk artificially contaminated with Map.

Results

The IS900 based real time assay was more sensitive (approx. 10-fold) than the assay targeting the F57 sequence, detecting < 4 cfu ml^{-1} in artificially contaminated milk and < 30 cfu g^{-1} in spiked cheese and milk powder samples. Based on calibration curves generated through spiked cheese samples, this quantitative real time assay correctly predicted the levels of contamination in cheeses manufactured with known concentrations of Map-infected cheese milk. Both assays showed 100% specificity based on a range of Map isolates, non-Map mycobacteria and non-mycobacterial microorganisms associated with dairy products.

Conclusion

The real time PCR assay combined with the Adiapure Map-DNA extraction kit represents a reproducible, sensitive and convenient method for detection of Map DNA from a range of raw and pasteurized dairy products. Its robustness has been confirmed through a ring trial within the FP6 EU ParaTBTools project.

OP–28

DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* USING INTRINSIC AUTOFLUORESCENCE OF THE BACILLI

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Introduction

Autofluorescence is an intrinsic characteristic of some microorganisms that allows detection without the need of staining. Here we report for the first time that the mycobacteria, including Mycobacterium tuberculosis, emitted autofluorescence in the cyano range of the visible spectrum. Purpose of the study: The strength of the autofluorescence and its potential use as a tool to improve the detection of the tubercle bacilli in clinical samples was evaluated.

Methods

Different mycobacteria, including M. tuberculosis, as well as other related bacteria were studied. The strength of the autofluorescence of un-labeled bacilli inside infected macrophages was compared with the green fluorescent protein (GFP)-labeled and fluorescein diacetate (FDA)-stained bacilli. Confocal microscopy was also applied to have insights into the nature of the intrinsic autofluorescence. The influence of standard sputum processing methods, usually applied in the diagnostic of Tuberculosis, on the autofluorescence of bacilli was also tested.

Results

The autofluorescence of the Mycobacterium species analyzed was easily visualized, without any processing, by simple direct observation in the fluorescence microscope or by fluorimetry, using selected excitation and barrier filter. Confocal laser scanning microscopy demonstrated that the mycobacteria exhibited an unique emission maximum at 475 nm when excited at 405 nm, suggesting that the autofluorescence of M. tuberculosis is most possibly due to the fluorescence emitted by the coenzyme F420. Inside macrophages, the M. tuberculosis cyan-fluorescence was as brilliant as bacilli expressing cytoplasmic GFP or FDA-stained.

Conclusions

Our results indicated that autofluorescence fluorimetry could be a successful method for the detection of M. tuberculosis. Favorably, the routinely heat inactivation (80?C during 20 min) as well as the addition of chemical reagents frequently used for the decontamination and liquefaction of the sputum specimens, appreciably increased the autofluorescence of M. tuberculosis. These treatments represent a great benefit because they reduce the risks of contamination between the health-care workers and increase the sensibility of the method.

OP–29

A MODEL OF PHENOTYPIC SUSCEPTIBILITY TO TUBERCULOSIS: DEFICIENT IN SILICO SELECTION OF *MYCOBACTERIUM TUBERCULOSIS* EPITOPES BY HLA ALLELES

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Background

HLA-DR allelic variants have been associated with TB susceptibility in different populations with risk ratios of 3.7-7.2. We hypoth-

esize that the genetic susceptibility to TB depends on the reduced capability of TB patients HLA-class II alleles in selecting the Mycobacterium tuberculosis (MTB) peptide antigen repertoire. To test this hypothesis, we developed a software that can predict HLA-DR restricted epitopes in the whole MTB genome or in specific groups of genes, based on quantitative peptide binding matrices. We analyzed the number of MTB epitopes recognized in two already described populations of TB patients and matched controls and in a control populations of beryllium exposed subjects.

Results

The number of putative epitopes capable of binding any HLA-DR allele in the whole MTB genome (HLA-DR immunome of MTB), is 405.422 out of 1.304.277 possible 9-mers, and it represents the 31.08% of the global capability and it is lower than the theoretical recognition of 35%. The HLA-DR alleles positively associated with TB susceptibility recognize a significant lower mean number of MTB-epitopes (7862±4258) respect to the MTB-epitopes recognized by HLA-DR alleles negatively associated with TB (11.376±1984, $p<0.032$), at an affinity level equivalent of the 1% of the best binder peptides for those alleles. The TB patients can bind at high affinity in the context of their HLA-DR alleles and in the whole MTB genome a number of epitopes [TB-population 1: 11341±908 (mean+SEM); TB-population 2: 15303±657] lower than matched healthy controls (CTR-population 1: 13587±605, $p<0.03$ vs TB-population 1; CTR-population 2: 16841±555, $p<0.04$ vs TB-population 2). This difference is not detectable in the beryllium hypersensitive subjects respect to their matched controls (beryllium-hypersensitives: 17593+447; beryllium-exposed controls 18014±421; $p=0.57$).

Conclusions

The MTB has less epitopes than expected if its genome presented a normal distribution of composition. There is a general reduced capability to present at high affinity T-cell epitopes in TB patients.

OP–30

THE MAPAC COMBINED *MICOBACTERIUM AVIUM* SUBSPECIES PARATUBERCULOSIS AND MYCOBACTERIUM AVIUM SUBSPECIES AVIUM GENOME MICROARRAY REVEALS VARIABLE DUPLICATIONS OF LARGE GENOMIC REGIONS (VCP`S) WITH LOW GC% FLANKED BY TRANSPORTABLE ELEMENTS

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Purpose of the study

Investigate mechanisms for variability in *Mycobacterium avium* subsp. *paratuberculosis*

Methods:

A 60mer oligonucleotide microarray (MAPAC) covering both genomes of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and *Mycobacterium avium* subspecies *avium* strain 104 was used to perform comparative genomic studies on a panel of MAP isolates with variant morphologies isolated from a variety of hosts.

Results

The MAPAC microarray was able to define a set of large sequence polymorphisms (LSP's) diagnostic for each of the three major

types of MAP and deleted LSP's associated with attenuation in MAP vaccine strains.

In addition, some strains contained up to 17 genomic regions (VSP's) giving signals suggestive of a series of large genomic regional duplications. VSP's were predominantly subsets of LSP's, with significantly low GC% regions and immediately flanked by insertion sequences or integrases. VSP's were present in only a proportion of any one culture.

Confirmation of MAPAC differential signal increases was made using quantitative PCR of a selection of genes within VSP's (including several virulence determinants and the rrn operon). Associations could be made between VSP duplications and colony growth rate / morphology.

An increase in expression of transposase associated with VSP's and active transposition resulting in significant alteration of the MAP proteome was demonstrated after infection into a human macrophage cell line.

Conclusion

The control of transposase activity, possibly associated with host cell entry, may be a mechanism for variation in genome plasticity and phenotype switching in MAP.

OP–31
ANTIBODIES TO LOCALIZE CELL DIVISION PROTEINS IN MYCOBACTERIA

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Introduction

InEscherichia coli, amodel bacterium, the assembly of the divisome involves the recruitment of no less than 10 essential proteins into a cell

division ring at midcell. Prior to their assembly, site position mechanisms as the MinCDE system, prevent polymerisation of FtsZ, a Tubulin analog, at other positions different from the cell center. Mycobacteria lack some of the components of theE. coliMin system and some elements of thedivisome. It is nevertheless crucial for the study of bacterial proliferation to obtain a detailed picture of the assembly of the divisome components. Moreover, in Mycobacteria, these elements are likely to play an important role in the establishment and exit from latency.

Purpose of the study

To develop tools to study the assembly and function of the mycobacterial divisome.We have developed antibodies that recognise the FtsZ, FtsQ, and DivIVA proteins from mycobacterial origin. These are valuable tools to study the cell division cycle of Mycobacterium tuberculosis having a major advantage over the use of fusions to fluorescent protein tags, as the latter ones may influence the physiology of the cell.

Methods

The ftsZ, ftsQ and divIVA genes from Mycobacterium tuberculosis were cloned into expression vectors that were transformed into a host adapted for the expression of unfrequent codons in E. coli. The proteins were purified and used to raise polyclonal antibodies in rabbits.

Results

Expression of the cloned ftsZand divIVAM. tuberculosisgenes in E. colihave toxic effects leading to the selection of clones showing production of partial or faulty gene products. In the case of ftsZwe circumvented this problem by introducing a his tail tag at the C-terminal end of the proteinand purified it by affinity chromatography. In all cases wefully sequenced the clones and discarded those harbouring undesired mutations.The antibodies have been tested for

their ability to detect the FtsZ, FtsQ and DivIVA proteins in western blots of M. tuberculosis and M. smegmatis total protein extracts.

Conclusions

Antibodies have been obtained against the three division proteins. The antibody against the DivIVA protein has already been used to localise it in permeabilisedM. smegmatiscells showing, as expected, both a polar and midcell localisation, consistent with its potential role in division site selection and polar development. This confirms that antibodies can be used advantageously to study the mycobacterial cell cycle.

OP–32

ACTIVITY OF DRUGS AGAINST AEROBIC AND DORMANT *MYCOBACTERIUM TUBERCULOSIS*

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Introduction

Nonreplicating (dormant) *M. tuberculosis* (Mtb) likely resides in the hypoxic areas of lung tuberculomas; dormant cells may be generated *in vitro* by adaptation of aerobiosis to anaerobiosis (Wayne model). Some drugs display partial activity against dormant Mtb including rifampin (R), metronidazole (M), moxifloxacin (Mx), amikacin (A).

Purpose of the study

The purpose of the study was to find drug combinations killing both aerobic (A) and hypoxic (H), dormant, Mtb.

Methods

Mtb H37Rv was grown in Dubos-Tween-Albumin in tubes incubated in A or H (Wayne) conditions, and growth monitored as CFU/ml. R, M, Mx, A (final concentration 8, 8, 4, 8 μg/ml, respectively) were added on day 5, 12, 19, 26 on A (A5) or H (H5-H12-H19-H26) cultures. Viability of 7, 10 or 14 days drug-exposed, washed, cells was monitored as CFU/ml and Day-To-Positivity (DTP), i.e. the positivity day in BACTEC460 (GI≥10) or MGIT960 (GU≥75).

Results

Among single drugs, increasing DTP values (from 12 to 28 days for A5 and H26, respectively, after 7 days exposure) were seen for R. M was active against late stages (H19-H26 cells) while Mx and A were active against early stages (A5-H5 cells). R+M sterilized (DTP >100 days) H19 and H26 cells in 10 days. R+Mx was active (DTPs 14-28 days) on both A and H cells, but did not sterilize them. R+M+Mx was more active than R+Mx and R+M. R+M+Mx+A sterilized A5 and H19 cells after 14 and 7 days of exposure, respectively; a good (DTPs 26-32 days) but not sterilizing activity was seen against H5 and H12 cells, which were the most refractory to drug treatment.

Conclusion

The combined use of drugs inhibiting synthesis/functions of RNA (rifampin), DNA (metronidazole, moxifloxacin) and proteins (amikacin), may attack/sterilize both aerobic and dormant cells (This work was supported by the 7th EC Project StopLatent-TB, Grant 200999).

OP–33

MUTATIONAL CHARACTERIZATION OF TLYA – GEN IN CAPREOMYCIN-RESISTANT MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATES

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Purpose of the study

With the worldwide emergence of extensively drug-resistant tuterculosis (XDR-TB), resistance mechanisms to second-line antibacillary drugs have gained an increased importance. So is the case for capreomycin (CAP), a cyclic peptide that binds to ribosome 16S and 23S subunits, effective against *Mycobacterium tuberculosis*. Mutations occurring in specific methylation sites of the 16S or 23S ribosome subunits and, in TLYA gene were shown to confer CAP resistance. Given the high XDR-TB rates in Lisbon Health Region, 29 CAP-resistant isolates were screened for TLYA mutations to identify the mutations responsible for CAP-resistance in the region.

Methods

The entire TLYA ORF was amplified in the 29 clinical isolates, recovered in 2005, and subjected to sequencing analysis. The isolates were also genotyped by mycobacterial interspersed repetitive unit – variable number of tandem repeats (MIRU-VNTR) to assess their genetic relatedness.

Results

We have identified nucleotide polymorphism present in all isolates, which classified as a silent mutation. Moreover, non-silent mutations were found in 55.5% of the isolates. Fifteen (51.7%) had a GT insertion at position 755 of the tlyA gene and all, belonged to the same MIRU-VNTR cluster.

Conclusions

We conclude that the majority of CAP resistance in the region may be due to mutations occurring in **TLYA** gene. The remaining resistance may be explained by mutations occurring in the 16S or 23S ribosome subunits and further studies are on the way. More concerning is the fact that 51,7% of the analyzed strains belong to the same cluster and had the same mutation. This suggests a high and recent XDR-TB transmission rate.

OP–34

DESIGN OF IMMUNOGENIC MULTIEPITOPIC *MYCOBACTERIUM TUBERCULOSIS* PEPTIDES FROM GENES SELECTED USING IN VITRO MODELS OF MYCOBACTERIAL MACROPHAGE INFECTION

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Background

Mycobacterium tuberculosis (MTB) is an intracellular pathogen that evokes a strong Th-1 immune response. *In vitro* diagnosis of MTB-infection by blood testing uses MTB proteins coded for by genes of the region of differentiation 1 (RD1) of the MTB genome. This study wants to test if proteins preferentially expressed by during MTB intracellular growth might provide new targets for the diagnosis of MTB infection.

Methods

Seventy-five multiepitopic HLA-promiscuous MTB-peptides were designed by quantitative implemented peptide binding motif analysis from three MTB-protein genes expressed in activated human macrophages (MA), four genes expressed during growth in non-activated human macrophages (MN-A), twelve housekeeping genes (HKG) and 6 genes of the RD1 region (RD1) as control. Patients with active TB (22 untreated and 23 under chemotherapy) and controls (9 TST-negative and 25 TST-positive) were enrolled. ELISpot for IFN-γ were performed on PBMCs stimulated with the selected peptides.

Results

In active TB patients, the mean response to RD1 derived peptides (21.3 ± 10.8) was higher than that to either MA (7.1 ± 3.5; $p<0.01$), MN-A (6.9 ± 1.1; $p<0.008$) or HKG (8.2 ± 3.2; $p<0.01$) derived peptides.

In TST-positive subjects all selected peptides elicited significant IFN-γ T-cell responses (MA 7.6±3.5; MN-A 8.9±3.5; HKG 7.9±4.5; RD1 12.9±6.8; p<0.02 compared to TST-negatives), but without differences between the subgroups. Further, T-cell responses to RD1 peptides were lower in the 23 active-TB treated patients (SFC 155±129) than in the untreated ones (RD1 peptides SFC 251±116, p<0.01). The response to MA peptides in active-TB treated (SFC 121.8±44.6) was higher than untreated ones (SFC 52±29; p<0.01).

Conclusion

The data demonstrate that the use of *in vitro* models of MTB-intracellular infection to select MTB gene products for further *in silico* and *in vitro* assessment of their immunogenicity has the potential to identify novel antigens and eventually be amenable to the design of new diagnostic tools.

OP–35
THE DYNAMIC HYPOTHESIS OF LATENT TUBERCULOSIS INFECTION OFFERS A NEW RATIONALE TO DEVELOP FUTURE THERAPEUTIC STRATEGIES

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Summary

Classically, it is considered thatonce infected by *Mycobacterium tuberculosis*, its latent form can be retained for the whole life, dormant in old lesions. With the aid of resuscitation factors these bacilli

can reactivate towards active tuberculosis. These assumptions raise at least three relevant questions to answer: (1) how can dormant bacilli remain in the lungs for the whole life? Considering the constant cellular turnover and healing of injured tissues; (2) How are the resuscitation factors provided to the dormant bacilli, while immersed in old lesions?; (3) why can a 9-month treatment with isoniazid eliminate the dormant bacilli? As isoniazid is active only against growing bacilli, this treatment should be provided for life, to avoid the reactivation of dormant bacilli.

Using experimental models of latent tuberculosis infection (LTBI) in mice, we have demonstrated that the granulomas are characterized by: (1) the drainage of nonreplicating bacilli by the foamy macrophages towards the alveolar spaces; (2) the constant formation of new ones; (3) the presence of local immunodepression, characterized by a high apoptosis, lack of lymphocytic proliferation and anergy; (4) the reduction of the immunological response and foamy macrophages accumulation after a short-term chemotherapy, and the bacilli reactivation once is finished; (5) administration of a vaccine based on fragments of *M. tuberculosis* (RUTI) allows the control of this reactivation by inducing a polyantigenic response against secreted and structural antigens.

The "dynamic hypothesis" suggests that LTBI would be caused by the constant endogenous reinfection of nonreplicating bacilli. This hypothesis is the only one that may explain the efficacy of the 9-month isoniazid treatment, and supports a therapy based in the elimination of the local immunosuppression by a short-term chemotherapy, followed by a therapeutic vaccination able to generate immunity against structural antigens to detect the resting nonreplicating bacilli and to avoid its reactivation. So far, a phase I clinical trial with RUTI has already finished showing a safety and immunogenic profile.

OP–36

FREQUENT HOMOLOGOUS RECOMBINATION EVENTS IN *MYCOBACTERIUM TUBERCULOSIS* PE/PPE MULTIGENE FAMILIES: POTENTIAL ROLE IN ANTIGEN VARIABILITY

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Background

The PE and PPE (PE/PPE) multigene families are particularly GC-rich and share extensive homologous repetitive sequences. We hypothesized that these homologous repetitive sequences could be the substrate for homologous recombination events, a mechanism rarely described in the natural evolution of mycobacteria.

Methods

To test our hypothesis, we developed a specific oligonucleotide-based microarray targeting nearly all of the PE/PPE genes, aimed at detecting signals for homologous recombination. Such a microarray has never before been reported due to the multiplicity and the highly repetitive and homologous nature of these sequences.

Results

Application of the microarray to a collection of *M. tuberculosis* clinical isolates, representing prevalent spoligotype strain families in Tunisia, allowed successful detection of six deleted genomic regions involving a total of 2 PE and 7 PPE genes. Some of these deleted genes are known to be immunodominant or involved in virulence. The four precisely determined deletions were flanked by 400 to 500 bp stretches of nearly identical sequences which served as substrate for homologous recombination, eliminating the intervening sequence to yield in-frame fusion gene. The finding of a high prevalence (~45% and ~58%) in at least two of the genomic deletions suggests they likely confer advantageous biological attributes.

Conclusion

Our data suggest that homologous recombination of PE/PPE genes likely increased antigen variability and diversity which may have profound implications for these families in pathogenicity and/or host adaptation.

OP–37

OVER-EXPRESSION OF DEVR TRANSCRIPTIONAL REGULATOR SHOWS DIFFERENT MULTICELLULAR BEHAVIOR IN SLOW AND FAST GROWING MYCOBACTERIA

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Summary

DevR is a transcriptional regulator that mediates the genetic response of *M.tuberculosis* and *M. bovis* to a wide range of stresses including oxygen limitation, and nitric oxide exposure. In these strains and, under hypoxic condition, DevR regulates the coordinated expression of around 48 genes related with dormancy. DevR is part of an operon including the genes DEVS and Rv3134c whose transcriptional regulation is complex. The locus is partially conserved in *M. smegmatis*, being present in all the sequenced strains. With the aim of identify the effect of DEVR over expression in both slow and rapid growers mycobacteria, the entire gene was cloned in the expression vector pMV261 under the control of hsp60 promoter. *M.smegmatis* and *M. bovis* were transformed with the episomal version of the plasmid and the level of expression was determined by qRT-PCR. Colony morphology and exopolysacharide production displayed differences between both strains. Assays performed to identify biofilm formation and lipid profile also showed noticeable differences in these strains. Bioinformatic comparison of the genomic regions shows remarkable differences especially in the promoter regions of the two strains analyzed in this study. These results demonstrate that although DEVR is present in both strains, probably some of its targeted genes are different in both strains.

OP–38
STRUCTURE AND STRATEGY OF THE NEW STOP TB WORKING GROUP ON NEW DIAGNOSTICS

Ruth Mcnerney on behalf of the STOP TB WG on New Diagnostics

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Summary

The STOP TB Partnership is a network of international organizations, countries, donors, governmental and nongovernmental organizations and individuals. It was established in 2000 to realize the goal of eliminating TB as a public health problem and, ultimately, to obtain a world free of TB. Seven Working Groups have been established to provide a focus for coordinated action. Each of the Working Groups have independent governance mechanisms, but work under the umbrella of the Stop TB Partnership. The Working Group on New Diagnostics was established in 2001. The mission of this WG is to advocate and implement research and/or operational activities in pursuit of the development and implementation of TB diagnostic tools and to collaborate with other elements of the Partnership so as to create synergy and add value to actions taken in pursuit of the aims of the Partnership. Improving case finding and access to anti-tuberculosis treatment is at the core of efforts to control TB and the development of new diagnostic tools is considered a priority. The WGND has recently been restructured. Membership is open to all individuals with an interest in the development and assessment of new diagnostic tests for tuberculosis. A core group has been established, with representation from major interest groups. To complement this, eight subgroups with specific tasks have also been created. Each subgroup has two joint co-ordinators who will develop work plans and targets

for the subgroup. There are five subgroups with primary responsibility for advancing technology and the remaining three provide necessary information around specific issues that should guide tool development and implementation. These subgroups will work together to achieve WGND goals. A new draft strategy has been developed. This presentation will describe the structure of the WG, outline its strategy and invite the participation of ESM members in its activities.

OP–39
PREVENTION AND CONTROL OF TB EXPOSURE IN HEALTH CARE FACILITIES

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Summary

Tuberculosis is an important public health problem because of its frequency, attributable morbidity and mortality and cost. Nearly one-third of the world's population is infected with Tuberculosis (TB), and it kills almost 3 million people per year. Higher incidence rates are reported in hospitals in developing countries. One of the main goals of health care system is the reduction of the national TB case rate. For this purpose protection from tuberculosis must be stabile structured and professional organized. We present the principles of prevention.

