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[1]. World Health Organization. The use of liquid medium for culture and DST. WHO <http://www.who.int/tb/dots/laboratory/policy/en/index3.html> August 2009

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Izdajatelj
Bolnišnica Golnik - Klinični oddelek za pljučne bolezni in alergijo
Univerza v Ljubljani, Veterinarska fakulteta

Urednica zbornika
Darja Kušar
Urška Bidovec-Stojkovič

Organizacija srečanja
Matjaž Ocepek
Manca Žolnir-Dovč
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WELCOME MESSAGE

Dear colleagues,

It is our honour and great pleasure to welcome you to Bled and the 31st Annual Congress of the European Society of Mycobacteriology (ESM 2010).

The scientific programme covers all aspects of mycobacteriology: epidemiology, immunology and up-to-date diagnostic methods, clinical aspects of tuberculosis and non-tuberculous mycobacteria, quality control assurance and human/veterinary biosafety issues. Lectures and perspectives from the leading European and world scientists engaged in research on mycobacteria will be presented – an opportunity for all mycobacteriologists and clinicians not to be missed! In addition, more than 170 excellent abstracts were selected for presentations, which will hopefully provoke challenging scientific discussions.

Slovenia lies at the heart of Europe, where the Alps meet the Dinaric Karst and the Mediterranean meets the Pannonian plains. It is sometimes called the picture-perfect paradise due to its varied geography which encompasses lush, green forests, hills with vineyards, crystal clear mountain lakes, fairytale castles, mysterious cave underworlds, many natural and landscape parks, rivers and the sea and much more on only 20,273 km^2.

Bled and its renowned alpine lake with the island are a pearl of the Julian Alps that hosts thousands of tourists from all over the world each year. It is an ideal place that offers a great chance to boost your energy which you need for the busy everyday life – and to fight against tuberculosis.

We hope the oral presentations and poster sessions will strengthen our interactions, generate new ideas and that you will enjoy friendly gathering in the idyllic Bled resort!

Matjaž Ocepek and Manca Žolnir-Dovč

Dear colleagues,

It is our pleasure to welcome you on behalf of the European Society of Mycobacteriology (ESM, http://www.esmycobacteriology.eu) to the 31st Annual Congress of ESM in Bled, especially as this is the first congress after the official registration of the ESM as a non-profit international scientific association.

The ESM was founded in 1980 and the first meeting was held in the same year in Borstel (Germany). Since then, thirty meetings took place annually in different European countries. During these thirty years of excellent and enthusiastic work, the ESM has become a reference point in the area of mycobacteriology and related diseases and gained a primary position among the most active international scientific societies.

The association is committed to the promotion of science and research in the field of mycobacteriology, thus fostering a better understanding of mycobacteriology and thereby preventing mycobacterial diseases. During the next years we will support dissemination of knowledge on all aspects of mycobacteriology and related diseases through scientific meetings and publications and provide high standard trainings for interested health care providers. Furthermore, the ESM will be a natural partner to establish, review and revise guidelines on all aspects of mycobacteriology and to advise and cooperate with government and non-government agencies in matters of common interest.

We wish you a fruitful and exciting congress.

Enrico Tortoli (Italy)
President of the ESM

Stefan Niemann (Germany)
Vice President of the ESM

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EUROPEAN SOCIETY OF MYCOBACTERIOLOGY
http://www.esmycobacteriology.eu
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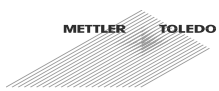

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ABSTRACTS OF GUEST LECTURES (GL)

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EPIDEMIOLOGY OF TUBERCULOSIS AT THE GLOBAL LEVEL

Véronique Vincent

UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Geneva
Contact: vincentv@who.int

WHO estimates that 9.27 million incident cases of TB occurred in 2007 (9.24 M cases in 2006, 6.6 M cases in 1990). Most of the estimated number of cases were in Asia (55%) and Africa (31%). The five countries with the highest case burden are India (2.0 M), China (1.3 M), Indonesia (0.53 M), Nigeria (0.46 M) and South Africa (0.46 M). Among the new cases 1.37 M (15%) were HIV-positive; 79% of these HIV-positive cases were in the African Region and 11% were in the South-East Asia Region. Although the total number of incident cases of TB is increasing in absolute terms as a result of population growth, the number of cases per capita is falling. The rate of decline is slow, at less than 1% per year. Globally, rates peaked at 142 cases per 100 000 population in 2004.