The prevention includes the following common safety and health topics: Program to control exposure; Identification, screening and medical surveillance; Training and education ; Isolation and isolation rooms; warning signs and tags; Respiratory protection . We discuss the reference's centre experience of prevention measures investigation in hospitals and TB labs.

Important solution for prevention of employers with employee exposure to TB will be development of effective TB infection control program which minimizes exposures to TB including: early identification, isolation, and treatment of persons with TB , provide and practice early patient screening in the Emergency Department, to identify potentially infectious patients, and prevent employee exposures, the use of engineering and administrative procedures to reduce the risk of exposure and the use of respiratory protection.

OP–40
GLOBAL WATER MYCOBACTERIUM PROJECT: PRELIMINARY RESULTS

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Introduction

Nontuberculous mycobacteria are usually described as environmental saprophytic species with limited capability to cause disease in humans. Recently, an increased number of cases of human disease due to nontuberculous mycobacteria have been described. Some of the cases are directly related to water reservoirs.

Purpose of the study

This project was started in 2007 with the aim of word-mapping the environmental mycobacteria present in tap and natural waters.

Methods

Samples were taken from Athens (at the ESM meeting), Belfast, Lisbon, United States, Afghanistan, Uzbekistan, Zambia, Cuba and Luxemburg. Water samples were filtrated and residues were cultured on solid and liquid media at 27-35 °C. Samples were then identified by the Inno-Lipa reverse line blot.

Results

We found positive cultures from all samples except the Athens and Uzbekistan sample. Among the results were M. avium complex and M. gordonae in the Zambezi reiver (Zambia), M. gordonae, M. intracellulare and M. chelonae from the Lisboa water distribution system and M. fortuitum complex in municipal pump water in Hayratan (Afghanistan). An up to date summary of results will be presented.

Conclusion

NTM are present in the environment worldwide and regional differences may lead to regional differences in human disease.

OP–41
EVALUATION OF CAPILIA TB ASSAY IN ZAMBIA AND THE IMPACT ON THE DIAGNOSIS OF TUBERCULOSIS IN A RESOURCE LIMITED SETTING

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Purpose of the study

Compare sensitivity and specificity of Capilia TB assay and niacin strip using GenoType Mcobacterium CM Assay (CM) as golden standard. Measure cost effectiveness of Capilia TB for diagnosis of TB.

Methods

Overall 403 archived acid-fast isolates collected during the TB prevalence survey in Zambia 2005/2006, have been subjected to Niacin strip, Capilia TB and CM assay. All identification tests were performed according to the protocol of the manufacturer.

Costs were established primarily by expenditure reviews and observed times spent on processing cultures. Based on 2006 prices, they represent full economic costs (including consumables, opportunity costs of staff and equipment and apportioned overhead costs).

Results

Using CM Assay 79 (20%) were identified as MTB complex and 324 (80%) as NTM. Capilia TB identified 78 out of 79 MTB complex. The sensitivity and specificity of Capilia TB were 98.7% and 99.4% respectively. Only 74 MTB complex isolates showed sufficient growth for the niacin strip. Niacin identified 62 out of 74 MTB complex isolates. The sensitivity and specificity of niacin strip were 83.8% and 92.6% respectively.

Incremental costs per culture of Capilia and Niacin as a confirmatory test to the overall cost using MGIT 960 were estimated to be 0.30 USD and 1.75 USD, respectively. This is equivalent to approximately 1% of the total culture cost for Capilia vs. 6 % for Niacin. Costs per identified MTB were estimated to be 1.86 and 10.95 USD, respectively.

Conclusions

We recommend the use of Capilia TB for identification of MTB in resource poor settings. Compared to Niacin, Capilia TB performs considerably better in terms of sensitivity, specificity, overall costs, duration of the test and simplicity. Additionally, Capilia TB can be used as stand-alone test whereas it is generally recommended to use Niacin in combination with another identification test.

OP -42
B CELL INFECTION BY PATHOGENIC AND NON-PATHOGENIC MYCOBACTERIA.

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Introduction:

Most of the studies on the pathogenesis of mycobacterial diseases rely on the macrophage-mycobacteria interaction. However, recently, other cells have been implied in the mycobacterial diseases, among

them are epithelial, endothelial and fibroblast cells. Studies related on the interactions mycobacteria-host cell are neccesary.

Purpose of the study:

Human B lymphocytes (Raji cell line), were analyzed for their ability of performing macropinocytosis, the endocytic pathway responsible of bacteria internalization in nonphagocytic cells.

Methods:

Early times of B-cell Infection with Mycobacterium smegmatis and Mycobacterium tuberculosis were study and PMA was used as a chemical inductor of macropinocytosis. Intracellular survival was evaluated by CFU determination, structural changes were studied by scanning electron and transmition microscopy. Fluid phase uptake triggered by mycobacterial infection was evualted by plate spectrofluorometry. Nitric oxide production was observed by fluorescence microscopy

Results:

Scanning electron microscopy revealed significant changes on cell membrane topography with both bacteria and PMA, abundant lamellipodia surrounding the bacteria and all around the cell was observed. Cytoskeleton rearrangements were observed, with filamentous actin accumulation beneath lamellipodia in infected and PMA treated cells. Confocal microscopy and transmission microscopy revealed intracellular Mycobacterium smegmatis and Mycobacterium tuberculosis. Fluid phase uptake induced by the infection and by PMA was quantified and several inhibitors were tested along to the inductors; amiloride, cytochalasin and wortmanin abolished totally fluid phase uptake induce by bacteria and PMA. Mycobacterium smegmatis was efficiently eliminated contrary to Mycobacterium tuberculosis.

Conclusions:

This report gives evidences that: a)B-lymphocytes are able of uptake mycobacteria being macropinocytosis the mechanism responsible of this capability; b) M. tuberculosis survives intracelullarly in this cell, contrary to M. smegmatis; c) B cells produce nitric oxide in response to mycobacterial infection

OP-43

USEFULNESS OF THIN LAYER PLATE AND "IN HOUSE 7H9 TUBE" FOR THE RAPID DIAGNOSIS OF TUBERCULOSIS

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Introduction:

Actual TB situation including MDR or XDR-TB has pointed to a change in tuberculosis or micobacteriosis diagnosis. In the recent years multiple efforts to improve TB-diagnosis have been reported; those proposals with applications in laboratories with low income and high number of clinical samples are ideal. Among them, are methodologies

like thin layer plate, that in combination with "in house 7H9 tube" could reduce costs and time for bacterial recovery from clinical samples.

Purpose of the study

To determine the usefulness of thin layer agar and "in house 7H9" for the recovery of mycobacteria from pulmonary samples.

Methods

To determine the usefulness of thin layer agar and "in house 7H9 tube", a total of 520 pulmonary samples from the same number of patients, were decontaminated and seeded on both culture mediums and Lowestein-Jensen. Plates and culture tubes were checked frequently for any mycobacterial growth evidence. Culture purity was determined and a multiplex PCR was performed to confirm that recovered bacteria belonged to Mycobacterium genus and to MTB-complex, in case of non-tuberculosis mycobacteria recovery, speciation was done by sequencing. Drug susceptibility of each isolate was performed by microplate alamar blue assay.

Results

A total of 50 clinical isolates were recovered, 49 of them were identified as MTB-complex, and the only one non-tuberculous mycobacteria isolate was identified as Mycobacterium abscessus, The combination of thin layer plate and "in house 7H9 tube" allowed the maximum bacterial recovery in the shortest time period in comparison with Lowestein-Jensen medium. Only one of the MTB-complex clinical isolate was MDR and the Mycobacterium abscessus isolate was sensitive to clarithromycin and moxifloxacin.

Conclusions

The combination of thin layer plate and "in house 7H9-tube" allowed a rapid and efficient mycobacteria recovery hence improving TB-diagnosis.

IV. POSTER PRESENTATIONS (PP)

PP–01
TUBERCULOSIS AND MYCOBACTERIOSES IN HIV POSITIVE PATIENTS

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Summary

Statistical data on HIV/AIDS in the Czech Republic are collected and evaluated within a framework of the National Programme. In the period from 1986 to 2007 a total of 1042 HIV seropositive subjects and 244 AIDS patients (both Czech citizens and naturalized foreigners) were recorded. Medical care of these patiens is secured by 7 specialized institutions.

Department for Diagnostics of Mycobacteria of the Regional Institute of Public Health performs microbiological examinations for patients hospitalized in the AIDS Centre of the Faculty Hospital in Ostrava which serves several districts of Moravia.

In the 1997-2007 period mycobacteria were identified by culture and other techniques (LCR, MTD, PCR, MB/BacT, MGIT, BioFM and hemoculture) in 13 patients, 10 males and 3 females: M. tuberculosis in 2, M. xenopi in 3, M. kansasii in 1, M. avium in 2 and M.celatum in 1 patient; M. fortuitum was detected in four culture negative patients: in one by PCR, LCR and MTD, in the other by LCR and MTD and in the third one by LCR only. In one female patient M.

fortuitum was identified once, M. gordonae once, MTD was positive but M. tuberculosis was not confirmed by culture.

PP–02

OUTBREAK OF NEW GENOTYPS OF MYCOBACTERIUM TUBERCULOSIS COMPLEX IN ALGIERS LINKED TO THE IMMIGRATION FLOW

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Purpose of study

The aim of this study is to emphasize the installation of new genotyps of *M.tuberculosis* complex isolates at an infectious disease reference hospital in Algiers during a period study of 6 years (from 2000 to 2006), a total of 312 were analysed.

Methods

The molecular typing of the M.tuberculosis isolates was performed by spoligotyping for 162 strains and by IS6110 restriction fragment length polymorphism for 97 strains.

Results

The phylogenetic analysis has shown a biodiversity of *M.tuberculosis* complex strains isolated, they were mainly represented by 3 major families: the family H(Haarlem), family T and the family LAM(for Latin America and the Mediterranean).

However, new genotyps have been highlighted

For the first time the profile ST 61 or LAM 10-CAM has been identified for four strains isolated from patients of subsaharian origin, three of whom were HIV positive, this profile is frequently seen in Cameroun and west Africa.

The profile ST1 or«Beijing» has been identified in one isolate obtained from an Algerian patient HIV positive, the strain was resistant to isoniazid (INH) with double mutation(arg463leu and ser315thr) in KATG gene. On the spoIDB4 , one strain was declared by Pasteur Institute of Algeria.

Algeria is witnessing illegaly immigration flow mostly from subsaharian and Asian countries.

Conclusion

Tuberculosis cases in Algiers are the result of reactivation or transmission of previously acquired infectio. However, this study provides an insight into the genotyps newly appeared in Algiers, therfore efforts should focus on the surveillance of *M.tuberculosis* strains circulating in Algeria.

Keywords

M. tuberculosis; genotyping; spoligotyping; RFLP-IS6110

PP–03
EXTENSION OF AN OUTBREAK OF INFECTIONS BY MYCOBACTERIUM MASSILIENSE IN LAPAROSCOPIC SURGERIES TO SIX STATES IN BRAZIL

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Introduction

Surgical-site infections by rapidly-growing mycobacteria were detected in more than 300 patients submitted to laparoscopic surgeries from 2004 to 2006 in the city of Belém (PA), in the North of Brazil. Isolates were identified by PRA-HSP65 and sequencing of the HSP65 and RPOB genes as *Mycobacterium massiliense***,** a recently described new species belonging to the *M. chelonae-M. abscessus* group. These isolates presented two PFGE patterns, differing in a single restriction band. Since 2006, similar infections in patients submitted to laparoscopic, artroscopic and implant surgeries were detected in five other states from Brazil: 1051 cases in Rio de Janeiro (RJ), 185 in Vitória (ES), 121 in Goiânia (GO), 61 in Santo Ângelo (RS) and, recently, cases were also detected in Curitiba (PR).

Purpose of the study

To evaluate if surgical RGM infections in different states in Brazil were caused by the same *M. massiliense* strain.

Methods

Isolates from the different sites were collected and analysed by PRA-HSP65, RPOB sequencing and PFGE.

Results

76 isolates from Rio de Janeiro (RJ), 21 from Vitória (ES), 18 from Goiânia (GO), and 10 from Santo Ângelo (RS) presented the same PRA-HSP65 pattern, RPOB sequence, and PFGE patterns found in surgical isolates from Belém.

Conclusions

Infections related to surgical procedures are emerging in Braziland isolates studiedso farbelongto a unique strain of *M. massiliense***,** suggesting a single infection source. The source of these infections has not been identified, but common aspects were the re-utilization of discardable material and disinfection of surgical equipment by submersion in 2% glutaraldheyde for 20 min between surgeries.

PP–04

TUBERCULOSIS IN THE FRENCH DEPARTMENTS OF THE AMERICAS: EPIDEMIOLOGY, DRUG RESISTANCE AND GEOGRAPHICAL TRACKING OF THE MAJOR GENOTYPIC LINEAGES OF MYCOBACTERIUM TUBERCULOSIS

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Summary

A previous long-term genotyping study focused on *Mycobacterium tuberculosis* clinical isolates from the 3 French Departments of the Americas (Guadeloupe, Martinique and French Guiana; January 1994 – December 2003, n = 744 isolates), based on spoligotyping and 5 loci-VNTRs (Variable Number of Tandem DNA Repeats). In the present work, we extended this study to isolates collected during January 2004 – December 2005 (n= 176) using spoligotyping with a newer 12-loci MIRUs (Mycobacterial Interspersed Repetitive Units) format. The genotypes obtained were compared to an updated spoligotype-MIRU-VNTRs international database developed at the Pasteur Institute of Guadeloupe with information on 68000 isolates from about 160 countries of origin. The epidemiological and drug-resistance data were studied both for clustered versus unclustered isolates, and underlined the continued impact of imported cases of TB. We further compared clustering rates and genetic diversity to underline the benefits of 12-loci format. Not surprisingly, the clustering rate with spoligotyping alone in the present investigation (73.9% – 95% IC ± 6.5) was similar to the previous study (77.3%; 95% IC ±

3.4). The combination with 12-loci significantly decreased clustering rate to 37.4 % (95% IC ± 7.6; p<0.001), with a significantly higher genetic diversity (number of different patterns observed) than spoligotyping alone (116 patterns among 154 strains studied so far, versus 76 patterns among 176 strains; p<0.001). Among the major genotypic lineages observed – Latin American and Mediterranean (LAM), Haarlem, and ill-defined T clades predominated, with a smaller proportion of East-African Indian (EAI), X, and Beijing clades. We also developed a specialized software to detect the most pertinent MIRU loci combinations while simultaneously reducing the number of loci to be used for typing. For LAM and Haarlem lineages which cause most of the TB in our setting, the Hunter and Gaston Discriminative Index (HGDI) scores of the full typing scheme (12 MIRU loci) were 0.918 and 0.841 respectively. Reducing the MIRU typing scheme to only 2 loci (loci 26 and 40) for LAM and 4 loci (loci 10, 20, 23 and 40) for Haarlem lineages allowed to retain 93.6% (HGDI = 0.857) and 91.3% (HGDI = 0.768) of the discriminatory power of the full typing scheme.

PP–05

GENOTYPIC ANALYSIS OF M. TUBERCULOSIS STRAINS ISOLATED IN THE CITY OF RIO GRANDE, BRAZIL, PERFORMED BY SPOLIGOTYPING AND MIRU-VNTR

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Summary

Genotypic analysis of 50 *Mycobacterium tuberculosis* strains obtained from patients in the city of Rio Grande, Brazil, was performed by the standard spacer oligonucleotide typing (spoligotyping) and the 12-locus MIRU-VNTR assays. The MIRU-VNTR assay detected 42 distinct patterns with 15 strains distributed in seven different clusters and 35 strains with unique patterns. According to the allelic diversities, seven of the 12 MIRU-VNTR loci were moderately or highly discriminative. Spoligotyping was performed on 48 strains identifying 26 different patterns, with 32 strains grouped in ten clusters and 16 had unique patterns. The Hunter-Gaston discriminatory index (HGDI) was 0.992 and 0.959 for MIRU-VNTR and spoligotyping respectively. When the two methods were applied in combination, the HGDI was 0.995 whereas 44 different patterns were discriminated. The predominant genotypes belonged to the Latin American-Mediterranean (LAM) and Haarlem families and clades T1 and T3; however, one strain was characterized as belonging to the East Africa-Indian lineage. This lineage is exotic in the Americas being endemic in South-East Asia, South-India, and East-Africa. The results highlight the importance of molecular characterization to monitor the dynamics of tuberculosis transmission, and warn on the possibility that the port city of Rio Grande is allowing the entrance of new tuberculosis genotypes in Brazil.

PP–06
MOLECULAR CHARACTERIZATION OF MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM TB PATIENTS

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Introduction

Introduction:Molecular typing methods have been used in epidemiologic studies of Mycobacterium tuberculosis isolates in various areas of the world. Purpose of the study:The aim of this study was to determine the antibiotic susceptibility and spoligotyping pattern of M. tuberculosis strains isolated from TB patients

Methods

The isolated M. tuberculosis strains have been characterized by performing susceptibility tests against four first-line antituberculosis drugs and were then subjected to spoligotyping characterization.

Results

The resistance rate for Isoniazid (INH), Rifampicin (RIF), Streptomycin (STM) and Ethambutol (EMB) were 20%, 20.5%, 32.4% and 17.4% respectively. Resistance to INH+RIF was 12.7%. The most prevalent spoligotype detected in this study was SR3 belonging to the group 2, and SR19 belonging to group 1. Additionally, 220 strains were subclassified into previously described families; 34% belong to the Haarlem family, 17.1% belong to the T family, 14% to the EAI familly, 8.6% to the CASI family, 6.3% to the W-Beijing family, and 1.3% to the Manu family.

Conclusion

This study showed the spread of spoligotyping family and antibiotic resistance in the clinical isolates. It is also important to undertake studies to identify which factors are the most significant to consider in tuberculosis control program.

PP–07

THE POPULATION STRUCTURE OF MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATES FROM WESTERN SWEDEN DEFINED USING SPOLIGOTYPING AND MIRUS IN A LONG-TERM STUDY

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Summary

Mycobacterium tuberculosis clinical isolates (n=357) collected over a period of 5 years (2001 to 2005) in western Sweden were studied in order to investigate the molecular epidemiology. Genotyping was performed using spoligotyping and 12-loci MIRUs (Mycobacterial Interspersed Repetitive Units), and the data were compared to the international genotyping database of Pasteur Institute of Guadeloupe.

The overall clustering after spoligotyping was 73.4% (46 clusters containing a total of 262 strains, 2 to 43 strains per cluster). This high clustering rate was related to a high proportion of imported cases of TB from well-defined geographical regions. However, when spoligotyping was combined with MIRU-typing, the final clustering rate decreased to 25 % (30 clusters containing a total of 89 strains, 2 to 10 strains per cluster). Using the n-1 formula, the ongoing transmission rate was calculated to be 17%. Phylogeographical analysis showed that genotypic lineages found among foreign-born group were significantly different than for Swedish born patients (Beijing: 9% vs. 5%, EAI: 17% vs. 3%, Bovis: 0% vs. 1%; Manu: 1% vs. 0%, CAS: 13% vs. 2%; Haarlem 16% vs. 18%, LAM 9% vs. 9%, X: 1% vs. 1%, S: 2% vs. 5%, T: 28% vs. 36%, Unknown: 5% vs. 20%, $p<0.001$), reflecting the respective origins of the patients. Most of the ancestral and/or evolutionary older lineages (Principal Genetic Group 1, PGG1) were found among foreign born patients (38.6% vs. 11.4%; $p<0.001$), while evolutionary-recent lineages of the PGG2/3 genogroup were more common among Swedish patients. Despite a high proportion of Somali patients in our study, no strains with *M.africanum* signature were found.

Our results show that in spite of a relatively lower TB transmission among Swedish born patients, ongoing TB epidemic is largely maintained due to imported cases of disease.

PP–08

CONTAINMENT OF TUBERCULOSIS IN PRISONS – A KEYPOINT OF THE GLOBAL POLICY

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Summary

Prisons play a crucial role in epidemiology of tuberculosis (TB): an air-born infection, with a higher prevalence among lower socio-economical strata, HIV-infected and addicts. Conditions in prisons, incl. overcrowding, facilitates the spread of disease to other prisoners, personnel and visitors. Relative rate of MDR- and XDR- TB, and conversion of latent TB to an active, is much higher than in society. Released prisoners before accomplished treatment become a source of TB in the society.

In Bulgaria the legislation for TB-care in prisons is acting since 2003 and the National Program for Prevention and Control of TB (supported by the WHO, EuroTB, ECDC and the Global Fund), was accepted in 2007.

The item 4 under the Action Plan focuses on prisons, aiming at identification of TB cases, strengthening the infrastructure of hospitals and medical centers with the prisons, delivering new medical apparatus, associating the Healthcare sector and facilities of the Ministry of Justice to the information TB system, building an equip to plan, coordinate, manage and control these activities.

The control of TB in prisons is difficult and complex. It requires a prompt identification of disease upon the entrance (screening), isolation, treatment (DOTS) and control of cases upon release out of prison. Microbiology diagnosis should be as rapid as possible, through real-time PCR, FISH diagnostic and other contemporary methods, incl. for susceptibility testing. The questions regarding the Infection Control in prisons are open: new or renovating facilities are required, with negative ventilation (Air-born Infection Isolation Rooms), air-born personal protective equipment (such as N95 respirators) etc. Care should be taken for the health of guards, the medical personnel and to the environment. The total program in prisons will require much more organization, education, coordination, ensuring appropriately trained staff and well equipped facilities, and obviously, more resource.