Among new TB cases in 2007, WHO estimates that 1.3 M deaths occurred among HIV-negative (20 per 100 000 population) and 456 000 deaths among HIV-positive (23% of the estimated 2 M HIV deaths in 2007). The numbers of HIV-positive TB cases and deaths are estimated to have peaked in 2005, at 1.39 M cases and 480 000 deaths.

There were an estimated 0.5 M cases of multidrug resistant TB (MDR-TB) in 2007. There are 27 countries (of which 15 are in the European Region) that account for 85% of all such cases. The five countries with the highest numbers of MDR-TB cases are India, China, the Russian Federation, South Africa and Bangladesh. The emergence of extensively drug resistant TB (XDR-TB) detected in all continents is a major concern, outcomes for patients are much worse than for MDR-TB patients.

Globally, the rate of treatment success for new smear positive cases treated in DOTS programmes in 2006 reached the target of 85% first set by the WHA in 1991. The Eastern Mediterranean (86%), Western Pacific (92%), and South-East Asia (87%) regions met the target. However the treatment success rate was below the target in the other regions.

GL-2

EPIDEMIOLOGY OF TUBERCULOSIS IN SLOVENIA

Petra Svetina Šorli

University Clinic of Pulmonary and Allergic Diseases Golnik, Slovenia
Contact: petra.svetina-sorli@klinika-golnik.si

Tuberculosis (TB) remains a global health problem, because in certain parts of the world, despite the World Health Organization help and implementation of disease control in the context of national programs for tuberculosis (NPTB) in individual countries, the incidence of this disease is still very high. The reasons are mainly poorly designed and / or implemented national programs for tuberculosis (NPTB) in individual countries and the epidemic of HIV infection.

For Slovenia just the opposite is true, since in the last fifteen years in the control of tuberculosis epidemic considerable progress has been made, which lead to a fall in the incidence of TB.

Control of TB epidemic in Slovenia is guided by a Register of Tuberculosis, which was established in 1952 and has always been an integral part of the University Clinic of Pulmonary and Allergic Diseases Golnik. Beside for cases of active TB, supervised inspections of contacts with TB patients and other persons who require treatment for latent tuberculosis infection are integrated in Register of Tuberculosis. In collaboration with the National Reference Laboratory for TB micro-epidemics are supervised and occurrence of resistance to certain drugs for TB is controlled. In the event of the occurrence of micro-epidemics Register of Tuberculosis is working together with doctors and other medical staff on site to clarify all the important links between patients and, consequently, people with latent tuberculosis infection.

In Slovenia since 1998 sensitivity testing for 1st line antituberculosis drugs is performed for all the culture positive patients automatically. These tests are carried out in the National Reference Laboratory at the University Clinic of Pulmonary and Allergic Diseases Golnik. Since 2000 all genotyping of isolates is performed in culture positive patients with TB. That same year we began to notify regularly people who have been in contact with infectious tuberculosis patients (so called contacts) to the Register of TB. Register of tuberculosis is obliged to provide a list of persons-contacts to the regional pulmonologists or pediatrician-pulmonologists (in the case of a child contact), which then contact the person and invite them for visit. Pulmonologists or pediatrician-pulmonologists are required to provide information on examination of those persons, which include information on preventive treatment of latent tuberculosis infection. We are also preparing guidelines for the contacts management, in which the unified treatment of contacts, use of tests for the detection of latent tuberculosis infection, indications and contraindications for

prophylactic treatment of latent infection and follow-up to these people, are going to be reviewed.

Since 2004 TB-Gold interferon test is used to demonstrate latent TB infection. This test should be performed in all patients to be treated with inhibitors of TNF-alpha, before organ transplantation and all contact persons who are candidates for preventive treatment of latent tuberculosis infection.

In Slovenia, in 2005 we stopped non-selective BCG vaccination at birth. Slovenia has several years earlier met international recommendations of IUATLD (International Union Against Tuberculosis and Lung Disease) for the suspension of non-selective vaccination at birth. The situation in the Balkans and the disintegration of the former Republic of Yugoslavia and, consequently, increased migration of people from these areas and temporary resettlement of refugees in Slovenia lead to implement these some years later. From January 01 2005, BCG vaccination is carried out selectively at birth and is mandatory for newborns in families whose parents moved from countries with high incidence of TB (>31/100,000) in the last five years before the birth of a child. In Slovenia, the treatment of TB is required by law. All contagious patients should be treated in hospital in TB designated wards, where administrative, technical measures and personal protection is implemented. In a culture positive TB patients or patients with non-pulmonary form of TB treatment is ambulatory, although generally treatment is started at the hospital to identify potential treatment complications. All patients with TB, either pulmonary or non-pulmonary, are supervised by pulmonologist.