PP–09
GENOTYPING ANALYSIS OF TUBERCULOSIS CLINICAL ISOLATES FROM A HOSPITAL IN LISBON BY MYCOBACTERIAL INTERSPERSED REPETITIVE UNIT

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Purpose of the Study

Driven by multidrug resistance and the dissemination of the human immunodeficiency virus (HIV), the worldwide resurgence of tuberculosis (TB) has led the World Health Organization to declare it as a global health emergence. Portugal has one of the highest TB notification rates of the European Union, 32.4 cases PER 100 000 inhabitants in 2006. Lisbon Health Region notification rate is well above the national average and in several areas within, an incidence rate of >100 cases per 100 000 inhabitants has been reported. In the present study we have analyzed the transmission, drug susceptibility and characteristics of a study population from a Lisbon's Hospital Pulido Valente.

Methods

One hundred and thirty-two *Mycobacterium tuberculosis (M. tuberculosis)* clinical isolates, each corresponding to a different patient in 2006, were collected from Hospital Pulido Valente in Lisbon.

All isolates were previously tested for drug susceptibility to first-line antibacillary drugs. Genotyping was performed by MIRU-VNTR.

Results

The clinical isolates analyzed belonged to a study population of 132 patients, 27 females and 105 males. Furthermore, 26 (26.5%) patients were positive and 72 (73.5%) were negative for HIV serology. The remaining had an unknown HIV serology. The MDR resistance rate was found to be 3.0%, while 13.6% were resistant to one or more first-line antibacillary drugs. Fifty-three (40,2%) out of the 132 isolates were found to be distributed through 17 MIRU-VNTR clusters of two or more isolates.

Conclusions

We conclude that transmission of resistant and susceptible *M. tuberculosis* strains is occurring, with special concern for the MDR strains. We have also verified the existence, among the patients analyzed, of a high HIV co-infection rate and a very likely association with clustering. Attention must be payed to this problem, or otherwise it will continue to hamper the control of tuberculosis in the region.

PP–10

GENOTYPE MTBDRPLUS FOR DETECTION OF RIFAMPIN AND ISONIAZID RESISTANCE IN CLINICAL SPECIMENS OF M. TUBERCULOSIS

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Introduction

The development of molecular assays based on knowledge of the mutations conferring resistance to rifampin (RMP) and isoniazid (IHN) has enabled *M. tuberculosis* genotype susceptibility testing to be performed. The new Genotype MTBDRplus assay was evaluated for its ability to detect mutations in the 81-bp hotspot region of the rpoB gene, mutations in codon 315 of the katG gene and alterations in the inhA promoter region.

Purpose of the study

The present study sought to evaluate the use of the new Genotype MTBDRplus assay directly from positive clinical specimens, comparing the results with those obtained by conventional phenotypic resistance studies.

Methods

86 bacilloscopy-positive sputum samples from patients with suspected tuberculosis were subjected to the new Genotype MTBDRplus assay. This technique is based on DNA-strip technology, consisting of a multiplex PCR in combination with reverse hybridization using nitrocellulose strips for detecting mutations related with Rifampin (RIF) and Isoniacid (INH) resistance comparing the result with those obtained phenotypically in Bactec MGIT 960.

Results

Of the 86 M. tuberculosis strains tested, 36 were phenotypically sensitive to INH and RMP, and displayed no resistance-linked mutations in 32; in four of them a mutation at inh-A promoter region was found, but no phenotypically resistance was detected. Among the 50 sputum specimens showing some form of phenotypic resistance, 13 displayed RMP resistance, 14 INH resistance and 23 Multi-drug resistance (MDR). All of the phenotypically RIF resistances were detected by

the test and only two of the INH resistant strains could not be detected. Sensitivity for RIF resistance is 100% in our study and 93,02% for INH.

Conclusions

The new Genotype MTBDRplus assay is a valid technique for detecting resistance to RMP and INH, providing within 6-8 hours a result that enables a more effective orientation of patient treatment. Since the detection of resistance-related mutations did not cover 100% of possible cases of resistance, use of this new assay does not obviate the need for conventional phenotypic resistance testing. Mutation located at the inhA promoter region could not award phenotypically resistance in some cases.

PP–11

USE OF THE GENOTYPE MTBDRPLUS ASSAY FOR RIFAMPIN AND ISONIAZID SUSCEPTIBILITY TESTING OF MYCOBACTERIUM TUBERCULOSIS CLINICAL STRAINS ISOLATED IN GREECE

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Introduction

Purpose of the study: To evaluate the performance of the MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) assay as

a rapid diagnostic tool for the detection of rifampin and isoniazid resistance in Mycobacterium tuberculosis isolates

Methods

243 clinical strains recovered from patients' specimens admitted to the NRCM "Sotiria" Hospital and 26 strains admitted from other laboratories, have been analyzed with the MTBDRplus assay, since September 2007. Drug susceptibility testing (DST) was performed using the proportion method on Lowestein-Jensen medium. Several strains were also analyzed by means of the Bactec MGIT960 system.

Results

Two strains gave faint hybridization signal to various rpoB bands and excluded from the analysis. 227/267 strains (85, 01%) were found not to harbor any detectable mutation in the rpoB hot spot region, in the KatG 315 codon and in the inhA promoter.

1. 3 strains (1, 12%) had mutations conferring rifampin resistance whereas 24 strains (8, 98%) had mutations conferring isoniazid resistance.
2. 13 strains (4, 86%) harbored mutations conferring resistance to both rifampin and isoniazid (MDR).
3. The mutations identified and their prevalence was: For rpoB; 10 S531L, 2 H526Y, 2 ΔWT7, 1 ΔWT8, whereas 1 strain had both the ΔWT3 and ΔWT4 mutations. Twenty-four strains had the KATG S315T1 mutation, 13 the C(-15)T in the INHA promoter, 2 stains harbored both the S315T1 and the C(-15)T and 1strain had both the KATG S315T1 and a ΔWT2 mutation in the INHA promoter.
4. Regarding Rifampin a 100% agreement between the MTBDRPLUS and the DST data was found. Regarding Isoniazid a 99, 5% concordance was found. One strain having no detectable mutation with the MTBDRPLUS assay was found resistant to Isoniazid and most likely harbored mutation(s) outside the genomic regions analyzed.

Conclusions

The above findings substantiated the usefulness of the MTBDRplus assay for the rapid detection of drug resistance.

PP–12

PRELIMINARY STUDY OF *MYCOBACTERIUM TUBERCULOSIS* CLINICAL ISOLATES RESISTANT TO ISONIAZID AND RIFAMPICIN BY MAS-PCR SYSTEM

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Introduction

Timely detection of drug resistance (DR) and multidrug-resistance to anti-tuberculosis agents is important in the effective management of tuberculosis (TB) cases.

Purpose of the study

The aim of this study was to detect point mutations conferring isoniazid (INH) and rifampicin (RIF) resistance in clinical isolates from patients with TB

Methods

Drug susceptibility testing (DST) to first-line drugs were performed by indirect proportion method on Löwenstein Jensen (ILJ)

and MGIT 960. A total of 30 laboratory strains plus the reference strain H37Rv were subcultured on LJ and included in the study.

A multiplex allele-specific polymerase chain reaction (MAS-PCR) involving the genes RPOB**,** KATG, and the MABA-INHA promoter region was used to detect the point mutations presumably related to the DR profile of the isolates.

Results

Seventy nine DR clinical isolates were recovered from LJ and MGIT 960 media: 71 isolates were resistant to INH (INH-R) and 42 to RIF (RIF-R). Using MAS-PCR we found point mutations in 55 (77.5%) of the INH-R strains: 36 (50.7%) with mutation in codon 315 of KATG; 19 with a mutation in the promoter region (-15) of INHA (27.0%); and 16 (22.5%) without mutations detected. Thirty four (81.0%) of the RIF-R strains showed mutations in the RPOB gene studied codons. Both drug-susceptible and DR laboratory strains were correctly identified by MAS-PCR system. In clinical isolates 22 (52.4%) mutations were found in codon 531, 12 (28.6%) in 526, and no mutations appeared in codon 516 of RPOB gene.

Conclusion

The MAS-PCR used in this preliminary study allowed us to detect mutations conferring resistance to INH and RIF from pure cultures, within a working day and with a low cost. Therefore it could be a useful tool for a rapid detection of drug-resistance at clinical level and for describing frequent point mutations in our geographical area.

PP–13
PYRAZINAMIDE MONORESISTANT MYCOBACTERIUM TUBERCULOSIS IN MANISA, TURKEY

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Introduction

Introduction:Pyrazinamide (PZA) is a primary antituberculous drug. BACTEC 460TB is recommended method as reference method for detection of PZA resistance in Mycobacterium tuberculosis. This method is more expensive than conventional susceptibility methods and therefore, it is recommended that according to conditions of their community the laboratory directors should decide whether PZA would be included in panel of primary drugs tested. One of most important factors to make a decision is the prevalence of PZA resistance, particularly PZA monoresistance in the community.

Purpose of the study

The aim of the present study was to determine the extent of PZA monoresistance in M. tuberculosis kompleks (MTBC) isolates in our region.

Methods

In this study, PZA susceptibility testing of 109 MTBC strains (susceptible against to isoniazid, rifampicin, ethambutol and streptomycin) isolated from Manisa city in Aegean region of Turkey was performed by using the BACTEC 460TB radiometric system.

Results

Among the isolates, two (%1.8) of 109 isolates which were susceptible to all primary drugs showed monoresistance against PZA. It was also determined that one of the PZA-monoresistant isolates was Mycobacterium bovis.

Conclusions

The results of the study indicate that the rate of PZA monoresistance was low; therefore, susceptibility testing of a panel of primary drugs without PZA may be an economical alternative in our region.

PP–14
MULTI-DRUG RESISTANT TUBERCULOSIS: RAPID MOLECULAR DETECTION WITH MTBDRPLUS ASSAY

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Introduction

Nowadays, the greatest concern of tuberculosis control programs is the appearance of multidrug-resistant (MDR) tuberculosis and extensively drug-resistant (XDR) tuberculosis. Rapid determination of drug resistance in clinical samples of *Mycobacterium tuberculosis complex* (MTC) is the prerequisite for the initiation of effective chemotherapy ensuring successful treatment of the patient and preventing further spread of drug-resistant isolates.

Drug susceptibility testing (DST) by conventional methods takes several weeks and so, more rapid results could be achieved by direct testing of patient specimens by molecular methods. These methods are based on the knowledge that resistance to Rifampicin and Isoniazid in MTC is most often attributed to mutations in the RPOB, KATG and INHA genes. The GenoType MTBDRplus assay (Hain Lifescience GmbH) has the ability to detect a broader variety of mutations in these genes.

Purpose of the study

The aim of our study was to determine the sensitivity of the new MTBDRplus assay in comparison to culture, identification and classical DST directly from smear-positive clinical specimens.

Methods

A total of 48 smear-positive sputum specimens were processed by both the classical micobacteriological methods and the molecular assay, MTBDRplus.

Results

MTBDRplus assay allowed an accurate identification of MTC species by detection of the specific band in all samples, from which we also isolated and identified MTC strains by cultural methods.

In the samples from which we isolated susceptible strains (54.2%), wild type patterns were found using MTBDRplus assay. The samples from which we isolated resistant strains (45.8%) showed specific mutations associated with the correspondent resistant phenotype.

Conclusion

The great sensitivity of MTBDRplus assay is due to the presence of targeted genes for INH resistance, mostly INHA which is the main responsible for the INH resistant phenotype among Portuguese

stains. We found that this assay allows rapid detection of resistance, always in agreement with the classical methods, improving our ability for detecting MDR strains.

PP–15
POLYMERASE CHAIN REACTION (PCR) BASED GENOTYPING AND DRUG RESISTANCE STUDIES OF MYCOBACTERIUM TUBERCULOSIS USING PRIMARY CLINICAL MATERIAL

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Summary

Tuberculosis (TB) incidence in Latvia decreased from 74 per 100.000 population in 1998 till 47.2 in 2007. However, drug resistance (DR) is still high (>10%). Therefore are actual studies of DR, evaluation of recent transmission. PCR based analysis of primary clinical material (PCM) may give a rapid answer on above mentioned questions. The goal of our study was to investigate such possibility.

Clinical material – sputa, bronchial washings from 118 tuberculosis (TB) acid fast specimen positive patients (2005-2008) have been studied. NALC decontaminated samples were processed by Qiagen DNA isolation kit and applied for **RPOB**, **KATG** and **INHA** gene analysis by Hain Lifescience GenoType commercial PCR kit , spoligo-

typing by standardised method, and in house method for quinolone resistance was introduced. Additionally drug resistance was analysed by BACTEC MGIT 960 method when culture was later available.

35 different spoligotype patterns among 118 samples studied were found, 34 of them of Beijing genotype, 8 original, 3 mixed culture. Repeated spoligotyping in 14 cases gave discrepant picture and waas not interpretable therefore. 56 samples were analysed for DR. Evaluated by molecular method all 18 isoniazid (H) resistant samples were confirmed by BACTEC, with additional evaluation of 6 resistant isolates. More discrepant were rifampin (Rif) resistance where of total 14 resistant samples only 9 were both positive by BACTEC and **RPOB** method. Among total 14 samples two mutations were not confirmed by BACTEC and vice versa.

It should be concluded, that spoligotyping of DNA from PCM should be developed and introduced in clinical laboratories, since different genotypes, esp. Beijing genotype with the highest proportion of drug resistant representatives may be clearly detected and appropriate medical measures undertaken therefore. The same is true about H and Rif resistance by PCR based methods and an urgent need for commercial PCR kits evaluating fluoroquinolone resistance is actual.

PP–16

EVIDENCE FOR AN EXCEPTIONALLY HIGH RATE OF MULTIDRUG-RESISTANCE AMONG RECURRENT TUBERCULOSIS PATIENTS IN DOMINICAN REPUBLIC: EMERGENCE OF A CIRCULATING CLONE (H3-HISPANIOLA) OF TUBERCLE BACILLI AND ITS SPREAD TO THE AMERICAN CONTINENT

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Summary

We studied *Mycobacterium tuberculosis* clinical isolates from recurrent tuberculosis (TB) patients in the Dominican Republic (timeframe 2003 to 2006, n=89). The study sample was characterized by pulmonary TB (98.9%), male gender (male to female sex-ratio of 2), younger age (67.4% cases among the age group 17 – 39 yrs), and a very high proportion of MDR-TB (41.6%). Genotyping was performed using spoligotyping and MIRUs (Mycobacterial Interspersed Repetitive Units), and the data were compared to the international genotyping database of Pasteur Institute of Guadeloupe. The epidemiological and drug-resistance data were studied both for clustered versus unclustered isolates. Among the major genotypic lineages observed – Latin American and Mediterranean (LAM), and Haarlem predominated, with a smaller proportion of the ill-defined T superfamily, X and S clades. Interestingly, all these belong to evolutionary-recent TB lineages, as opposed to evolutionary older Central-Asian (CAS), Beijing

and the ancestral East-African Indian (EAI) clades; all the three being completely absent in our study sample. MIRU typing and database comparison revealed the emergence of a new clone (SIT457-MIT 222) of tubercle bacilli. Worldwide, the countries of origin are known for 88% of the patients and in 7/10 cases, it corresponded to a patient originating from Dominican Republic. We show that this clone belongs to the Haarlem linage, and hereby designate it as a new sublineage named "H3-Hispaniola" due to its specific signature (absence of spacer 28 in addition to the Haarlem-3 signature). We present data underlining its evolution from possible variants, including a precursor clone that was tracked to a TB patient from Dominican-Republic, diagnosed with TB in 1998 in Rhodes Island, USA. We show that the H3-Hispaniola clone has spread to the American continent; indeed 71% of SIT457 strains worldwide are today found in USA, and 64% all such patients in USA originated from Dominican Republic. Lack of compliance during prior treatment appears as the single largest selection factor among our patient population, favoring the emergence of MDR-TB in Dominican Republic.

PP–17

SECOND-LINE DRUG SUSCEPTIBILITY OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS ISOLATES IN GEORGIA

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Objectives

Resistance to standard first-line antituberculosis agents is not uncommon in Georgia (FSU). A country-wide drug resistance survey in 2006 found that the prevalence of multi-drug resistant *M. tuberculosis* (MDRTB) to be 6.8% in new and 27.4% in re-treatment TB cases. The purpose of the present study was to investigate the susceptibility of MDRTB isolates to second-line antituberculosis drugs in Georgia.

Methods

MDRTB isolates obtained from 462 patients of the NCTLD were tested for their susceptibility to standard second-line drugs and several quinolones using the proportion method on Lowenstein-Jensen media and Middlebrook 7H11 agar.

Results

Ethionamide resistance was present in 325 (70%) of the 462 isolates tested. Resistance to amikacin, capreomycin, cycloserine and para-aminosalicylic was found in 110 (24%), 101 (22%), 79 (17%), and 62 (13%) of the isolates respectively. Resistance to ofloxacin, levofloxacin, moxifloxacin and gatifloxacin was as follows: 105 (23%), 20 (4%), 55 (12%), and 4 (1%) of the isolates. The breakpoint MIC for ofloxacin was 2μg/ml and was 4μg/ml for the other quinolones. All of the isolates were resistant to streptomycin. Extensively drug resistant TB (XDRTB) was found in 44 (10%) of the isolates evaluated.

Conclusions

Resistance to various second-line antituberculosis agents was found to be present in a large proportion of MDRTB isolates in this selected sample of patients. The presence of resistance to multiple second-line antituberculosis agents adds more complexity to the

treatment of patients. Quinolones resistance is comparatively low at the present time; however, increased resistance to this class of drugs will likely continue to develop. It is of interest that resistance to the second-line agents occurred in a setting where these agents were not available through the National TB Program. In order to decrease the emergence of additional drug resistance it is important that availability of tuberculosis drugs be restricted to appropriate settings.

PP–18
MOLECULAR INSIGHT INTO DRUG RESISTANT TUBERCULOSIS IN BULGARIA

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Purpose of the study

In Bulgaria, the rate of MDR-TB is much higher as compared to the neighboring countries (WHO, 2008). Here, we report results of a first study on the molecular basis of drug resistance in M. tuberculosis clinical isolates currently circulating in Bulgaria. We compared distribution of drug resistance mutations within the main genotypic clusters identified by spoligotyping and VNTR typing.

Methods

The study panel included 133 *M. tuberculosis* isolates from newly diagnosed, epidemiologically unlinked, pulmonary TB patients from different regions of Bulgaria. Spoligotyping and 24-loci MIRU-VNTR typing were used for strain typing. Mutations were detected in RPOB hot-spot, KATG315, INHA promoter region, and EMBB306.

Results

The study collection included 37 drug-resistant and 96 susceptible *M. tuberculosis* strains. Sixteen strains (12.0%) were MDR. This latter figure is close to the MDR-TB rate (10.7%) among all new TB cases diagnosed in Bulgaria in 2006 (WHO, 2008) which supports the representativeness of our sample. Three types of the RPOB mutations were found in 20 of 27 RIF-resistant strains while RPOB S531L was the most frequent. Ten (45.5%) of 22 INH-resistant isolates had KATG S315T mutation. INHA -15C>T mutation was detected in 3 INH-susceptible strains and one INH-resistant strain that also had KATG315 mutation. A mutation in EMBB306 was found in 7 of 11 EMB-resistant strains. No mutations in KATG315, RPOB, EMBB306 were found in susceptible strains.

Conclusions

RPOB and EMBB306 mutations may serve for rapid genotypic detection of the majority of the RIF and EMB-resistant strains in Bulgaria. The results for INH resistance are complex and further investigation of more genes is needed. Comparison with genotyping data did not reveal a statistical difference in distribution of drug resistance between clustered and non-clustered isolates. Emergence and spread of drug-resistant and MDR-TB in Bulgaria is not associated with any specific spoligotype or MIRU-VNTR cluster.

Acknowledgments

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PP–19

MOLECULAR EPIDEMIOLOGY OF RIFAMPICIN-RESISTANT MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM MANISA, TURKEY

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Introduction

Drug-resistant tuberculosis is a serious health problem throughout the world.

Purpose of the study:The aim of the study is to investigate the molecular epidemiology of rifampicin resistant Mycobacterium tuberculosis strains isolated from Manisa in Western Turkey.

Methods

We evaluated the mutations in a 329 base pairs located in 481-589 codons of the rpoB gene by automated sequencing of 43 strains. Results:In 43 rifampicin resistant strains, mutations were identified in 93% strains. No mutation was detected in the three resistant strains. The most common mutation (63%) was Ser531Leu. Overall,

15 different mutations and 13 alleles affecting 12 codons were identified. One new mutation (Ile572Leu), which is localized outside the RRDR was identified. The codon numbers of the most frequently encountered mutations 531 (67%), 516 (9%), and 526 (5%).

Conclusions

It was concluded that the results from this study can serve as a basis for monitoring of molecular epidemiology of drug resistant M. tuberculosis strains isolated from our region.

PP–20

COMPARATIVE ASPECTS OF MYCOBACTERIUM TUBERCULOSIS DRUG RESISTANCE IN SPUTUM AND LUNG RESECTATES OBTAINED FROM PULMONARY TB PATIENTS IN GEORGIA

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Purpose of the study

Analysis of drug-resistance in M.tb recovered from both patient sputa and lung tissue resectates at the National Center of TB (NCTLD).

Methods

M. tuberculosis isolates were obtained from a total of 27 patients undergoing surgical intervention therapy in the NCTLD. Standard sputum microscopy and cultural analyses were performed both before and after surgery. Lung tissue resectates were taken during surgery and designated as either 'I' for internal wall of cavitating granulomas, 'E' for external wall, 'C' for caseous mass, or 'N' smaller peripheral nodulus in distinct lung tissues. Studies were supported by the U.S.CRDF Grant No. GEX1-002712-TB-06.

Results

Out of 27 patients before operation, 11 (40.8%) were sputum-smear positive, and 16 (59.2%) were sputum-smear negative. *M. tuberculosis* was successfully cultured from sputa for 19 patients (70.3%). M.tb were also cultured from 14(51.8%) lung tissues. The frequency of successful culture differed in different types of pulmonary lesions, with 92.8% culture positive from C, 78.5% for I, 71.4% for E, and 41.6% for N. Estimates of the relative numbers of viable bacteria on positive slants also differed, with 150-180 CFU for I, 80-90 for C, and 50-60 for both E and N specimens. Cultures from resectates were : 1- monoresistant; 3- polyresistant, 13- MDR, and 9 resistant to all drugs. Notably, antibiotic resistance differed in sputum isolates from the same patients. This indicates that resistance was arising independently in patients during infection, and likely also at different tissue sites during a single infection.