At present, adult patients with TB can be treated in six hospitals while children are treated in one hospital. Most adult patients with infectious TB are treated in the University Clinic of Pulmonary and Allergic Diseases Golnik. Hospital treatment is completed, when sputum becomes negative for *Mycobacterium tuberculosis* on microscopy, and treatment was in place for at least three-weeks and at the same time clinical and radiological improvement is noted. Before discharge visits with family doctor and regional pulmonologist are arranged. With DOT (Directly Observed Treatment) we ensure regular consumption of drugs, monitoring of potential side effects and ensure completion of treatment. The frequency of monitoring depends on patient cooperation. Observations are carried out by nurses in health centers, in nursing homes, in schools, clinics. If adequate treatment control can not be achieved or the patient lives in inadequate social conditions, a prolonged hospitalization is administered. Prolonged hospitalization is mainly needed with the homeless, alcoholics or in patients with history of discontinuation of previous treatments.

In 1996, when the incidence of TB was 28.28 cases per 100,000 inhabitants, renovation of NPTB was made. Already in 2000 for the first time, the incidence of TB dropped below 20 cases per 100,000 inhabitants (19.6 cases per

100,000 inhabitants), which ranked Slovenia among countries with low incidence of TB. The incidence of TB dropped continuously from year to year and reached a value of 10.7 cases/100,000 inhabitants in 2006, and leveled at that range for next three years. According to preliminary data for 2009, the incidence of TB in Slovenia for the first time dropped below 10 cases/100,000 population (9.25/100,000).

In all these years we noted the decrease in incidence of new cases of TB as well as relapses. In 1996 the relapses accounted for 17% of all TB patients registered during the year. Over the past five years the proportion never exceeded 10% of all cases of TB patients annually.

In Slovenia men are involved more often and proportion (male/female ratio) over the past ten years ranged between 1.4 and 1.7, except in 2006 when the ratio was 1.2.

Among patients with TB less than a third were immigrants from other countries, mainly from the former Republic of Yugoslavia. Slovenia – borne patients were mostly men in the age group 55 to 64 years and women older than 65 years. In the group of immigrants, men and women between 25 and 44 years of age, were the predominant group.

Although the incidence of TB in Slovenia declined with years, micro-epidemics in particularly susceptible populations such as homeless people, prisoners, immigrants, drug addicts, persons with impaired immunity as a result of underlying disease or treatment with immunosuppressive drugs, the elderly and persons living in nursing homes and health-care workers, become increasingly important.

In Slovenia patients with TB and concomitant infection with HIV are not common. From 2000 till 2007 we have treated 10 such patients (0.3%) and none in the last three years.

Over the past ten years, we have confirmed the disease by microbiological cultivation and / or with appropriate histology in 90.98% cases. In all detected patients pulmonary tuberculosis was most common. In a third of all cases non pulmonary or non-pulmonary with pulmonary TB was detected. Among non-pulmonary forms of TB disease pleurisy (approx. 40% of all non-pulmonary TB) and TB lymphadenitis (approx. 30% of all non-pulmonary TB) were most common, while other forms of non-pulmonary tuberculosis were rare.

Most patients (two thirds) were treated with standard 6 months regimen - two months triple or quadruple treatment (rifampicin, isoniazide, pyrazinamide, etambutol) following with 4 months of two-track therapy (isoniazide and rifampicin). In other cases, we decided to tailor treatment regimes because of intolerance for certain substances or drug resistance. Multidrug resistant forms

of the disease occur sporadically, in the last ten years we have treated 8 patients with MDR-TB (0.28% for all the cases of patients with TB) and 1 patient with XDR-TB (0.03% for all the cases of patients with TB). Among the isolates that show resistance to individual drugs, resistance to streptomycin (0.2% in 2009) and isoniazide (0.15% in 2009) were most commonly detected during the past 10 years.

Over the past nine years, 81.8% of all patients with TB completed the treatment. In the period from 2000 – 2008 the mortality due to TB was 2.69%, annual mortality ranged from 1.3% to 4.2%.

Due to new knowledge that has emerged in recent years in the fields of prevention, detection and treatment of TB and current epidemiological situation in Slovenia, we are preparing updates of NPTB.