Conclusion

M. tuberculosis capable of forming colonies on L-J slants were most readily detected in necrotic caseous material, to a lesser extent in both inner and outer cavity walls. Less frequently, live bacteria were found in small nodules. Further investigation will allow to compare patterns of *M. tuberculosis* gene expression during growth in the human lung.

PP–21
RESISTANCE OF M. TUBERCULOSIS IN A REFERENCE CENTER FROM 1997 TO 2007 IN SPAIN

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Introduction

Tuberculosis is considered a serious public health problem. Some factors ,such as HIV infection and immigration, are contributed of the increase of the resistance. In recent years , the problem is increased with the Multidrug-resistance (MDR, XDR and XXDR).

Purpose of the study

The purpose of this study is study the level of resistance of the strains of M. tuberculosis received in our Micobacteria Reference Center, in the last 10 years.

Methods

894 M. tuberculosis strains are studied. In these years , different techniques with rapid methods to determinate the susceptibility testing,are used: Bactec 460 TB, Esp II system and Bactec MGIT 960. The antimicrobial agents evaluated are: Streptomycin, Rifampin, Ethambutol, Isoniazid and Pirazynamide. When the resistance to any of these occur, the sensitivity of second-line drugs were studied.

Results

From the 894 strains studied, a 13,87 % of resistance were detected (streptomycin 1,9%, rifampin 9,28%, ethambutol 0.67 %, isoniazid 10.85

% and pirazynamide 1.0%), and a 6,59% of the strains were MDR (multi-drug resistance).Of them a 71 % were resistant to rifampin + isoniazid.

Conclusion

The higher percentage of resistance were obtained with isoniazid and with rifampicin . Lower, with ethambutol and streptomycin and pyrazinamide are around 1%. In general, no increase of multidrugs resistant are observed in this years in comparison with the previous years. So they are a trend to increase this resistance in strains isolated from patients from other countries.

PP–22
COUNTING THE COST OF DRUG RESISTANCE IN CLINICAL ISOLATES OF TB

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Purpose of Study

The acquisition of antibiotic resistance has been shown to result in a fitness cost which depends on the position and nature of the chromosomal mutation. The frequency at which resistant *Mycobacterium tuberculosis* strains are isolated *in vivo* is correlated with the fitness of different chromosomal mutations *in vitro* (Billington, McHugh, Gillespie. Antimicrob. Agents Chemother. 1999;43:1866-9). We have provided evidence that strains adapt *in vivo* to their host (Gillespie *et al*. Microb. Drug Resist. 2002;8:273-9).

Methods

In order to investigate the fitness cost of drug resistance in clinical strains, a novel rapid *in vitro* method using liquid culture was developed. Strains were inoculated into BacT/ALERT MP bottles and once this signaled positive, diluted 1:10 and 1:10,000 and inoculated in triplicate into fresh MP bottles. A modification of the Youmans and Youmans method (J. Bacteriol. 1949;58:247-255) was used to determine the generation time (G) of the strain. The mutations conferring rifampicin and isoniazid resistance were investigated.

Results

The findings were that multiple drug resistant clinical isolates have a longer G (45.9hr) than sensitive clinical isolates (19.5hr) and monoresistant isolates (19.4hr). Laboratory strains have the shortest G (15.1hr).

Conclusions

The fitness deficient depended on the genetic background and the ability of the strain to transmit. We have shown that strains of *M. tuberculosis* which have acquired antibiotic resistance have undergone a fitness detriment, however successful strains have little fitness deficit. The differences demonstrated in clinical isolates may be due to compensatory mutations ameliorating the fitness deficit.

PP–23

PHYLOGEOGRAPHY OF THE MULTI DRUG-RESISTAN MYCOBACTERIUM TUBERCULOSIS COMPLEX ISOLATED IN SPAIN

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Introduction

The population of *M. tuberculosis* can be subdivided according to different molecular markers in families, groups or lineages (*Filliol, Sreevetsan and Gagneux*). The genetic diversity observed is associated with the geographical origin of TB cases. During the 10-year period of the study of the MR-TB cases in Spain, we have seen a progressive increase in the number of isolates from foreign patients. In 1998, only 5% of patients were immigrants while in 2007, near 75% of the isolates were from immigrants patients.

Purpose of the study

To study the philogeography of the isolates comming to our country

Methods: The analisys of the Spoligotype and the the genetic polymorphism in the gene codon 463 KatG, used in the routine screening, permited us both, to identify families and differentiate between the three large groups in the tuberculosis complex.

Results

Of the isolates from 171 immigrant patients, only one isolate belongs to the West African lineage, which was obtained from an African patient. Twenty four isolates were from the Beijing Family, or East Asian lineage which were isolated from 15 patients who came from Eastern Europe (Russia, Lithuania, Ukraine, Latvia), 4 from Latin America (Peru, Colombia), 4 from Asia (Tibet, Vietnam, China), and 2 from North Africa. No isolates from Indo-Oceanic lineage, or East African-Indian lineage were found. The remaining 126 isolates, were consider to be included in the Euro-American lineage and were LAM, Haarlem, X, T, or unasigned spoligotype family.

Conclusions

We could speculated that all isolates of immigantspatients from the last ten years in Spain, except those of the Beijing family and the *M. africanum*, belong to the Euro-American lineage described by Gagnoux *et al*.

PP–24

BIOFILM DEVELOPMENT BY NON-PIGMENTED RAPIDLY GROWING MYCOBACTERIA

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Introduction

Biofilm development has been claimed as a pathogenic factor form Non-pigmented Rapidly Growing Mycobacteria (NPRGM), but no studies have been performed using a high number of clinical strains.

Purpose of the study

To evaluate the ability of clinical strains of NPRGM to develop biofilm.

Methods

Clinical significance of the strains was evaluated according to commonly accepted criteria. Biofilm test was performed in polystyrene microtiter plates with Middlebrook 7H9 during 25 days. One well for each strain and medium was stained and photographed to measure the biofilm development in previously defined days. Surface covered by bacteria was calculated. Motility test were realized using Middlebrook 7H9-0.3% agar. A logistic regression analysis was performed to evaluate the relationship between biofilm development and clinical significance.

Results

168 clinical strains (9 *M. abscessus*, 2 *M. alvei*, 30 *M. chelonae*, 90 *M.fortuitum*, 5 *M. mageritense*, 8 *M. mucogenicum*, 22 *M. peregrinum*, 1 *M. porcinum* and 1 *M. septicum*) were tested. Forty-one of them were clinically significant (8 *M. abscessus*, 14 *M. chelonae* 17 *M. fortuitum* and 2 *M. peregrinum*). Biofilm was formed by 123 strains, while 45 of them were not able to develop it (1 *M. abscessus*,

9 *M. chelonae*, 22 *M. fortuitum*, 2 *M. mucogenicum* 10 *M. peregrinum* and 1 *M. septicum*). 76 of the biofilm-positive and 25 of the biofilm-negative strains showed sliding motility. There was a statistically significant relationship between biofilm formation and clinical significance, both for all strains (p=0.003), and also more specifically for M. fortuitum (p=0.01), and almost for M. chelonae (p=0.05).

Conclusion

Not all the NPRGM tested were able to develop biofilm. No differences were detected in biofilm development among the different species. A significant relationship was found between the clinical significance and the biofilm development in all species and *M. fortuitum*, and a trend was detected for *M. chelonae*.

PP–25
PULMONARY INFECTION DUE TO MYCOBACTERIUM MARINUM

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Summary

Mycobacterium marinum has long been recognized as a cause of swimming pool or fish tank granulomata. Most M.marinum

infections do not invade beyond the superficial cooler regions of the skin. The organisms grow well at 30 to 32°C but usually poorly or not at all at 37°C. In 2005 however, it was first time reported that these organisms produced pulmonary infection in an immunocompetent patient (Lai C-C et al. 2005). We present the second case of pulmonary M.marinum infection. A 46-year-old woman was admitted to the Municipal Hakodate Hospital, Hokkaido, Japan, with a 4-year history of dystrophy due to the nervous anorexia. The patient had fever at admission. Chest radiography revealed cavitations surrounded by infiltration of both upper lung fields. Acid-fast stains of three of four sputum samples (April 26-May 7) revealed numerous acid-fast bacilli and one mycobacterial colony was recovered on 2% Ogawa egg medium incubated at 37°C. Isolate was identified as M.marinum using DNA-DNA hybridization kit (DDH MYCOBACTERIA KYOKUTO). The patient was successfully treated with combination therapy of isoniazid, rifampicin and ethambutol for 6 months. The mycobacterium developed smooth photochromogenic colonies after 7 days on 2% Ogawa egg medium incubated at 32°C. Organisms grew on 2% Ogawa egg medium at 25 and 32°C, but not at 37 and 42°C and grew in presence of TCH (1,10μg/ml), but not in Nacl (5%), PNB (500μg/ml) and EB(5μg/ml). The mycobacterium was negative for nitrate reduction, semi-quantitative catalase and iron uptake, but positive for arylsulfatase (3day), 68°C catalase, Tween hydrolysis (5,10day), urease and pyrazinamidase. The characteristics of the isolate were 100% consistent with that of M.marinum ATCC927T, and 99.7% to "M.ulcerans subsp. shinshuense" ATCC 33728T by RIDOM analysis of 16S rDNA sequence (E.coli positions 54-510).

PP–26
MOLECULAR TYPING OF MYCOBACTERIUM INTRACELLULARE ISOLATED FROM INFECTIONS AND COLONISATIONS IN HUMANS

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Introduction

Mycobacterium intracellulare(MIN), a species in the avium complex, can cause various pathologies. In this study, we report the first genotyping study using the MIRU-VNTR technique of genotypic and phenotypic diversity within the MIN species.

Methods

We studied 42 strains: 41 were isolated at Bordeaux University Hospital and one was the reference strain *M. intracellulare* ATCC 13950. These strains were isolated in 35 patients between 2001 and 2008. The mean age of these patients was 68 years and the ratio of the sexes (M/F) was 0.94. Identification was carried out using Genprobe® probe. We tested 44 MIRUs, of which 16 were new MIRUs that had been identified from the *M. avium* genome 104 and 17 from the genome of the ATCC strain, which were available in the form of contigs. The amplification products obtained following amplification and migration were sequenced and showed a link between molecular mass and number of repetitions.

Results

Using the MIRU technique, MIN is shown to be widely diverse. Of the 44 MIRUs tested, only 7 were present and discriminant and

6 MIRUs had not been observed before and were determined using MIN contigs. The discrimination index, established using the profile of 7 MIRUs and of all 42 strains, was 0.96. The stability study was carried out on 4 strains, and no difference was noted before and after 10 processes. BioNumerics® analysis enabled 6 clonal complexes to be distinguished. Clonal complex I is made up of 12 strains, 8 of which are identical. This complex contains only those strains that are of pulmonary origin. Complexes II and III contain 12 and 6 strains respectively, which are of pulmonary or extra-pulmonary origin. A breakdown of strains by pathogenicity and various demographic criteria was also carried out.

PP–27
FIVE YEAR SURVELLANCE OF MYCOBACTERIUM AVIUM POLYMORPHISM IN PIG FARMS

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Summary

Infections with non-tuberculosis *Mycobacteria* are registered among human patients in Latvia, nevertheless the source of infection is unclear. At the same time, caseous necrosis in mesenteric lymph nodes was found among pigs bred and slaughtered in Latvia. Studies were started from 2003, isolates obtained from animals and identified as *Mycobacterium avium* (MAV). The goal of this study was genetic characteristics of infectious agents.

149 pigs were examined within 5 year period. 24 mycobacteria isolates were obtained from 95 pigs (36%) in 2003, but in 2007 these numbers were 8 (33%) from 54 pigs. Genotypes of isolates were analysed using IS1245-PVUII restriction analysis (RFLP), sequencing of the 5'– and 3'– terminal fragments of HSP65 and polymorphism of GYRB gene.

The sequence of 5'-region of HSP6**5** gene was that of the MAV with genetic identity among isolates except one from 2003, containing mutation at the 135 (C/T). The 3'-regions were similar with single mutations, including an isolate of MAV from human. Negative IS901 test excluded presence of MAV subsp. *paratuberculosis*. Analysis of IS 1245 marker revealed similar RFLP (Dice coefficient >70%) patterns characteristic to MAV subsp. *hominissuis*. Some small clusters were registered. One isolate found in 2007 was quite similar with 3 ones obtained in 2003 from the same farm. Additionally, one similar cluster with 2 isolates was found in another farm; however difference was in 1 or 2 bands in comparison with 2003 2-isolate clusters. 3 identical isolates to them were found in 2 distant farms.

We conclude that isolates being isolated from pigs are of MAV subsp.*hominissuis* type only. It seems that they are genetically related and some of them are permanently present in piggeries monitored within this 5-year period. Genetic analysis of isolates from animals and in human clinic will discover possible ways of infection transmission.

PP–28

TEN YEARS OF ATYPICAL MYCOBACTERIA ISOLATION IN SPAIN

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Opportunistic infections caused by mycobacteria other than *M. tuberculosis* have increased as a consecuence of several factors such as AIDS epidemic, inmunosupressed therapy, transplantation etc.

Purpose of the study

The objectives aimed in this study was to describe the atypical mycobacteria indicence referred to our Mycobacteria Reference Center for a period going from 1997 to 2007.

Mehods

Our study has been carried through from positive cultures to atypical mycobacteria from all samples received in our Reference Center.

Processing has been made from rutinary sowing in solid and liquid mediums. Identification was made by Biochemical, genetic (Probes, Genotype) and/or HPLC.

Result

During this ten year period a total of 408 atypical mycobacteria (36 different species).

were isolated. The most frecuently was *M. avium* (107), *M. fortuitum* (98), *M. chelonae* (53), *M. gordonae* (52),*M kansasii* (32), *M. marinum* (22), *M. szulgai* (16), *M. peregrinum* (15), *M. intracellulare* (14), *M. xenopi* (13), *M. simiae* (9), *M. flavescens* (9), *M. phlei* (8), *M. lentiflavum* (7), *M. abscessus* (6), *M. scrofulaceum* (6), *M. smegmatis* (5), *M. gastri* (4), *M. terrae* (3), *M. gadium* (3), *M. malmoense* (3), *M. diehrnoferii* (3), *M. interjectum* (2), *M. shimoidei* (2), *M. duvalii* (2), *M. mucogenicum* (2), *M. rhodesiae* (1), *M. oubense* (1), *M. aichiense* (1), *M. neoaurum* (1), *M. falax* (1), *M. termorresistibile* (1), *M. nonchromogenicum* (1).

Conclusions

The genetic techniques allow identified a great number of atypical mycobacteria quickly and that is important for the clinic interest in some patients.

PP–29
MYCOBACTERIUM CAPRAE AND MYCOBACTERIUM BOVIS ISOLATES IN SLOVENIA – A 1996-2007 NATION-WIDE STUDY

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Summary

Among the members of *Mycobacterium (M.) tuberculosis* complex (MTc), *M. tuberculosis* is predominantly a human pathogen, whereas *M. caprae* and *M. bovis* have a broad host range and are responsible for tuberculosis (TB) in domestic and wild mammals. However, they can also infect humans. Zoonotic TB is clinically indistinguishable from TB caused by *M. tuberculosis* but accurate diagnosis is essential for epidemiological reasons and adequate treatment. The aim of this study was to evaluate the frequency of human and animal *M. caprae* and *M. bovis* isolates in Slovenia in the period of 1996 – 2007.

All MTc isolates were identified with AccuProbe culture identification kits (Gen-Probe, CA, USA). Until 2005, differentiation of MTc members was performed on the basis of biochemical tests (nitrate reduction, niacin test, TCH), colony morphology and PZA susceptibility testing. In 2005, Genotype MTBC test (Hain Lifescience, Germany) was introduced and also used for differentiation of MTC members.

During a twelve-year period 4311 TB patients were registered at Central Registry for TB Golnik, Slovenia. 3510 (81.4%) were culture

positive. In 3505 cases (99.86%), active TB was proven by isolation of *M. tuberculosis* while *M. caprae* and *M. bovis* BCG were isolated in 4 cases (0.11%) and 1 case (0.03%), respectively. The average age of 4 patients (3 males, 1 female) with TB caused by *M. caprae* was 79 years (age limits 61-88 years); all of them had pulmonary and extrapulmonary TB. In animals, bovine TB caused by *M. caprae* was diagnosed in cattle (n=4) and in 3 animals (bisons and camel) from a zoological garden.

Slovenia is one of the countries with a high rate of bacteriologically confirmed human TB. The majority of TB cases were caused by *M. tuberculosis* while *M. caprae* isolates were rare. *M. bovis* has not been identified, neither in humans nor in animals.

PP–30

MIRU-VNTR-BASED DIFFERENTIATION OF MYCOBACTERIUM AVIUM ISOLATES FROM ANIMALS AND HUMANS – A FEASIBILITY STUDY

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Summary

In the past, IS-based RFLP analysis has been most extensively applied to investigate the genetic diversity of *M. avium* strains. Recently, PCR-based typing methods, such as MIRU-VNTR, have been developed to allow faster and easier differentiation among the

strains. Previous reports have indicated the feasibility of this method to differentiate *M. avium*. The aim of our study was to assess the usefulness of the method to subtype Slovenian *M. avium* subsp. *avium* (MAA) and *M. avium* subsp. *hominissuis* (MAH) strains that have been previously genotyped with RFLP.

A panel of 30 strains (17 MAH isolates from humans and pigs and 13 MAA isolates from cattle, poultry and pigs) was tested against 8 typing markers identified previously (Thibault et al., 2007) and designated as TR 292, 25, 47, 3, 7, 10, 32 and X3. Primer sequences and annealing temperatures according to the abovementioned reference were used. PCR products were analyzed on a 1.5% agarose gel by their amplicon size to detect differences in tandem repeat numbers.

Two of the markers (X3 and TR 292) failed to give any or clearly interpretable results, respectively. Other six markers revealed remarkable homogeneity of the strains. All MAA isolates shared the same MIRU-VNTR code. The same finding was observed in MAH isolates from pigs while MAH isolates from humans exhibited 2 MIRU-VNTR types. However, the codes of the respective groups of strains (MAA/MAH from pigs/MAH from humans) differed.

These preliminary results indicate that MIRU-VNTR typing seems to have considerably limited usefulness for differentiation of *M. avium* strains. However, in the scope of our further studies, the PCR conditions for two of the markers will be optimized and more strains and markers will be tested to reliably assess the usefulness of the method for *M. avium* genotyping.

PP–31
MULTIDRUG RESISTANT MYCOBACTERIUM BOLLETII INFECTIONS

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Summary

RpoB sequencing identified multidrug-resistant *Mycobacterium bolletii* in four patients including three patients fulfilling ATS criteria for pulmonary disease. Primary clarithromycin resistance was found in 100% *M. bolletii* isolates and 0% non-*M. bolletii* organisms of the *M. chelonae-abscessus* complex. In *M. bolletii* isolates, no mutations could be detected in the domain V of the 23S rDNA and in the riboproteins L4 and L22, previously associated with macrolide resistance in gram-positive bacteria.

PP–32
THE REAL-TIME PCR: APPLICATION FOR DETECTION IN CLINICAL SAMPLES OF MYCOBACTERIA

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Introduction

After the resurgence of tuberculosis in early 1990 due to the emergence of HIV, there is stagnation in France in the rate of incidence. However, the analysis shows a sharp disparity as immigration status. Indeed, among the migrant population the incidence is thirteen times higher than the rest of the population, increases of 8% per year since 1997 and multidrug resistance to antibiotics although limited (1.4%) were found to 82% in this category population. These

data prompt us to use diagnostic techniques that are rapid, sensitive and easy to implement.

Purpose of the study

The purpose of this study was to develop a rapid diagnostic technique using real-time PCR (LightCycler 2.0 – Roche Diagnostics), easy to use and which allowed both to identify Mycobacterium tuberculosis complex (MTBc) and the other species of mycobacteria (NTM).

Methods

The **HSP65** gene was amplified. Two specific FRET probes were used for the detection of **MTBC** and of *Mycobacterium* genus. The identification of NTM was carried out by sequencing of amplicon product directly from the capillary of the LightCycler. Finally, the real negativity of samples was verified by using of an internal control.

Results

For MTBc, the assay sensitivity was 5x103 CFU / ml or 5 CFU/5 µl of *M. tuberculosis* spiked into human sputum.

The sensitivity from the analysis of 955 clinical samples (37.3% pulmonary and 62.7% extra-pulmonary) was 69.6%, specificity 98.1%, PPV 88.8%, 2% and VPN 93.7%. The sensitivity from the analysis of 701 patients was 73.2%, specificity 98.6%; PPV 91.8% and NPV 94.5%. For NTM:14 different species were detected, among them *M. avium*, *M. chelonae*, *M.ulcerans*, *M. leprae* and *M.genavense*

Conclusions

We report here an easy in-house real-time PCR assay for fast and reliable detection of mycobacteria within two hours after DNA extraction from clinical samples.

PP–33

EVALUATION OF RAPID TESTS BASED ON LIPOARABINOMANNAN IN URINE AND SPUTUM FOR THE DIAGNOSIS OF TB

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Introduction

Effective control of TB depends on early detection and successful treatment of cases. One of the biggest challenges to Tuberculosis (TB) control programs is the poor performance of the TB diagnostic techniques used in identifying TB cases in resource constrained settings where more than 98% of TB cases occur. In most cases, diagnosis is limited to smear microscopy and clinical work up only, which identifies less than 50% of TB cases.

Purpose of the study

To evaluate the sensitivity and specificity of two lipoarabinomannan (LAM) antigen based tests (Chemogen and Lionex) in detecting the presence of TB disease using urine and sputum specimens

Methods

TB suspects with a cough of 3 weeks or more and registering smear positive patients were recruited into the study. Suspects were investigated for TB by clinical examination with the aid of chest radiography and improvement to antibiotic treatment and smear and culture with 2 months clinical follow up.

Spot and morning sputum and urine samples were collected from participants. Sputum smear microscopy and culture were carried and new tests were carried out on both sputum and urine. Confidential HIV testing was also carried out on the participants. The sensitivity and specificity of the new tests were evaluated by comparing to overall TB diagnosis (smear status, culture status and clinical work up). The sensitivity and specificity were also stratified by HIV infection status.

Results

TBA before conference date

Conclusions

TBA before conference date

PP–34

COMPARISON OF THREE METHODS FOR DETECTION OF MYCOBACTERIA: THREE YEARS PERIOD EXPERIENCE

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Purpose of the study

The aim of the study was to evaluate the diagnostic value of various methods widely used in microbiological diagnosis of tuberculosis: direct smear examination for acid-fast bacilli, cultural identification in Lowestein-Jensen (L-J) medium, the radiometric BACTEC 460 system. Also the susceptibilities of four antituberculous drugs (isoniazid, rifampin, streptomycin and ethambutol) were examined.

Methods

During the period of January 2005- April 2008 in Kocatepe University Hospital Microbiology laboratory, one thousand six hundred and twenty three samples (sputum 709, gastric aspirate 174, pleural fluid 91, bronchoalveolar lavage 161, urine 253 and other samples 235) were processed for detection of *Mycobacterium spp.* by Ziehl Nielsen (ZN) smear examination, L-J medium culture and BACTEC 460 radiometric culture. Susceptibilities of four primary antituberculous drugs were evaluated by BACTEC 460.

Results

Forty eight of 1623 clinic specimens (3.3%) were found positive with all three methods. Direct smear examination for acid-fast bacilli had a diagnostic value of 64.5%. Cultural identification in L-J and BACTEC 460 TB radiometric system media had sensitivity for the diagnosis 81.2% and 93.8%, respectively. Thirty nine (81.2%) of 48 isolates were identified as *M. tuberculosis* complex and 9(18.8%) were identified as *Mycobacteria* other than tuberculosis bacilli. The rates of contamination were 1.3% and 2.3% in the BACTEC 460 system and L-J media, respectively. *M. tuberculosis* complex strains were not resistant to any major drugs which is tested by BACTEC 460 system.

Conclusions

For the detection of Mycobacteria spp., BACTEC TB 460 was found the most useful methodhology according to these results.

However, it might be more useful to performance all methods together for precise detection of Mycobacteria.

PP–35
FIRST EXPERIENCE WITH SPOLIGOTYPING IN HONDURAS

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Introduction

In recent years new tools have been developed to improve tuberculosis (TB) case detection and control. Molecular typing techniques have been used to characterize the *Mycobacterium tuberculosis* complex (MTC), supporting conventional epidemiological methods and allowing the study of transmission dynamics. Spoligotyping, a robust polymerase chain reaction (PCR)-based method, is one of the most commonly used methods to carry out molecular characterization of MTC.

Purpose of the study

To provide an insight of the genetic biodiversity of in Honduras using spoligotyping for the analysis of clinical isolates collected between 1994 and 2002.

Methods

A total of 157 *M. tuberculosis* strains isolated from Honduran pulmonary TB patients were characterized using spoligotyping. The patterns obtained were compared with the International Spoligotyping database (SpolDB4) from the Institute Pasteur in Guadeloupe

Results

Spoligotyping grouped 79% of the strains into 22 clusters. Twenty-six shared international types (SITs) from SpolB4 were identified among the Honduran isolates. However, 23 not previously described patterns were found, which represents a potential contribution from our study to the international database.

The most common genotype was the Latin American Mediterranean (LAM) since 48% of the strains were included in this lineage. This finding is consistent with previous reports from South American countries. Other genotypes found were: X clade, ill-defined T clade and Haarlem.

Conclusions

Our study provides an overview of the *M. tuberculosis* population in Honduras, giving valuable information that can be used for further monitoring of the TB transmission in the country and the Central American region.

Key words

Mycobacterium tuberculosis, spoligotyping, Honduras.

PP–36
GENOTYPE CM AND AS ASSAY FOR DETECTION OF CLINICAL MYCOBACTERIAL SPECIES FROM CULTURES

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Introduction

The use of culture in liquid media in clinical mycobacteriology laboratories improves the ability to detect growth of Mycobacterium spp. Identification of a mycobacterium growing on solid medium can be done by biochemical methods, such as thin-layer chreomatography, gas-liquid cromatography and HPLC. Reverse hybridation assays can be performed directly from liquid culture for rapid identification of Mycobacterium species. Genotype CM/AS assay allows for the identification of most important mycobacterial species isolated in clinical laboratories.

Purpose of the study

The goal of this study was to evaluate the Genotype® CM and AS assay for the identification and differentiation of specific mycobacterial species directly from positive liquid and solid cultures comparing the results with those obtained by conventional studies.

Methods

141cultures (124 liquid and 17 solid medium) were subjected to the Genotype® CM and AS assay and to Biochemical, GenProbe® and/or HPLC testing. Genotype CM (Common Species) is able to

identify 20 mycobacterial species while Genotype AS (Additional Species) can detect another 17 species. Both are based on a multiplex PCR with botinilated primers and a reverse hybridization in a DNA-Strip.

Results

136 of the 141 mycobacteria cultures were correctly identify by Genotype® CM or AS, corresponding to: 60 M. tuberculosis complex (MTB), 11 M. avium, 8 M. intracellulare, 9 M. gordonae, 4 M. kansasii, 14 M. fortuitum, 8 M. chelonae, 6 M. peregrinum, 4 M. marinum, 2 M. xenopi, 4 M. abscesus, and 1 M. lentiflavus. 5 cultures were positives for two different mycobacterias: 2 MTB+M. fortuitum, 1 MTB+M. avium, 1 MTB+M. kansasii and 1 MTB+M.intracellulare. 5 cultures could not be identified because they were not in the species included: 2 M. interjectum, 2 M. diernohferi and 1 M. gadium.

Conclusions

Genotype CM and AS are valid techniques for correctly identification of the more commonly mycobacterial species isolated from human patients. It is an easy to interpret and rapid test to perform in clinical setting.

PP–37

BACTERIOLOGICAL EVALUATION OF THE ROTOR GENE REAL TIME PCR FOR RAPID DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS COMPLEX IN PULMONARY AND EXTRAPULMONARY SPECIMENS ; A RETROSPECTIVE STUDY

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Summary

Despite effective treatment strategies, tuberculosis is a major health problem in the world. Rapid detection of *Mycobacterium tuberculosis complex* plays a key role in control of infection. The purpose of the present study was to evaluate the routine use of Real Time PCR for recovery of M. tuberculosis from pulmonary and extrapulmonary specimens in our laboratory retrospectively. A total of 1914 specimens (1104 pulmonary and 810 extrapulmonary) were tested using artus® *M. tuberculosis* RG PCR Kit in our laboratory from February 2005 to April 2008. This method consists of a system for detection of DNA of all members of the M. tuberculosis complex, that amplified a specific 159 bp region of mycobacterial genome. The amplified products were detected by fluorescent dyes. The same specimens were inoculated in the solid medium Lowenstein Jensen (LJ) and stained EZN method for microscopy. Using culture results as gold standard, the sensitivity, specifity, positive (PPV) and negative (NPV) predictive values of Rotor Gene Real Time PCR assay and EZN staining were found 55%, 97%, 45%, 98 % and 50%, 97%, 55%, 97 % for pulmonary samples, 55%, 99%, 76%, 98% and 39%, 99%, 93%,

97 % for extrapulmonary samples respectively. In conclusion, there was no a major differences between the Rotor Gene Real Time PCR and EZN staining results when the culture was taken as reference standard; if the Rotor Gene Real Time PCR results were evaluated together with the clinical status of patients, the performance of the test would be increased. Although it is a fast method for identifying M. tuberculosis complex isolates from clinical specimens, the Rotor Gene Real Time PCR method is found to be less sensitive than culture techniques and much more expensive than microscopy. We propose therefore that it should only be used in combination with culture results in the clinical diagnosis of tuberculosis .

PP–38
TUBERCULOSIS DIAGNOSIS: MOLECULAR METHODS

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Introduction

The World Health Organisation (WHO) estimates that each year 8.8 million new cases of tuberculosis occur, transforming it into the main infectious cause of death worldwide. The increasing incidence of tuberculosis resistant to first line of antibacilares, its association with HIV and that it can be easily spread, makes it urgent the improvement of laboratory diagnosis methods. Thus the application of molecular biology in the hospital routine should be a priority meas-

ure, which is necessary to optimise these methods, so they can be applied directly in the sample.

Purpose of the study

This study aims to assess the viability of applying the PCR in the hospital routine for tuberculosis diagnosis, directly in the sample, compared with conventional techniques.

We studied 30, random samples of bronchial and gastric secretions, out of 337 received at the Laboratory of Microbiology, Hospital Infante D. EPE Pedro de Aveiro, between January and August 2007.

Methods

To each sample, a direct exam was performed (Ziehl-Neelsen) as well as cultural examinations after homogenization (Lowenstein-Jensen and Middlebrook – BacT / ALERT ® 3D). The identification of positive cultures was developed at the Laboratory of Microbiology of the University Hospitals of Coimbra. The PCR was carried out directly in the sample after decontamination.

Results

The PCR has proven to be efficient in the amplification and detection of mycobacteria of *Mycobacterium tuberculosis complex* in this type of samples. Also, although more expensive than traditional methods, PCR is more sensitive, specific and mainly a lot faster, allowing a rapid diagnosis that would lead to a decrease in the risk of spread of Tuberculosis and costs with isolation of patients.

Conclusions

The implementation of this technique in hospital, overcoming the difficulty of diagnosis dependent on the slow growth of settlements and focusing new studies under the multi-resistance to antibacilares has a high interest.

PP–39
META-ANALYSIS OF RAPID DIAGNOSIS PROCEDURES FOR TUBERCULOSIS

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Purpose of the study

A meta-analysis was performed to evaluate the accuracy of nucleic acids amplification test for the tuberculosis diagnosis.

Methods

We searched published studies in the Medline electronic database. The search terms were: "tuberculosis", "Mycobacterium tuberculosis", "nucleic acid amplification test", "polymerase chain reaction", "ligase chain reaction", "direct amplification test", "sensitivity and specificity" (1996 – 2006). Two independent reviewers valued the works and their results: study design (cross sectional, cohorts), patient sampling size and method (prospective, retrospective) … Disagreements were resolved by consensus. The global indexes were calculated as pondered averages of sensibility and specificity. Analysis were done using Meta-DiSc ver 1.1.1

Results

Most of the studies are cross sectional design and prospective patient sampling. The summary estimates in 53 studies were: sensitivity 0,82 (0,80-0,83 CI 95%) and specificity 0,98 (0,97-0,99 CI 95%). For respiratory processes (0,89; 0,87-0,90 IC 95%) a bigger one was obtained sensibility in front of extrapulmonary processes (0,72; 0,70-0,74 IC 95%). The nucleic acids amplification test for the tuberculosis diagnosis were most sensibility for smear positive specimens that for smears negative specimens

Conclusion

These techniques provide a similar specificity to the conventional techniques, they supplement them and they shorten the time of diagnostic. The decision of its use could be based among other approaches, in the cost or simplicity of the technique

PP–40

EVALUATION OF POLYMERASE CHAIN REACTION-ENZYME LINKED IMMUNOASSAY (PCR-ELISA) FOR DIRECT DETECTION OF MYCOBACTERIUM TUBERCULOSIS IN CLINICAL SPECIMENS

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Introduction

In controlling of tuberculosis (TB), early diagnosis of disease caused by Mycobacterium tuberculosis complex (MTBC) is vital. Hence, nucleic acid amplification methods for rapid diagnosis of TB methods came to be used widely in recent years. PCR-ELISA is among nucleic acid amplification based methods that are used for the direct detection of MTBC in clinical samples.

Purpose of the study

The aim of the present study was to evaluate the PCR-ELISA (ProDect *Mycobacterium tuberculosis*- ProDect GEN E.I.A., bcs Bio-

tect S.p.A., Cagliari, Italia) for detection of MTBC in pulmonary and extrapulmonary clinical samples.

Methods

In this study, 100 clinical samples (76 pulmonary and 24 extrapulmonary) from patients suspected of TB were assessed by microscopy, culture and PCR-ELISA methods.

Results

When PCR-ELISA test results were compared to culture results taken as the gold standard, the sensitivity, specificity, positive and negative predictive values (PPV and NPV) for whole specimens were found to be 68.2%, 94.1%, 95.7% and 60.4%, respectively. The sensitivity, specificity, PPV and NPV were 77.1%, 93.5%, 94.1%, 69.0% for pulmonary specimens and 61.9%, 100%, 100%, 27.3% for extrapulmonary specimens, respectively. For smear positive pulmonary specimens, the sensitivity and specivity were found to be 83.3% and 100%, respectively.

Conclusions

In conclusion, PCR-ELISA method can be utilized cost-effectively, providing results with the use of less equipment, as a routine nucleic amplification test for the rapid diagnosis of tuberculosis in a mycobacteriology laboratory, specifically for smear positive pulmonary samples.

PP–41
EVALUATION OF THE GENOTYPE MYCOBACTERIA DIRECT ASSAY FOR DETECTION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX IN CLINICAL SAMPLES

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Summary

In controlling of Tuberculosis (TB), early diagnosis of disease caused by Mycobacterium tuberculosis complex (MTBC) is vital. Hence, nucleic acid amplification methods for rapid diagnosis of TB methods came to be used widely in recent years. GenoType Mycobacteria Direct (GTMD), a commercial assay based on nucleic acid sequence-based amplification technology is used for the detection of MTBC, M. avium, M.intracellulare, M. kansasii, and M. malmoense directly from clinical specimens.

We evaluated GTMD for the detection of Mycobacterium tuberculosis complex directly from pulmonary and extrapulmonary clinical samples. A total of 354 respiratory and extrapulmonary samples (242 pulmonary and 112 extrapulmonary) from 309 patients suspected of TB were processed.

When GTMD test results were compared to culture results taken as the gold standard, the sensitivity, specificity, positive predictive and negative predictive values (PPV and NPV) for whole specimens were found to be 44, 98, 73, and 93%,, respectively. The sensitivity, specificity, PPV and NPV were 71%, 98%, 75%, 98% for pulmonary specimens and 21%, 98%, 67%,86% for extrapulmonary specimens,

respectively. For smear positive specimens, the sensitivity and specivity were found to be 77% and 88%, respectively.

The GMTD technique can be useful, reliable, and rapid when used during the normal routine of a clinical laboratory, specifically for smear positive pulmonary samples.

PP–42

COMPARISON OF SPOLIGOTYPING, RFLP AND MIRU-VNTR GENOTYPING FOR DISCRIMINATION OF MYCOBACTERIUM BOVIS ISOLATES

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Summary

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DNA fingerprinting of *Mycobacterium bovis* isolates by RFLP analysis has been of considerable benefit to epidemiological investigations in the Republic of Ireland, particularly for studies of possible transmission of infection between domestic animals and wildlife. However, while RFLP analysis with IS6110, PGRS and DR probes has given good differentiation of strains the procedures are laborious and time-consuming. Spoligotyping has not proved to be an adequate substitute for RFLP typing because of inadequate discriminatory power; approximately 50% of *M. bovis* isolates in the Republic of Ireland are spoligotype SB0140. MIRU-VNTR typing is now being used instead of RFLP because it is an easier procedure to perform. The objective of this study was to compare the discriminatory power of all three typing methods.

A total of 250 *M. bovis* isolates were typed by RFLP analysis with IS6110, PGRS and DR probes, by spoligotyping and by MIRU-VNTR analysis of six loci (2163a, 2163b, 2165, 2996, 4052 and 1895). The Hunter-Gaston discriminatory index (HGDI) was used to calculate the discriminatory power of each typing method.

The HGDI of the three methods combined was 0.926. RFLP produced the greatest discrimination of strains (HGDI = 0.906), with slightly less resolution attributed to MIRU-VNTR (HGDI = 0.885), while spoligotyping produced a relatively low level of discrimination (HGDI = 0.680). The study has shown that *M. bovis* RFLP genotyping can be replaced by MIRU-VNTR or a combination of MIRU-VNTR and spoligotyping without any appreciable adverse effect on strain discrimination.

PP–43: DIFFERENTIATION OF M. AVIUM SUBSP. PARATUBERCULOSIS ISOLATES FROM GERMANY USING MLSSR IN COMPARISON TO RFLP AND MIRU-VNTR-TYPING

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis (Johne's disease), a chronic gastroenteritis mainly affecting cattle, goat, sheep, and other ruminants around the world. MAP strains exhibit a very limited genetic diversity. Dif-

ferent molecular typing techniques were examined separately or in combination to differentiate isolates and investigate potential routes of trans-mission. In Europe the techniques used are analysis of Restriction Fragment Length Polymorphism based on IS900 (IS900-RFLP), macroresticti on analysis using Pulsed Field Gel Electrophoresis (PFGE), and Mycobacterial Interspaced Repetitive Unit -Variable Number of Tandem Repeats (MIRU-VNTR) – typing. In the United States MAP was differentiated by Multilocus Short Sequence Repeat (MLSSR) analysis.

The purpose of the study

The objective of this study was to compare the discriminatory power of MLSSR, RFLP, and MIRU-VNTR typing and provide information on the MLSSR genotypes of MAP isolates from Germany to compare these with genotypes from the United States.

Methods

Analysis of MLSSR at four loci, MIRU-VNTR-typing based on 10 markers, and analysis of IS900-RFLP using BstEII and PstI digestion was applied to 17 MAP (type II) isolates originating from red deer (from one region) and cattle (from ten different herds) in Germany.

Results

Five MLSSR genotypes, more than six MIRU-VNTR-, and six RFLP patterns were identified. A combination of all methods resulted in 13 genotypes for the 17 isolates. For isolates from cattle and red deer no host specificity of genotypes was detectable, independent from the typing techniques used. The number of examined isolates has to be enhanced to assess the usefulness of different typing methods.

Conclusion

Results of the different typing techniques were not associated. Despite the restricted set of German isolates, for MLSSR the most

frequently detected alleles at SSR loci 1 (7Gs), 2 (11Gs), 8 (5GGT), and 9 (5TGC) were analogue to results published for isolates from the United States.

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PP–44
DETECTION OF MYCOBACTERIUM TUBERCULOSISFROM PAUCIBACILLARY SPECIMENS BY REAL TIME PCR

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Purpose of the Study

Tuberculosis remains a public health problem in Portugal. Rapid detection of *Mycobacterium tuberculosis* plays a key role in control of infection. Rapid diagnosis of extrapulmonary tuberculosis has a greater impact on patients‘ management than on limiting spread of the disease.Our objective was to evaluate the routine use of Real Time PCR for recovery of *M. tuberculosis* from extrapulmonary specimens and to compare the results with culture.

Methods

A total of 193 (82 LCR and 111 other extrapulmonary specimens) were tested using artus *M. tuberculosis* RG PCR Kit. This method consists of a system for detection of DNA of all members of the *M. tuberculosis* complex, that amplified a specific 159 bp region of mycobacterial genome. The amplified products were detected by fluorescent dyes. The same specimens were inoculated in the solid medium Lowenstein Jensen (LJ) and in the liquid media MGIT (Mycobacteria Growth Indicator Tube).

Results

Of the 193 specimens 15 (7,8%) were positive on Real Time PCR, being 12 LCR and 3 other extrapulmonary specimens. All LCR samples were negative on culture. The PCR positive extrapulmonary samples (synovial fluid, knee biopsy and purulent fluid) were also positive on cultures in both systems LJ and MGIT.All positive PCR results were confirmed by clinical diagnosis.

Conclusion

Real Time PCR is a sensitive and specific method, which permits a rapid diagnosis of tuberculosis in particular cases of paucibacillary specimens. The application of the technique to extrapulmonary samples, although not recommended by manufactures is an important field. In this work we demonstrate the useful application of a real time PCR kit to extrapulmonary samples, particularly to LCR with a very good sensitivity.

PP–45
P-NBA TEST IN MGIT MEDIUM FOR IDENTIFICATION OF M.TUBERCULOSIS COMPLEX

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Conventional biochemical methods for identification of Mycobacteria are labour-consume and slow. Sensitivity test to para-nitrobenzoic acide (p-NBA) in Mycobacteria Growth Indicator Tube (MGIT) is rapid and easy to made. The basis of the test is the ability of p-NBA to suppress M.tuberculosis complex growth. The aim of this study was to evaluate this assumption.

Methods and results: At mycobacteriological laboratory , University Hospital – Pleven, for 12 months period were tested 56 strains of mycobacteria. Forty seven strains were sensitive to p-NBA and their growth was suppress in MGIT medium. These strains were aditionaly tested for M. tuberculosis complex: growth rate and pigment formation, nitrate-reduction test, and niacin test. Nine strains showed resistance to p-NBA and grew in MGIT media for 7 to 9 days. Genetic identification at National reference laboratory for tuberculosis found 6 of them as M. gordonae and 3 as M. fortuitum-M. peregrinum complex.

Conclusion: These data support the opinion that p-NBA test in MGIT gives us an opportunities for rapid identification up to M. tuberculosis complex.

PP–46
ANTIBACTERIAL ACTIVITY OF MYRTUS COMMUNIS AGAINST MYCOBACTERIUM TUBERCULOSIS STRAINS

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Introduction

Mycobacterium tuberculosis is the etiological agent for tuberculosis. Today the increase of multidrug-resistant TB (MDR) and emergence of extremely drug-resistant-TB (XDR) provide the rationale to search for new antimycobacterial drugs.

Purpose of the study

In this study we investigated the antimicrobial properties of the essential oil of *Myrtus communis* against *M. tuberculosis***.** The essential oils from *Myrtus communis* leaves have been obtained by hydrodistillation method with Clevenger-type apparatus according to the Italian Official Pharmacopoeia and has been analysed by GC/MS.The strains tested are H37RV (reference strain) and seven clinical isolates of *M. tuberculosis* resistant to one or more drugs: two clinical strains are rifampicin resistant, three strains are resistant to two drugs (one streptomicin and rifampicin resistant, one streptomicin and isoniazid resistant and one isoniazid and rifampicin resistant), one is resistant to three drugs (streptomicin, isoniazid and ethambutol). The last one is an Extensively Drug-Resistant (XDR) strain.

Methods

All the strains are collected in the Dept. of Biomedical Sciences, Microbiology, University of Sassari, Italy. The antibacterial activity of the oil was assessed by the proportional method used for *Mycobacterium tuberculosis***.** Isolate suspensions of *M. tuberculosis* in 7H9 broth were adjusted to an optical density of 1 McFarland, and a 10^{-2} and 10^{-4} dilution was plated onto 7H10 agar with different concentration of essential oil (16%, 8%, 4%, 2%, 1% and 0.17%).

Results

All the strains have shown a sensitivity to each concentration tested (MIC 0,17% of essential oil). Our results are encouraging, but additional in „vitro" testing and large clinical study are needed to characterise the efficacy of this essential oil against *M. tuberculosis*.

Conclusions

The essential oil of *M. communis* contain a different polyphenolic classes, the leaves are rich source of flavonoid; all these compounds can contribute to display the antibacterial activity that we demonstrated against mycobacteria.

PP–47

EVALUATION OF MYCOBACTERIAL ACETYL-COA CARBOXYLASEB SUBUNIT (ACCD6) AS A POTENTIAL TARGET FOR NEW ANTIMYCOBACTERIAL AGENTS

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Introduction

Mycolic acids are major component of mycobacterial cell wall. Their biosynthesis is the target of antibiotics such as isoniazid and ethionamide. The biosynthesis of mycolic acids is linked to the unusual presence of two fatty acid synthases in mycobacteria: FAS I and FAS II. Inactivation of FAS II system in *Mycobacterium tuberculosis* leads to cell death. ACCD6 (Rv2247) gene is a member of FAS II operon in *M. tuberculosis*. As confirmed by *in vitro* studies AccD6, together with AccD3 reconstitute an active acetyl-CoA carboxylase and could play a crucial role in mycolic acids biosynthesis, providing malonyl-CoA – the building block for FAS I and FAS II reactions.

Purpose of the study

In this study we have checkedwhether ACCD6 gene can be deleted from the *M. tuberculosis* and *M. smegmatis* chromosome. Although we would expect both genes to be essential, no definitive genetic proof has been established.

Methods

A two step, homologic recombination method was used to remove ACCD6 gene from the chromosome of *M. tuberculosis* Ra and *M. smegmatis* mc2 strain.

Results

We demonstrate that *M. tuberculosis* ACCD6 gene can only be deleted from chromosome when a second functional copy is provided elsewhere, showing that under normal conditions this gene is essential. Surprisingly we managed to delete the ACCD6

(MSMEG_4329) from chromosome of *M. smegmatis* andΔACCD6 mutant shows significant decrease in cell viability and cell wall permeability.

Conclusions

Essentiality of *M. tuberculosis* ACCD6 gene means that FAS II acetyl-CoA carboxylase β subunit could be considered as a potential target for new antimycobacterial agents. Obtained ΔACCD6 mutant of *M. smegmatis* suggests that not all FAS II enzymes have essential nature. In some strains AccD6 activity can be taken over by other carboxylases. Mutants obtained in this study allow for better understanding precise mechanism of acetyl carboxylation in mycolic acids biosynthesis.

PP–48

CYTOLOCALIZATION OF THE CHROMOSOME PARTITION PROTEIN PARB IN MYCOBACTERIUM TUBERCULOSIS AND INTERACTIONS WITH OTHER PROTEINS

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Introduction

Despite being a widespread human pathogen, little is known about Mycobacterium tuberculosis cell cycle. The ParA/Soj (ATPase) and ParB/Spo0J (DNA-binding) proteins, and the cis-acting parS site,

participate actively in bacterial chromosome segregation and cell cycle progression. Genes homologous to parA/soj and parB/spo0J, and two putative parS sites have been identified near the chromosomal replication origin (oriC) in all the mycobacterial chromosomes sequenced to date. Moreover, M. tuberculosis has two soj-like genes, annotated as Rv1708 and Rv3213c.

Purpose of the study

To evaluate the function of the ParB protein in M. tuberculosis, this work focused on its cytolocalization and in the determination of ParB interaction with itself, ParA and other prominent candidates.

Methods

We localized this protein in exponentially growing cells by immunofluorescence microscopy of fixed and permeabilized M. tuberculosis H37Rv, labeled with anti-ParB antibodies. We also evaluated the localization of the ParBMtb-GFP fluorescent foci in unfixed M. smegmatis mc2155. To determine the interaction partners of ParBMtb we used the Bacterial Adenylate Cyclase Two-Hybrid System (Euromedex, France). This allowed us to evaluate in vivo protein-protein interactions of ParBMtb with itself, ParA, Rv1708, Rv3213c and Wag31, a DivIVA orthologue.

Results

ParBMtb was detected as discrete fluorescent foci, localized towards the cell poles in most cases and exhibited a distribution pattern expected for a protein involved in the organization and/or segregation of oriC. High levels of beta–galactosidase activity, as result of functional complementation between the hybrids, indicated that ParBMtb was able to interact with itself. Lower, but still significant, levels of interaction were detected with ParA and Wag31. Finally, no interactions between ParBMtb and the Soj-like proteins (Rv1708 and Rv3213c) were detected.

Conclusions

As expected, these results suggest that ParBMtb could be involved in chromosome segregation and that is able to interact in vivo with ParA and Wag31.

PP–49

STABILITY AND REPLICATIVE TRANSPOSITION OF THE MYCOBACTERIUM AVIUM SPECIFIC INSERTION SEQUENCE IS1245 IN MYCOBACTERIUM KANSASII

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Introduction

IS1245 is a *Mycobacterium avium* specific 1,414pb mobile element. It has been extensivelly used for identification of *M. avium* by PCR and for molecular typing by Restriction Fragment Length Polymorphism (RFLP). IS1245 amplicons were detected in pure colonies of *Mycobacterium kansasii*isolated from a bone marrow aspirate of an HIV-positive patient. A mixed culture of *M. avium* and *M. kansasii* was originally present in this specimen. The existence of one to two IS1245 copies in pure *M. kansasii* colonies from this specimen was confirmed by RFLP-IS1245.

Purpose of the study

To analyse the stability and transposition capacity of the IS1245 element in *M. kansasii* genome

Methods

Two *M. kansasii* isolated colonies containing one and two IS1245 copies, respectively, were cultivated in Middlebrook 7H9-ADC liquid medium. When OD600nm reached 0.6-0.8, bacteria were dilluted 1:10 and reincubated at 37°C. After 10 serial passages, the two cultures were dilluted and plated on Middlebrook 7H10-OADC solid medium, and isolated colonies from each plate were analysed by RFLP-IS1245. Five additional mixed cultures of *M. avium* and *M. kansasii* were investigated using PCR and RFLP-IS1245 to detect the IS1245 element in *M. kansasii*.

Results

The original RFLP-IS1245 bands were retained in isolated colonies analysed after 10 serial passages in culture. Replicative transposition of the IS1245 was observed with 11 colonies, generating 1-3 new copies of this insertion sequence. The IS1245 element was not detected in *M. kansasii* isolated colonies from the other five mixed cultures.

Conclusions

The detection of the IS1245 element in *M. kansasii*, never described, confirmed that this element can be dispersed by horizontal transfer to other non-tuberculous mycobacteria. The obtained results confirmed that the IS1245 sequence can replicate and transpose in *M. kansasii* genome. Horizontal gene transfer can play a role in virulence, evolution and genetic diversity of environmental mycobacteria.

PP–50
FACTORS INFLUENCING FRAGMENT ANALYSIS OF HIGHLY DISCRIMINATORY VARIABLE NUMBER TANDEM REPEAT (VNTR) LOCI IN MYCOBACTERIUM TUBERCULOSIS

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Introduction

Currently, in addition to the 12 MIRU (Supply et al., 2000) and 3 ETR (Frothingham and Meeker O'Connell, 1998) loci, HPA the MRU use 7 additional VNTR loci (2163B, 2347, 3232, 2163A, 1982, 3336 and 4052) to improve discrimination; some of these loci have been previously reported as too unstable for obtaining reproducible results in different laboratories.

Purpose of the study

This study explores the reproducibility and stability of two particularly useful loci, VNTR1982 and VNTR3232.

Methods

A panel of 16 *M. tuberculosis* complex strains was selected to include a wide range of copy numbers at MIRU26, ETR-B, VNTR1982 and VNTR3232. These 4 loci were amplified in each isolate using standard and hot-start enzymes from a range of suppliers. PCR products were analysed on agarose gel and by polyacrylamide gel capillary electrophoresis, where the conditions for denaturation and separa-

tion were varied. The resulting apparent molecular weights in each experiment were compared with expected values. The most efficient amplification and automated analysis conditions were used to type 265 sets of serial isolates prospectively to check for reproducibility.

Results

The control loci MIRU26 and ETR-B were amplified by all polymerases. VNTR1982 and VNTR3232 were amplified more efficiently by Qiagen (HotStartTaq and HotStartTaq PLUS) and by Bioline Diamond polymerases, respectively. The duration of denaturation had no influence on apparent molecular weights. However, variation of capillary temperature did affect apparent molecular weight leading to the miscalling of copy number. Diamond polymerase was used to amplify VNTR loci in serial isolates and automated analysis performed using the most efficient conditions; there were no disagreements between isolates from the same patient.

Conclusions

This study demonstrates VNTR loci with a higher discrimination can provide consistent data when amplification and automated analysis methods are optimized.

PP–51

CLONING AND EXPRESSION OF EARLY SECRETORY ANTIGEN TARGET 6 KD PROTEIN (ESAT-6) OF MYCOBACTERIUM TUBERCULOSIS

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Objective and Background

Tuberculosis remains a major infectious disease with over 8 million new cases and 2 million deaths annually .The ESAT-6 antigen from *Mycobacterium tuberculosis* is a dominant target for cell-mediated immunity in the early phase of tuberculosis (TB) in TB patients as well as in various animal models ESAT-6 was found to distinguish TB patients from BCG-vaccinated donors and an interesting candidate for the diagnosis of TB.

Methods

A DNA encoding the 6-kDa early secretory antigenic target (ESAT-6) of *Mycobacterium tuberculosis* was amplified with PCR then The PCR products were inserted into the pET102/D vector and transferred into *E. coli* strain TOPO10 . The recombinant plasmids purified from positive clones were prepared for nucleotide sequencing then recombinant plasmid transferred in *E.coli* strain BL21. *E.coli* strain BL21 transformed with pET102/D:esat-6 was plated on LB solid medium containing ampicillin (100 _g/ml), and grown overnight at 37 °C. An overnight culture was used to inoculate 200ml LB with ampicillin medium and grown at 37 °C. This plasmid was transformed into *Escherichia coli* strain BL21 effectively expressed. The expressed fusion protein was found almost entirely in the soluble form and the recombinant protein was purified by Ni–NTA column.

Results

In this study, we amplified esat-6 from *M. tuberculosis* H37Rv genome DNA by PCR. The PCR products were confirmed to contain 288 bp as expected by agarose gel electrophoresis and then cloned into the prokaryotic expression vector pET102/D followed by sequencing. The recombinant plasmid was transformed to expression

host E. coli strain BL21. After IPTG inducing, ESAT-6 was expressed at about 23kDa on SDS–PAGE. Recombinant protein almost entirely accumulated in the soluble phase. It was purified with Ni-NTA column.

Conclusion

The availability of sufficient amounts of ESAT-6 from M. tuberculosis is an essential step to studying the biochemical features of this antigen. In conclusion, our study clearly suggests that a specific antigen ESAT-6 can form the basis of an improved next-generation diagnostic reagent which can play a role in the diagnosis of active TB in humans and cattle as well as in epidemiological studies and disease control programs. Such a specific reagent would have the potential to play a much more important role in the prevention and control of TB than PPD has done in many years.

Keywords

ESAT6, Cloning, Mycobactrium tuberclusis.

PP–52

CLONING, EXPRESSION AND PURIFICATION OF 10 KD CULTURE FILTRADED PROTEIN (CFP10) OF MYCOBACTERIUM TUBERCULOSIS

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Background and Objective

Human TB is the most frequent cause of death from a single infectious agent, being responsible for eight million new cases and approximately two million deaths annually. Tests based on tuberculin purified protein derivative (PPD) cannot distinguish between tuberculosis infection and Mycobacterium bovis BCG vaccination, or exposure to environmental mycobacteria. It has been apparent, therefore, that a new diagnostic reagent with specificity for M. tuberculosis and M. bovis is needed to overcome the limitations of PPD. The recent identification of RD1 region of the M. tuberculosis new opportunity for the development of novel diagnostic tools. This region encodes the T-cell antigen CFP10, which is a potential diagnostic reagent which is highly specific for active tuberculosis diagnosis. Our main purpose of this study is cloning,expression and purification of CFP10 for use in serodiagnostic tests in futute**.**

Material and Method

DNA was extracted from *M.tuberculosis* H37Rv after growgh for 3 weeks in LJ medium. PCR performed by using specific primers to amplify CFP10 from M. tuberculosis H37Rv chromosomal DNA. PCR products were subcloned into the pET102/D vector and recombinant plasmid were initially transformed into *E.coli* TOP10 and were subsequently transformed into *E. coli* BL-21 host cells for expression.E. coli BL-21(DE3) was used for high-level expression. Recombinant (His-tagged) antigens were purified from the soluble supernatant by affinity chromatography with the Ni-nitrilotriacetic acid (NTA) agarose matrix. Fractions containing the recombinant antigen were pooled, dialyzed**.**

Results

In this study we amplified CFP10 from *M. tuberculosis* H37Rv genome DNA by PCR. The PCR products were confirmed to contain 303 bp as expected by agarose gel electrophoresis and then cloned into

the prokaryotic expression vector pET102/D followed by sequencing. The recombinant plasmid was transformed in expression host *E. coli* strain BL21. After IPTG inducing, CFP10 was expressed at about 27 kDa on SDS–PAGE. In this way most of recombinant protein accumulated in the soluble phase and was purified by affinity chromatography with the Ni-nitrilotriacetic acid (NTA) agarose matrix**.**

Conclusion

The availability of sufficient amounts of CFP10 from *M. tuberculosis* is an essential step to studying the biochemical features of this antigen In conclusion, our study clearly suggests that a specific antigen CFP10 can play a role in the diagnosis of active TB in humans and cattle as well as in epidemiological studies and disease control programs**.**

PP–53

MOLECULAR APPROACHES FOR VACCINE CHARACTERIZATION: STUDIES ON M.BOVIS BCG, SUBSTRAIN SOFIA SL222, BASED ON VNTR-TYPING AND WHOLE-GENOME DNA MICROARRAY

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Introduction

As it was stated by WHO, BCG vaccine will continue to play a considerable role in the battle against TB, either as a primer to be

boosted by new components or as an integral component of new vaccines. In the last years the importance of BCG characterization has been strongly emphasized, especially in the light of new knowledge.

Purpose of the study:

Here we report our results from the molecular typing performed on BCG Sofia SL222 as well as our attempts to find its specific molecular characteristics. In order to gain an insight to genetic properties of BCG Sofia SL 222 and to find its place into the BCG genealogical tree, we have performed a set of genotyping methods, including VNTR-typing and whole-genome DNA microarray.

Methods

VNTR-Typing DNA Extraction (method of van Soolingen); Sources of DNA: *M.bovis* BCG Sofia SL222; *M.bovis* BCG WSL7-93; Commercial Lots BCG and *M.tuberculosis* H37Rv; Amplification of DNA: primers for ETR-A through ETR-F; Automated Fragment Analysis: ABI Prism 377Ò DNA Sequencer DNA microarray analysis. The genomic DNA of *M.tuberculosis* H37Rv and *M.bovis* BCG Sofia SL222 were used as a template for direct incorporation of fluorescent nucleotide analogues (Cy3 and Cy5 dCTP). After hybridization the slides were scanned by dual-laser Affymetrix 428. Fluorescent spot intensities were quantified using ImaGene4.0 software; the data were further analyzed and visualized by GeneSpring 5.0 (Silicon Genetics).

Results

The VNTR profile of BCG Sofia was determined to be 5-5-5-2-3-3.1**.** The more distinguishing and discriminative locus between BCG strains is locus D (1-3 copies in different strains). Tracing the line SL222 – WSL7-93- Final Product reveals a remarkable homogeneity of the copies of locus D. This is a strong evidence for the genetic stability for a period longer than 30 years.

The whole-genome DNA analysis of BCG Sofia disclosed a range of deletions and a duplication that places BCG Sofia in the same lineage as BCG Russia in accordance with the BCG genealogy. Main characteristics of *M.bovis* BCG Sofia SL 222 could be summarized as follows:

– Presence of RD2 Region (typically for early BCG strains)
– DU2 Duplication (from Rv3299 to Rv3316)
– Novel 1.6-kb deletion that affects the Rv3697c and Rv3698 (related to membrane protein in the cell wall structure). This region was also deleted in BCG Russia but not in any other strain. Hence, this deletion occurred prior to the in vitro cultivation of BCG Sofia.

Conclusions

M. bovis BCG Sofia SL222 reveals the genetic characteristics which pertain only to the most closest to the original of Calmette and Guérin strains.

Using thesepowerful enabling technologies of postgenomic era, we have confirmed the genetic stability, identity and provenance ofBulgarian BCG vaccine that is given to over 40 million individuals a year.

PP–54
TRANSFORMATION OF MYCOBACTERIUM SMEGMATIS WITH EMBB306 CODON ALLELES CONFERS ETHAMBUTOL LOW-LEVEL RESISTANCE

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Purpose of the Study

Mutations occurring in codon 306 of EMBB gene in *Mycobacterium tuberculosis* have traditionally been associated with ethambutol (EMB) resistance. However, in the last years several EMB-susceptible

isolates with EMBB306 mutations have been encountered. This fact has generated a controversy wether or not EMBB306 cause EMB-resistance.

The present study addresses that question by analyzing the EMB minimum inhibitory concentration in *Mycobacterium smegmatis (M. smegmatis)* strains transformed with *M. tuberculosis* EMBB306 alleles.

Methods

We have cloned the entire EMBB ORF from *M. tuberculosis* in the multicopy vector pMind and, generated by *in vitro* mutagenesis four different EMBB306 alleles. *M. smegmatis* mc^2155 was transformed with each recombinant vector and their EMB MIC determined.

Results

We have verified that the transfer of EMBB306 alleles to *M. smegmatis* only resulted in a slight increase of EMB MIC when compared to the transfer of the wild-type EMBB gene. If EMBB306 mutations were to confer EMB high-level resistance in *M. tuberculosis*, a higher increase in the measured EMB MIC was expected.

Conclusions

Our results indicate that EMBB306 mutations only confer EMB low level resistance, stressing the fact that EMB resistance acquisition may be a multistep process. The acquisition of EMBB306 mutations may be a first step in that way.

PP–55
CHARACTERIZATION OF GIDB MUTATIONS IN STREPTOMYCIN-RESISTANT ISOLATES IN LISBON, PORTUGAL

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Purpose of the Study

Streptomycin (STP) was the first antibacillary drug introduced in the treatment of tuberculosis in 1944. With the development of further antibacillary drugs, streptomycin has become less used. Development of STP-resistance is usually explained by the acquisition of mutations in RPSL gene or in the RRS gene. Our laboratory regularly isolates STP-resistant strains without any mutation in the referred genes. Recently mutations occurring in a rRNA methyltransferase (encoded by GIDB gene) were shown to be involved in the acquisition and resistance to STP. In this study, we examined the GIDB gene of STP-resistant isolates in search of mutations that may explain acquisition and STP low-level resistance on these strains.

Methods

We have analyzed by sequencing analysis the GIDB gene of 19 STP-resistant clinical isolates, recovered in 2005 from different hospital units. Sequences were compared with the wild-type GIDB from *Mycobacterium tuberculosis (M. tuberculosis)* H37Rv. The entire

RPSL ORF of all isolates was amplified and screened for mutations by endonuclease and sequencing analysis.

Results

We have found two missense GIDB mutations present in all isolates. Both mutations are naturally occurring polymorphisms given the fact that both were detected in the wild-type GIDB gene of *M. tuberculosis* CDC1511 and F11. Other missense mutations were found in 6 out of 19 isolates. We have found that mutation A80P, present in 5 isolates, was only present in isolates without RPSL mutations.

Conclusions

We conclude that GIDB mutations may in fact explain the high number of STP-resistant strains with no mutation in RPSL or RRS, isolated in our laboratory. These are probably STP low-level resistant strains and this type of resistance is probably granted by GIDB mutations. A more extensive analysis is ongoing.

PP–56

A MAP-IMMUNOASSAY RECONFIRMS THE ASSOCIATION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS WITH TYPE-1 BUT NOT TYPE-2 DIABETES MELLITUS

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Introduction

Mycobacterium avium subspecies paratuberculosis (Map) is a zoonotic pathogen whose association with Crohn‘s disease in humans is under scrutiny.

Purpose of the study

To investigate its association with other chronic diseases (Diabetes)where the involvement of a persistent pathogen as Map could be the trigger

Methods

A total of 193 participants comprising of 57 with type 1 diabetic patients (T1DM), 57 with type 2 diabetic patients (T2DM) patients and 79 healthy controls were tested for the presence of MAP specific antibodies against HbHA, Gsd and Map lysate.

Results

Extremely significant humoral immune responses to recombinant HbHA and GSD proteins and the whole cell lysates of the Map bacilli were recorded in T1DM patients as compared to T2DM patients and healthy controls

Conclusions

Finding evidence of Map involvement in T1DM is perhaps a novel finding that might serve as a foundation stone in establishing an infectious aetiology for T1DM.

PP–57
THE UTILITY OF SINGLE NUCLEOTIDE POYLMORPHISMS (SNPS) TO STUDY M. TUBERCULOSIS

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Introduction

Purpose of the study:to investigate variation in M. tuberculosis strains from a region of Malawi using SNP analysis and spoligotyping

Methods

124 M. tuberculosis clinical isolates from the Karonga District of Malawi have been classified into approximately nine different clades using the spoligotyping system. SNP analysis was performed for nine SNPs that were found to be sufficient to classify M. tuberculosis strains into seven phylogenetically distinct "SNP cluster groups" (SCGs), as previously described by Alland and colleagues. Spoligotyping results were compared to the SNPs findings. Analysis was undertaken using MEGA 4 software.

Results

Cluster analysis identified five phylogenetically distinct SNP cluster groups (SCGs) and 3 subgroups. Two strains with unknown spoligotypes were not assigned to a SCG. The spoligotype-defined Beijing clade was exclusively present in SCG-3 subgroup c. The East African-Indian (EAI) clade were presented in SCG-3 subgroup b and SCG-6

subgroup b. Spoligotyping family T was rare in this study but was strongly associated with the distribution of SCG-6 subgroup a. The Latin American and Mediterranean (LAM) clade showed a remarkable predominance with SCG-5. Group SCG-2 and the SCG-3 subgroup b were the least well defined, containing a number of orphan strains, and those belonging to LAM, CAS and EAI spoligo clades.

Conclusions

Beijing clades were strongly associated with SCG-3 subgroup c. Two apparently unique strains were detected. However, further study will be required to firmly delineate the origin and global spread of M. tuberculosis and to definitively identify all M. tuberculosis SCGs.

PP–58
NOVEL CLASSES OF ANTIMYCOBACTERIALLY ACTIVE COMPOUNDS

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Introduction

The effort of our laboratories has been focused on development of compounds with the antimycobacterial activity for some years. Newly prepared compounds are screened in vitro against several mycobacterial strains and MIC values serve for searching structure–activity relationship.

Purpose of the study: The aim of our study is to evaluate MIC values in the group of benzylsulfanylpyridine-2-carboxamides and N-benzylsalicylamides and their sulphur analogues.

Methods

In vitro antimycobacterial activity of the compounds was evaluated against *Mycobacterium tuberculosis* CNCTC My 331/88, *Mycobacterium kansasii* CNCTC My 235/80, *Mycobacterium kansasii* 6509/96 and *Mycobacterium avium* CNCTC My 330/88 using the micromethod for the determination of the minimum inhibitory concentration (MIC). All strains were obtained from the Czech National Collection of Type Cultures (CNCTC), with the exception of M. kansasii 6509/96, which was a clinical isolate. The activities of the compounds were determined in the Šula semisynthetic medium (SEVAC, Prague). The compounds were added to the medium in dimethylsulfoxide solutions. MICs were determined after incubation at 37 °C for 14 and 21 days.

Results

Several derivatives showed to be active at low concentrations. The most active derivatives of N-benzylthiosalicylamides exhibited the MIC values 0.125-1 μmol/L. Replacement of atom oxygen by atom sulphur enhances the activity in both groups. There are no differences in susceptibility of M. tuberculosis and nontuberculous mycobacteria.

Conclusion

N-benzylsalicylthioamides represent a promising group of antituberculotic agents with activity against *M. tuberculosis* and nontuberculous strains. The new compounds have probably different mechanism of action than INH.

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PP–59
MUTATIONS IN THE *INHA* GENE OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES WITH RESISTANCE TO ETHIONAMIDE

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Objective

To investigate if mutations in the *inhA* gene of *Mycobacterium tuberculosis* clinical isolates in Japan are associated with resistance to ethionamide (ETH).

Design

A total of 1,112 *M. tuberculosis* strains isolated from different patients from various districts of Japan were studied. Ninety-six strains, which showed a resistance phenotype to isoniazid (INH) by the BACTEC MGIT 960 AST were subjected to susceptibility testing to ETH and sequencing of *inhA* gene.

Results and discussion

Of 96 INH resistant strains, 43 (44.8%) were also resistant to ETH, and 53 were susceptible. Analyses of the promoter of *mabA-inhA* revealed nucleotide substitutions in 48.8% (21/43) of ETH-resistant and in 7.5% (4/53) of ETH-susceptible strains. Forty-three ETH resistant strains were divided into three groups by the results of the susceptibility testing to INH with the proportion method using Ogawa egg medium; 1) Low-S (susceptible at 0.2μg/ml INH), 2)

Low-R (resistant at 0.2μg/ml and susceptible at 1.0μg/ml INH), and 3) High-R (resistant at 1.0μg/ml INH). They included 11, 15, and 17 strains, respectively. One hundred percent of strains of Low-S had mutations in the regulatory region of the *inhA* gene, while only 5.9% of High-R strains carried mutations in this region. Low-R strains (66.7%) showed intermediate frequencies of mutations among the Low-S and High-R strains. These findings suggest that for the low-level INH resistant strains, mutations in the regulatory region of the *inhA* gene are associated with resistance to ETH but not for the High-R strains. For the High-R strains, resistance to ETH may be conferred by another mechanism(s). On the other hand, four out of 25 strains having regulatory region mutations were susceptible to ETH, indicating that this mutation is not predicative of ETH resistance.

PP–60

APOPTOSIS INHIBITION AS A STRATEGY OF A PROSPEROUS MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS STRAIN TO EVADE THE IMMUNE RESPONSE

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Purpose of the study

The multidrug resistant *Mycobacterium tuberculosis* strain M of the Haarlem 2 sublineage has prevailed over other multidrug resist-

ant *M. tuberculosis* strains in Argentina since it started clonal expansion in the early '90s. We have previously observed that strain M induced less apoptosis than strain H37Rv in human monocyte-derived macrophages. This study was aimed to explore further the influence of strain M on macrophage extrinsic and intrinsic apoptotic pathways and on caspase3 activation, their common final effect.

Methods

Apoptosis was assessed on macrophages pulsed 5 hours with heat-killed bacilli employing Annexin V/PI assay in presence/absence of exogenously added apoptosis inducers/mediators: i) staurosporin (0.5μM) as intrinsic pathway stimulator, ii) rTNF-α (50ng/ml) as major mediator of mycobacteria-induced apoptosis via the extrinsic pathway and iii) PDTC (50 μM) as inhibitor of nuclear factor κB activation in response to TNF. Caspase3 activation was also measured.

Results

Upon stimulation with staurosporin, strain M induced less macrophage apoptosis than H37Rv (19.1±4.4% vs. 26.6±4.6%, $p<0.01$). Addition of rTNF-α did not improve the low apoptosis induced on macrophages by strain M (H37Rv=25.3±5%, M=8.9±1.3%; M+rTNF-α=8.6±2.0%). PDTC produced a similar apoptosis increase in macrophages pulsed with M as it did in macrophages pulsed with H37Rv. Strain M induced less activation of caspase3 than H37Rv (8.6±1.0% vs. 16.9±2.0%, $p<0.05$).

Conclusions

Strain M seems to inhibit apoptosis mainly by interfering with the intrinsic apoptotic pathway. Neither a deficit of TNF-α nor the activation of nuclear factor κB influences this interference. The success of strain M might be related to this ability to evade the immune response by preventing host cell apoptosis.

PP–61
THE PLACE OF "ENTEROSAN" IN THE COMPLEX THERAPY OF MYCOBACTERIAL INFECTIONS

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The infections, including the mycobacterial ones, cause severe damage to the organism and the antibiotic therapy is long and sometimes has a weak effect. The application of antibiotics is related to the manifestation of multiple side effects, among which the disbacterioses have the leading part. The cases of liver damage, allergic reactions, etc. are not rare. In the case of disbacterioses the immune defense of the organism is additionally weakened. In this way the patient encounters new problems related to the antibiotic therapy itself.

The probiotics "Enterosan" are being used efficiently for more than a decade in the complex therapy of bacterial infections in the digestive tract and the urinary system. Their intake during the treatment does not allow the advent of disbacteriosis, allergic effects and liver damage. This approach is also recommended in the treatment of mycobacterial infections.

The probiotic "Enterosan – basic" consists of active cells of lactobacilli and bifidobacteria with marked probiotic properties. The viable cells concentration in a tablet is above 10 billion, which guarantees good effect. In the case of allergic reactions is recommended the use of the probiotic "Enterosan 44", which has excellent anti-allergenic properties by stabilization of the capillaries and prevention of toxin and allergen penetration. In this way the toxic products are removed quickly through the urinary system. In the case of damaged liver functions is recommended the application of "Enterosan 55" in the complex therapy, which restores the liver cells and protects them from the action of the microbial toxins, antibiotics and other chemotherapeutics.

The probiotics "Enterosan" exhibit immunomodulating properties by promotion the immunoglobulin A production in children. In this way through the enhanced immune defense is secured a faster healing process in the case of mycobacterial infections.

PP–62

HIGHER PREVALENCE OF POSITIVE QFT-G AMONG THE CONTACTS IN THE HIGHER PREVALENCE OF TUBERCULOSIS THAN THE CONTACTS IN THE LOWER PREVALENCE OF TUBERCULOSIS

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Purpose of study

To clarify the magnitude of current tuberculosis infection in the high prevalence of tuberculosis compared to the positive rate of QFT-2G in the lower prevalence of tuberculosis

Methods

Contact investigations were done using QFT-G after two months after the index patients were diagnosed sputum smear positive pul-

monary tuberculosis in the two local health centers of the suburbs of Tokyo metropolitan, Tama-Tachikawa public health center and Kawasaki public health center in the Kanagawa prefecture.

Results

A total number of 308 contacts in the Tama-Tachikawa health center and 138 contacts in the Kawasaki health center were included to this study. Thirty-six in Tama-Tachikawa Health Center and forty-five contacts in the Kawasaki Health Center were excluded from analysis because of suspected remote infection. Positive rate of QFT-G was 8.1% and 18.8%, respectively. The difference was statistically significant ($P<0.01$).

Conclusion

Incidence of tuberculosis in the both areas is $29.2/10^5$, and $57.9/10^5$, respectively. More current tuberculosis infection is occurring in the higher prevalence of tuberculosis comparing lower prevalence of tuberculosis. Contacts investigation using QFT-G should be done and recommend isoniazid preventive therapy to the QFT-G positive contacts.

PP–63

THE COMPREHENSIVE EVALUATION OF THE LATENT TUBERCULOSIS PRESENCE IN PATIENTS WITH ACTIVE PULMONARY TUBERCULOSIS AND CONTACTED HEALTH WORKERS BY PPD, ELISA AND ELISPOT METHODS

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Introduction

At the diagnosis of latent tuberculosis infection, the clinically application of the tests based on enumerating of *M.tuberculosis*-specific interferon(IFN)– γ response as an alternative to tuberculin skin test (TST) has been a guide to decide the accurate kemo-prophylaxis.

Purpose of the study

It requires to define the relation between positive detected IFN-γ results and the risk of LTBI's progression to active disease for the evaluation of the utility of IFN- γ tests.

Methods

This study was conducted during a 10-month period from March 2007to January 2008 with 95 participants constituting 3 groups. The first group includes 37 patients with active pulmonary tuberculosis

diagnosed at Atatürk Chest Diseases & Thoracic Surgery Center. The second group includes 34 contacted health worker at tuberculosis clinic and some tb patients' relatives accompanying them. The third group includes 24 healthy people as control. The blood samples were collected from all the participants for the in vitro T-cell IFN-γ tests (Quantiferon-TB Gold (QFT-G) ve T- SPOT.TB test). Then tuberculosis skin test was applied. The correlation between these tests were evaluated statistically.

Results

When the cut -off value of the PPD test was regarded as ≥ 15 mm, the 43,2 % of the patients and the 12,5% of the controls were positivite. The sensitivity (43,2%) and the negative predictive value (NPV) (50,0%) of the test were lower than specifity (87,5%) and positive predictive values (PPV) (84,2%). The sensitivity and the PPV of the QFT-G test were (78,4% and 76,3% respectively) higher than the specifity (62,5%) and NPV (65,2%). The PPV (81,8) of the T- SPOT. TB test was higher than the sensitivity (73,0%), specifity (75,0%), and NPV (64,3%).

Conclusion

As the false negativity of the PPD test was higher than both QFT-G and T-SPOT.TB, we decided that the IFN- γ tests are very usefull at the diagnosis of latent tuberculosis infection and kemoprophylaxis. QFT-G test was more simple to apply in laboratory than T-SPOT.TB.

PP–64
MYCOBACTERIA MODULATES NITRIC OXIDE PRODUCTION BY HUMAN PRIMARY ENDOTHELIAL CELLS

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Introduction

Resurgence of tuberculosis resulted in renewed interest in the study of the biology of *M. tuberculosis* interactions with host cells. In the early stages of *M. tuberculosis* infection, macrophages play a key role in controlling the infection, however recently studies has been focused on the participation and responses of other cellular types. It is known that *M. tuberculosis* can invade and replicate in non-phagocytic cells as epithelial cells and fibroblasts. Invasion and intracellular replication into endothelial cell line and mouse lung endothelium by bacterial pathogens such as *S. pneumonia, S. aureus, H. influenzae* and some fungi have been also documented. It has also been hypothesized that non-phagocytic cells can potentially serve as reservoirs for persistent infections.

Purpose of the study

In this study, we reported the infection of human primary endothelial cells by mycobacteria and nitric oxide production induced by infection. We compare mycobacterial intracellular growth in human umbilical vein endothelial cells (HUVEC) of three mycobacte-

rium species: a) *M. tuberculosis* H37Rv; b) *M. abscessus* and c) *M. smegmatis*.

Methods

Nitric oxide production was monitored using the fluorescent probe, DAF, this reactive diffuses through cell membrane been hydrolyzed by intracellular esterases into the non fluorescent DAF, which is then rapidly oxidized to fluorescent DAF in the presence of nitric oxide. Colony forming units were determined after 1, 3, 6, 24, 48 and 72 post-infection

Results

M. smegmatis was eliminated after 24 h post-infection; *M. abscessus* had an accelerate intracellular replication after 24 h post-infection and *M. tuberculosis* did not replicate or was eliminated resembling a later "latency" stage. Nitric oxide production was induced differentially depending of each mycobacteria: *M. abscessus* induced high levels of nitric oxide at 75 min, but, this production decrease rapidly; *M. tuberculosis* induced a "nitric oxide" burst that was rapidly abolished and nitric oxide production by *M. smegmatis* was lower but sustained up to 24 hrs post-infection.

Conclusions

Our results indicated that infection of HUVEC by pathogenic bacteria induced a nitric oxide burst not observed with non-pathogenic mycobacteria.

PP–65
EPIZOOTY OF BOVINE TUBERCULOSIS (MYC.CAPRAE) IN VARIOUS SMALL BREEDS WITH JOINT PASTURES

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Introduction

In the period from 2006. until april 2008. epizooty of tuberculosis was affirmed in two villages od Sisak-Moslavina county in variuos individual cattle breeds. Most of the cattle breeders from these two villages practise joint in-season pasturing and tuberculosis was firstly identified in their breeds.

Methods

Skin tuberculin testing using method of monotest (bovine tuberculin) is prefered for diagnosis of tuberculosis in Republic of Croatia. Comparative tuberculinization with avian and bovine tuberculin is used after identification of positive of suspicious reactions with monotest method. Definitely positive bovines are slaughtered and samples are taken for bacteriological examination.

Results

Tuberculin skin test. In 2006. 49 cows from 16 breeds; in 2007. 117 cows from 55 breeds and 32 cows from 23 breeds up to april 2008. gave positive and suspicious reactions.

Pathoanatomical findings. During the before mentioned period, 179 bovines were slaugthered, and caracteristic pathoanatomical changes were identified in 164 bovines (91,6%). Visiable changes were found in mandibular lymph nodes of 12 (7,3%) animals, in lungs of 91 animals (55,5%), in mediastinal lymph nodes of 110 animals

(67%), in tracheobronchal lymph nodes of 116 animals (70,7%), in liver and portal lymph nodes of 13 (7,9%) and in mesenterial lymph nodes of 27 animals (16,4%).

Mycobacterium bovis subsp. *caprae* was isolated from gathered material using bacteriological methods. MIRU-VNTR typing confirmed the same code 226424153522 on 12 MIRU loci (2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40) for all isolates which points to a single source of infection.

Conclusions

Joint pasturing favours spreading of tuberculosis. However, since the disease was identified in closed breeds, where the contact with infected animals is not possible, other ways of infection broadening should be taken into consideration. Still, direct and fast identification with skin tuberculin test and its use thoughout the year, together with extraction of infected cattle from breeds should bring the situation under control.

PP–66

THE PREVALENCE OF MYCOBACTERIUM BOVIS INFECTION AND ATYPICAL MYCOBACTERIOSES IN CATTLE IN AND AROUND MOROGORO, TANZANIA

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Summary

Bovine tuberculosis (BTB) is highly prevalent in developing countries: About 85% of cattle and 82% of humans in Africa live in areas where BTB is not controlled. BTB infection has been demonstrated in cattle in Tanzania by using tuberculin tests with a prevalence varying from 0.2-14%. In this study the prevalence of BTB infection and atypical mycobacterioses was determined in and around Morogoro using the Single Comparative Intradermal Tuberculin Test (SCITT) for further study of its wildlife reservoir.

Between April and June 2005, a total of 728 bovines from 49 herds were tested for BTB infection and atypical mycobacterioses by using SCITT. Milk samples were taken from all SCITT-positive animals and analysed by culture and PCR for the presence of mycobacteria.

Total prevalences of 2.5% and 10.1% were found, using SCITT, for BTB infection and atypical mycobacterioses respectively. Significantly more BTB infection was found in cattle in the extensive sector (3.6% as compared to 0.7%) and more atypical mycobacterioses were observed in cattle in the intensive sector (13% as compared to 8.3%). From 8 out of 42 milk samples (19%) atypical mycobacteria were cultured. Three milk samples (7%) were positive by PCR.

The prevalence of BTB infection in cattle is in the same range as demonstrated previously in Tanzania. A higher prevalence of BTB infection in the extensive sector can be due to several factors: overcrowding of animals, introduction of newly purchased animals, different cattle species and bigger sizes of herds. Herd owners and their families in the extensive sector are at risk for contracting the disease because they often drink raw milk and the prevalence of BTB infection is high in the extensive sector. Therefore control of BTB, as well as education of cattle owners is important, especially in the extensive sector.

PP–67
GENETIC DIVERSITY AMONG MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS STRAINS OF GERMAN CATTLE HERDS SHOWN BY COMBINATION OF IS900 RFLP AND MIRU-VNTR TYPING

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Introduction

Mycobacterium avium subsp. paratuberculosis (Map) as etiologic agent of Johne's disease is endemic in cattle herds of Germany. This organism exhibits a very low level of genetic heterogeneity.

Purpose of the study

The purpose of this study was to select a workable procedure for strain differentiation at a resolution sufficient to investigate epidemiological links on a national scale.

Methods

Analysis of Restriction Fragment Length Polymorphism (RFLP) based on the insertion element IS900 using four restriction enzymes,

and ten markers of specific Mycobacterial Interspaced Repetitive Units (MIRUs) and Variable Number of Tandem Repeats (VNTRs) was applied to 71 bovine Map isolates originating from 14 herds of different regions in Germany.

Results

The detected diversity of isolates was higher as expected. Within the herds, the diversity was different depending on the frequency of animal purchase. Although all isolates belonged to the Map type II group, 17 RFLP genotypes were found consisting of a combination of seven BstEII-, eight PstI-, nine PvuII-, and four BamHI-restriction patterns. Novel RFLP-types were detected. Fifteen MIRU-VNTR patterns were identified. Results of RFLP and MIRU-VNTR typing were not associated. The most common RFLP-BstEII-type C1 (72%) was subdivided into 14 MIRU-VNTR types. A combination of fingerprint and PCR based techniques resulted altogether in 24 Map genotypes for isolates from the 14 German herds and achieved a discriminatory index of 0.997. Using only BstEII- and PstI-digestion together with MIRU-VNTR typing, a discriminatory index of 0.993 was achieved.

Conclusions

In conclusion, despite the high level of homogeneity of Map isolates, typing by use of the combination of IS900 RFLP and VNTR-MIRU analysis has shown sufficient sensitivity to distinguish strains of type II, even within the cattle population within one country. It offers the possibility of tracing the transmission of Map among individual animals in one herd, between herds, and between different host species.

PP–68
INVESTIGATIONS INTO THE SURVIVAL KINETICS OF MYCOBACTERIUM BOVIS DURING MANUFACTURE AND MATURATION OF CHEESE PRODUCED FROM UNPASTEURISED MILK

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Purpose of the study

Mycobacterium bovis is the principal cause of tuberculosis in a wide range of domestic and wild animals. Although there are established mechanisms for the control and monitoring of tuberculosis in UK dairy herds the prevalence of tuberculosis amongst cattle continues to increase. Concomitantly there is a growing market for artisanal cheeses produced from unpasteurised milk. The absence of a pasteurisation process removes a major barrier to possible *M. bovis* contamination of unpasteurised milk cheeses. There is presently a lack of knowledge regarding the survival kinetics of *M. bovis* during the manufacture, ripening and storage of unpasteurised milk cheeses. This investigation seeks to characterise the survival of *M. bovis* during the cheese making process.

Methods

Laboratory scale cheese manufacture will use unpasteurised milk inoculated with *M. bovis* culture. Two cheese varieties will be produced using three strains of *M. bovis* at a high and low level of inoculum.

Results

Methodology has been developed for the production of cheese under the restrictions imposed by containment level 3. Furthermore a preliminary assessment of culture media for the isolation and enumeration of *M. bovis* from unpasteurised milk and cheese has been conducted. The presence of the indigenous microflora of unpasteurised milk may inhibit the recovery of *M. bovis***.** This will necessitate the development of antibiotic supplement combinations to allow the isolation of *M. bovis* in the presence of other microorganisms. In addition it is hoped to develop molecular techniques such as real time PCR to facilitate the screening of *M. bovis* in unpasteurised milk cheeses.

Conclusions

This investigation should provide information on the survival kinetics of *M. bovis* in unpasteurised milk cheeses. This knowledge will facilitate a greater understanding of the risks posed to consumers by consumption of unpasteurised milk cheeses.

PP–69

PRESENCE OF MYCOBACTERIUM SPP. IN FROGS (RANA ESCULENTA) FROM ALBANIA

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Summary

EC and non-European countries for now import the frogs, traditional ingredients of Italian cuisine, in order to meet the internal requirement. Not very well known are the real diffusion of MOTT and the possible role of the amphibians on the maintenance of this zoonosis in the environment.

214 *Rana esculenta* collected from a Turin's Institute analysed. All the samples are of capture and were imported alive from Albania.

A sample of skin, muscle and liver was taken from every animal in sterility. These aliquots are processed, decontaminated and centrifuged, and then the sediment was spread on Stonebrink and Löwenstein-Jensen medium. The test-tubes were incubated at 28°C until the development of colonies or up till sixty day. All the colonies resulted positive at ZN stain were processed for CDC Manual's fenotyping tests.

The specimens turned out to be positive, at least for one aliquot, for *Mycobacterium* spp. are 94 (43.9%) on 214 frogs examined. In skin 34.1% (73 subjects) of samples were positive, while at liver level 20.6% (44 frogs) were positive and only one muscle sample resulted positive (0.5%). 118 strains were isolated: 48 *Mycobacterium chelonae* (40.7%), 23 *M. fortuitum* (19.5%), 20 *M. gordonae* (16.9%), 13 *M. flavescens* (11.0%), 6 *M. abscessus* (5.1%), 4 *M. peregrinum* (3.4%), 3 *M. kansasii* (2.5%), and 1 *M. marinum* (0.8%). In skin and liver, the species must representative was *M. chelonae* (26 strains, 35.6%, in skin; 29 strains, 65.9%, in liver). The only *Mycobacterium* isolated from muscle samples was classified as *M. marinum.*

Our results confirmed the hypothesis that frog's muscles present a lower level of bacterial contamination than the non-edible portions. The frequent finding of mycobacteria in skin's aliquots supports the hypothesis of external contamination of samples, due to the natural

environment where the amphibians live rather than to an effective infection.

PP–70
MYCOBACTERIUM MARINUM IN ITALIAN FARMED STRIPED BASS: ANATOMOPATHOLOGICAL AND HISTOLOGICAL FEATURES

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Striped bass (hybrid of *Morone* ssp.) is a fish raised in hot water; in Italy is not very common because its meat is not so appreciate. In USA and Israel *M. marinum*, *M. shottii*, *M. pseudoshottii* were the mycobacterial species more frequently isolated from *Morone* spp.

The aim of the present work is to describe the first *M. marinum* infection in a striped bass farm of Northern Italy.

In summer 2007 some gills of striped bass with yellow-brown nodules were sent to Istituto Zooprofilattico and the histopathological examination showed several granulomas with a large number of acid-fast bacilli.

After this finding, some fishes from the same farm were randomly selected, sacrificed and necropsied in order to identify the mycobacterial species. The anatomopathological exams revealed lesions

in various organs of all animals: ulcerative cutaneous lesions, yellow mucus with red nodules on the gills, splenomegaly and miliary granulomatous lesions in liver, kidney and spleen. At necropsy, liver, spleen, kidney, gills and muscle were collected and a portion fixed in formalin for the histological and immunohistochemical examination while another portion, not fixed, utilized for parasitological, virological and bacteriological investigations.

Microscopically granulomas presented a central necrotic area surrounded by inflammatory cells and enclosed by a thin capsule. All granulomas were associated with a great number of acid-fast bacilli resulting positive to immunohistochemistry with a polyclonal antibody against *M. bovis*. From all samples, with the exception of muscle, it was possible to isolate photochromogenic colonies and the purified isolates were characterised by phenotypical and biochemical identification. All the isolates were identified as *M. marinum.*

It is important to emphasize that, although granulomatous lesions were diffused in various organs, no fishes died for the infection and that the low death rate allowed the increase of human risk by manipulation and consumption of contaminated fishes.

PP–71
HEAVY-METAL RESISTANCE OF YELLOWSTONE MYCOBACTERIUM SPECIES

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Summary

The resistance of mycobacteria to external aggressions is already known for sometime, but the persistence of *Mycobacterium* species

in extreme environments was proved just a few years ago. The amazing diversity of *Yellowstone mycobacteria* reveals an extraordinary environmental adaptation: High temperatures, Low pH and High concentrations of heavy metals.

In this work, we tested several *Yellowstone* isolates with increasing concentrations of heavy metals, namely, *cadmium*, *nickel*, *mercury*, cobalt, *aluminium* and *chromium*. Isolates were tested in solid (Middlebrook 7H10) and liquid (Bactec system) medium. Most of the tested strains revealed a high resistance to most of the tested metals. Growth was detected in concentrations up to 500μM $HgCl_2$, 25mM $CdCl_2$, 30mM $AlCl_3$.

Yellowstone isolates reveal a higher resistance to all metal and other toxic substances than the tested standard strains, indicating an adaptation to the surrounding environment.

PP–72
MULTIRECIPIENT DONOR TRANSMITTED PRIMARY TUBERCULOSIS

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Summary

We report transplant related primary Mycobacterium tuberculosis infection in three patients who shared the same donor. The donor was an Indonesian lady who had moved to Saudi Arabia for employment two months prior to her illness. She was admitted to another facility with sudden onset of lower limb weakness, and while under

investigations became comatose and had respiratory arrest. MRI of the spine was normal but the brain showed generalized edema. CSF studies showed normal cells with high protein. CSF for bacterial and acid fast bacilli culture was negative.

Both kidneys and the liver were procured for transplantation. One kidney and the liver were transplanted to two patients at our hospital, and the second kidney to a patient at another hospital. The recipient of the kidney developed disseminated tuberculosis as evidenced by positive AFB culture from sputum and bone marrow. The recipient of the liver had multiple liver abscesses, and culture of the pus grew Mycobacterium tuberculosis. Both the patients were treated with first line antituberculsosis drugs and are doing very well.

The isolates from the two patients at our hospital were sent for identification by restriction fragment length polymorphism (RFLP) typing. By using the Hain Genotype assay both isolates were identified as Mycobacterium tuberculosis. By IS6110 restriction fragment length polymorphism (RFLP) typing the isolates showed identical 1-copy patterns. By 15 loci variable number of tandem repeats (VNTR) typing the isolates showed identical patterns.

There were no cases of tuberculosis admitted in the hospital around the time of their transplant.

The recipient of the second kidney died of disseminated tuberculosis in another hospital. Bone marrow biopsy there showed necrotizing granuloma suggestive of tuberculosis, but culture was negative. No tissue or other sample was available from this recipient for fingerprinting.

It is apparent that all three patients acquired Mycobacterium tuberculosis from the donor.

PP–73
RAPID DIAGNOSIS OF TUBERCULOSIS AND A CASE OF MDR TB

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Summary

One negative side of globalization is the increasing incidence of tuberculosis and

mycobaterioses together with the rising number of multiresistant mycobacterial strains.

This in time way unfavorably alter the hitherto low incidence of TB and the number of multiresistant mycobacteria in the Czech Republic. The use of rapid procedures for detection and identification of M. tuberculosis strains and drug susceptibility tests, particularly in cases of multidrug resistant TB.

Samples were regularly collected from patients in the Central Bohemian Region (2 million

inhabitants) and a part of Prague. A total of 1166 strains of M.tbc was isolated in the period Jan.1,2003 – April 30,2008. All strains were isolated by conventional methods and BACTEC MGIT 960,and identified by GenoType MTBC. Drug susceptibility tests were performed by conventional methods and BACTEC MGIT 960. (S.I.R.E. + PZA). The mean time for detection of M.tbc strains was 12.5 days by Bactec, and 21.4 on L-J days. Susceptibility to AT was performed on L-J medium and Bactec, results were ready in 3-6 weeks and 7.5 days, respectively.

Of the 1166 isolated strains of M.tbc tested for sensitivity to 5 basic AT (S.I.R.E. + PZA),

1006 strains were susceptible and 112 (11.13%) were resistant to varying combinations of

AT; however, further 48 strains (4.7%) were resistant to INH+RIF, i.e. MDR TB.

The increase in the number of MDR TB patients is becoming a problem in our country in view of the need of using alternate AT.

Patient H.K. (F). Sputum repeatedly negative on microscopy, cultivation and MTD-T2, x-ray

findings inconclusive. Since clinical picture remained unchanged after repeated antibiotic

therapy, biopsy was performed: positive on cultivation only, histology confirmed TB. The

strain was identified by GenoType MTBC and unexpectedly found to be MDR TB.

Targeted bronchoalveolar lavage was positive on microscopy, cultivation, MTD-T2 and

BACTEC MGIT 960 as in previous biopsy, including MDR TB.

It is therefore imperative to employ rapid diagnostic techniques in routine diagnostic

laboratories.

PP–74
"UNUSUAL CASES OF PULMONARY TUBERCULOSIS"

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The aim of this study was to show few cases of nontypical pulmonary tuberculosis diagnosed by fibrobronchoscopy , bronchoalveolar fluid and lung biopsies. Method and results. The first one was male patient with unilateral diffuse opacities on chest rentgenogram. Only transbronchial

lung biopsy showed tuberculous granulomas from samples. The second case was patient with unexplained , unresolved pulmonary infiltrate. From endobronchial biopsy sample was evident tuberculous changes. The third patient was male with lung cancer in bronchus intermedius. This patient was treated twice with endobronchial electrosurgery in 3 months period. The washing liquids after second session was positive on MGIT for tuberculous bacteria. Chest rentgenograms showed typical infiltate in right upper lobe. Conclusion. These examples propose a topic

PP–75
RAPID DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS ISOLATES BY MIRU-VNTR

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The emergence of difficult to treat multi drug-resistant tuberculosis currently threatens control efforts in all regions of the world. Genotyping *M. tuberculosis* strains has the potential to identify outbreaks and track transmission of drug resistant strains. To be of value to the public health practitioner genotyping must be discriminatory, rapid and readily available. One preferred genotyping method for *M. tuberculosis* is PCR-based MIRU-VNTR typing examining 15 loci. The implementation of this method requires the ability to accurately size the resulting amplicons. Analysis by traditional gel electrophoresis is tedious and slow and MIRU typing has so far been largely restricted to laboratories with sophisticated high-throughput analytical facilities. ScreenTape® (LAB901, Edinburgh, Scotland) is an automated, electophoritic device for rapid analysis of nucleic acids. It is

simple to use and provides accurate sizing of amplicons in under 10 min. We applied ScreenTape® and gel electrophoresis to MIRU-VNTR analysis of 20 drug resistant *M. tuberculosis* isolates and compared the results to those obtained by a commercial genotyping company (GenoScreen, Lille, France). Using ScreenTape® the assignment of alleles was in agreement with the results from GenoScreen on 97.1% of occasions, compared to 93.4% for traditional gel electrophoresis. Correlation coefficients for predicted fragment length were 0.9911 and 0.9919 for ScreenTape® and gel electrophoresis, respectively. Whereas the 20 isolates had previously been identified as having two spoligotype patterns, 14 MIRU patterns were observed demonstrating the superior discriminatory power of MIRU-VNTR typing for *M. tuberculosis*. ScreenTape® appears to offer rapid, discriminatory genotyping and may prove an attractive option for public health laboratories charged with controlling infectious tuberculosis disease.

PP–76
DIRECT TESTING FOR MULTI-DRUG RESISTANT TUBERCULOSIS PERFORMS AS GOOD AS INDIRECT TESTING. A META-ANALYSIS

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Purpose of the Study

Early detection of multi drug resistant tuberculosis (MDR TB) is essential in its management. Conventional indirect susceptibility testing requires 1-3 months. New rapid tests are urgently needed. We compared the sensitivity, specificity, time to results and cost of 4 tests for MDR TB applied directly on positive sputum.

Methods

Literature review and meta-analysis was conducted.

Results

Direct Testing: the pooled sensitivity and specificity for detection of resistance to rifampicin was 100% and 98% with Genotype MTBDR® (Hain Lifesciences, Nehren, Germany), 98% and 100% with nitrate reductase assay (NRA), 96% and 97% with Microscopic Observation Drug Susceptibility (MODS), and 91% and 94% with manual Mycobacterium Growth Indicator Tube (MGIT), respectively. For isoniazid it was 64% and 100% for Genotype MTBDR®, 97% and 100% for NRA, 93% and 95% for MODS, and 100% and 97% for MGIT Manual, respectively. The average time in days to 100% of the results was 1 for Genotype MTBDR, 28 for NRA, 22.7 for MODS, 15 for MGIT Manual.

Indirect testing

the pooled sensitivity and specificity for resistance to rifampicin was 99% and 98% with Genotype MTBDR®, 99% and 100% with NRA, 100% and 100% with MODS, 93% and 100% with manual MGIT. For isoniazid, it was 74% and 100% with the Genotype MTBDR®, 96% and 99% with NRA, 100% and 94% with MODS, 97% and 97% with manual MGIT, respectively. Time in days to 100% of the results was 1 day for Genotype MTBDR® 13.3 for NRA, 12.5 for MODS, 11.6 for manual MGIT.

Cost of tests was reported in 7 of 34 studies, but settings were not standardised.

Conclusion

Direct testing performed as good as indirect testing with all four tests. Genotype MTBDR® was best in performance and time to results. There was inadequate data to compare test costs.

PP–77

MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* IN ALBANIA REVEALS A COMPLEX POPULATION STRUCTURE AND A LOW RECENT TRANSMISSION RATE OF TUBERCULOSIS

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Introduction

The magnitude of the tuberculosis problem in Albania is not exactly known. Official statistics from the Ministry of Health show that tuberculosis is among the leading causes of morbidity in Albania. The number of young adults affected with tuberculosis indicates active transmission in the population. In 2006 the reported TB incidence in Albania was 15/100,000. This number varied highly between Districts (from 4/100,000 to 50/100,000 in the North Regions of the Country) but for the suboptimal health care system,

Aim

The goal of this study was to have a first insight on the genetic diversity of *Mycobacterium tuberculosis* complex (MTC) in Albania. In particular we wish to evaluate the recent transmission rate of tuberculosis in Albania and to study the population molecular genetics structure of MTC in the Balkan region, a complex anthropological area.

Methods

A set of 100 DNA extracted from 100 tuberculosis patients resident in Albania recruited during 2006-2007 was studied by a combined 24 MLVA-spoligotyping (Multi Locus VNTR Analysis or MLVA) strategy.

Results

Results based on spoligotyping showed the presence of 16 clusters containing between 2 to 22 isolates totaling 85 isolates, including the presence of three Beijing types. Further genotyping by VNTR-MIRU-12 analysis provided more discrimination with a total of 15 subclusters totaling 49 isolates. Including three more loci (ETR-A to C) did not further improve discrimination. However, adding 9 more Mtub and QuB loci further reduced the total number of likely epidemiologically linked patients to 43 (min) to 49 patients (max) included in 15-16 clusters (recent transmission rate = 28-34%) depending on the definition stringency of clusters. 2 MDR Beijing isolates were also found in this study. We then studied the phylogeographical specificity of the MTC genotypes by comparing to SITVIT databases. Three new genotypes, designated as Albania-1 to 3, which are not found in SITVIT are described and their presumed origin and historical link to other genotypes is discussed.

Conclusion

This study suggests that TB is under control in Albania, with a moderate recent transmission rate. However, the possibility of MDR-TB expansion, through the presence of some MDR Beijing isolates in

Elbasan should be further monitored. Moreover, Albania and more largely the Balkan region provides an interesting setting in Europe to further study the molecular evolution of tuberculosis.

PP–78

PRELIMINARY RESULTS ON THE IMPLEMENTATION OF THE AUTOMATIZED MICROBEAD-BASED HIGH-THROUGHPUT LUMINEX SPOLIGOTYPING METHOD ON *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

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Introduction

Global Plan for TB suggests that TB will not be erdicated by 2050. However, molecular epidemiology can help to lower transmission chains and adequate well-funded centralized genotyping platforms, surveillance networks and/or rapid technology transfer can contribute to more efficient TB prevention measures world-wide.

Aim

A single report in 2004 evaluated the possibility to transfer the robust spoligotyping technique, a PCR membrane-based first-line molecular epidemiological method to the recently described high throughput microbead-based automatized format (suspension microarrays). Our goals were : (i) to implement a simplified (10 spacers) test-version Luminex assay (ii) to assess the scientific and economical interest of this suspension assay (iii) to assay the suitability of the Luminex format for new high-throughput assay development.

Methods

We used a total of 60 test-DNA from Albania (n=10) and France (n=50). The Luminex 200 was used in parallel to commercial spoligotyping membranes.

Results

On a set of 50 DNA of various genotypes, we found a basic correlation of 96% between the membrane-based and the suspension-based assay. The microbead suspension assay was easy to set up and no modification of the spoligotyping PCR procedure had to be introduced. Our results open the possibility to provide to National Mycobacteria Reference Laboratories a simple External Quality Control assay for large population-based spoligotyping studies. The new 10-spacers automatized spoligotyping format allowed to classify 35 of the 62 genotypes families described in SpolDB4 and allowed 184 of the 1024 theoretical signatures to be found. Such a simplified format will also decrease the workload of second VNTR typing for epidemiological purposes.

Conclusion

The microbead-based suspension array format and the Luminex platform appears to be powerful technologies, well suited to spoligotyping. As a next step, various extended spoligotyping assays (using

different geographically-based panels) are in development, as well as new high throughput assays on 3R SNPs and drug resistance genes.

PP–79
FIRST CASE OF MULTI DRUG-RESISTANT TUBERCULOSIS CAUSED BY *MYCOBACTERIUM TUBERCULOSIS* BEIJING GENOTYPE DIAGNOSED IN BOGOTA – COLOMBIA

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On November 14, 2007, a 15-year-old girl presented to the Hospital Central de la Policia (HOCEN) (Bogotá) with diagnosis of Pulmonary Tuberculosis associated with sickle cell anemia. Ziehl Nielsen (ZN) staining of sputum demonstrated acid-fast bacilli (AFD). The patient had fever and oxygen dependence.

The patient was from Buenaventura and was sent from Cali to Bogotá. She has been hospitalized for 3 times on October and November 2007 in Cali Colombia, with Pneumonia. The patient had lost of weight and was contact of TB patient. The tuberculin skin test was 13 mm, antimicrobial treatment was started with RIF, INH, PZA, EMB and Folic Acid.

In the HOCEN the patient continued with the antimicrobial treatment, anti-TB and Moxyfloxacin, however she got worst and

was transferred to the PICU. She presented ventilation failure, multiple organic failure, suprarenal failure, myocarditis and myocardial dysfunction, even though the treatment in the hospital she died on November 30th 2007. Autopsy was performed in order to clarify the case, some tissue samples (lung, kidney, suprarenal glands, brain, and heart) were analyzed in the Mycobacteria Laboratory of the School of Medicine of National University in Bogotá.

Only in the lung tissue ZN staining demonstrated AFD. The specimens were macerated and concentrated by centrifugation and cultured in the solid Ogawa Kudoh medium. Three cultures were positive for *M. tuberculosis* complex (lung, kidney, suprarenal glands). All isolates were tested for susceptibility to antimicrobial agents according to standard proportional method and all were resistant to RIF, INH, EMB and SM. In an attempt to obtain a specific etiologic diagnostic, DNA was extracted and Spoligotyping was realized using standard methods (Van Soolinger, 2002, Kamerbeek et al., 1997). The comparison of spoligotypes with the international spoligotype database [SpolDB4 http://www.pasteurguadeloupe. fr:8081/SITVIT-Demo/index.jsp)] showed that the isolates belonged to the genomic family of *M. tuberculosis* Beijing.

PP-80

RECOMBINANT PROTEINS OF *M. TUBERCULOSIS* STIMULATE MACROPINOCYTOSIS OF HUMAN ALVEOLAR A549 CELL LINE

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Introduction

Mycobacterium tuberculosis is an intracellular pathogen that initiates its infection in the terminal bronchi and pulmonary alveoli after aerosol inhalation of numerous bacteria. It is known that *M. tuberculosis* can invade and replicate into human alveolar A549 cells, being macropinocytosis the mechanism responsible of its entry to this cells.

Purpose of the study

In this study, we analyzed the ultrastructural changes induced by several recombinants proteins of *M. tuberculosis* (Ag85, HBHA, P16, Ag14kD y APA) on A549 cell line.

Methods

Fluid phase uptake induced by these proteins was monitored using Dextran-FITC labeled. Scanning electron and confocal microscopic analysis were using to demonstrate cytoskeleton rearrangements and ultrastructural changes.

Results

M. tuberculosis recombinant proteins stimulate membrane ruffling on A549 cells and formation of irregular lamellipodia on apical surface. All proteins induced cytoskeleton rearrangements in comparison with un-stimulated cells. Fluid phase uptake was stimulated by all the studied proteins; however Ag85 and HBHA induced a sustained fluid phase uptake during all time curse observation. Control proteins (BSA, HSA, OVA) didn´t induce important changes. Also, we studied other bacterial proteins like heat shock proteins (*E. coli* and *K. pneumonia*) and we observed that these proteins also stimulated fluid phase uptake similarly to *M. tuberculosis* recombinants proteins.

Conclusions

All together our results indicate that structural and secretion proteins of *M. tuberculosis* induced important changes on fluid phase uptake and on actin filaments, these evidences suggest that studied proteins could be important during adhesion and internalization of *M. tuberculosis* into A549 cells.