GL-3

TOWARDS ERADICATION OF BOVINE TUBERCULOSIS IN THE EUROPEAN UNION: AN OVERVIEW OF THE EU POLICY ON BOVINE TUBERCULOSIS ERADICATION

Valentina Piazza

Veterinary Administrator, Head of Sector: Veterinary, Unit 04: Veterinary Control Programmes, Health and Consumers Directorate-General, (DG SANCO),Rue Belliard 232, 1140 - Brussels, Belgium
Contact: valentina.piazza@cec.eu.int

The presentation reviews the developments and the progress towards eradication of bovine tuberculosis (TB) in the European Union (EU). A historical view of the EU legislation aimed at mainly cattle intra-community trade explains the present EU policies. The current situation of TB in the EU Member States is summarised.

Eradication of TB is the target at EU level as laid down in Community legislation and should be feasible in the long term despite the fact that different epidemiological situations in the EU, due to the variety of cattle breeding systems and environmental conditions, pose certain difficulties that should nevertheless be addressed through specific reinforced measures.

Requirements of Community legislation are to be considered in the context of the eradication programmes as the absolute minimum level of measures to be implemented. Effective eradication programmes should include additional measures aimed at addressing the different constraints to eradication in each epidemiological situation.

Full involvement of all stakeholders and optimum use of the abattoir as a surveillance resource that is more fully integrated in the eradication programme should be considered as necessary issues to be specifically dealt with in the context of eradication programmes.

It would be appropriate for the MS involved to rank the measures in order of priority/effectiveness when allocating the funds available for TB eradication. The diversity of the situations in different MS means that different emphasis must be made on the various measures.

Finally, a short presentation of the important role played by the "Task Force for Monitoring Animal Disease Eradication" (TF), tuberculosis sub-group (TBTF), as additional technical support for Member States, is made.

GL-4

ERADICATION OF BOVINE TUBERCULOSIS IN SLOVENIA

Matjaž Ocepek

University of Ljubljana, Veterinary Faculty, Slovenia
Contact: matjaz.ocepek@vf.uni-lj.si

In Slovenia, bovine tuberculosis (BTB) was described for the first time in 1851 by Dr. Simon Strupi. First records on the spread of tuberculosis among cattle are from the period between 1891 and 1900. The disease was detected in 0.19% of animals in abattoirs. On the other hand, the first tuberculin (TB) skin testing, performed on some large farms in 1922, showed that 70% of animals were positive. In general, the proportion of positive animals in abattoirs did not exceed 1% in the period between the world wars.

After the Second World War, BTB was recognized as a serious disease of animals and humans in most European countries. Consequently, a planned TB skin testing in community farms in Yugoslavia started in 1947. As a result, 8.05% positive cattle were found among the 14.196 tested.

In 1954, the Federal Veterinary Administration introduced basic measures for suppression and eradication of BTB. This led to the development of a federal eradication program, which was performed in the same year and included monitoring, compulsory slaughtering of all positive animals, prohibition of movement of animals and trade with milk and products of animal origin from the infected holdings.

Due to the organizing problems, the first general TB skin testing was not performed until 1962. Since then, systematic monitoring of the disease has been carried out. Almost all cattle were included in the test in 1962 and 1963. Among the 404,766 tested, 0.41% animals from 0.95% of the herds were positive. Because of the eradication of foot and mouth disease, which mobilized the majority of the veterinarians, less than a third of the animals were tested in 1964 and 1965. Despite this fact, all confirmed outbreaks of BTB were successfully controlled.

During the 36th general session of the OIE in Paris (1968), animal health code was adopted, which served as a model for basic principles of BTB eradication in Yugoslavia. As a result, in only three years (1974-1976) the proportion of positive herds fell under 0.1%. A decade later it dropped to 0.01% and BTB was practically eradicated.

Even though Slovenia fulfilled the main condition for becoming a country officially free of BTB (less than 0.1% infected herds in six consecutive years) already in the late seventies, it lasted 30 years before it obtained the status in 2009. The main reason for the delay was that Slovenia adopted the rules on the identification and registration of bovine animals in 2003 and EU administration did not admit any results of TB skin testing before 2003, in spite of the fact that there was only one case of BTB confirmed in Slovenia in the last 20 years.

DIAGNOSTIC PROBLEMS OF TUBERCULOSIS AND NTM DISEASES IN THE 21ST CENTURY

Wiel de Lange

University Medical Center Groningen, Department of Tuberculosis, Beatrixoord, Dilgtweg 5, Haren, P.o.box 30 002, 9750 RA Haren, The Netherlands
Contact: Tel. 00 31 50 533 84 80, Fax. 00 31 50 533 86 50, w.c.m.de.lange@cvr.umcg.nl

It was not even ten years ago that the time between starting a treatment for suspected tuberculosis and the arrival of the definitive diagnosis and the resistance pattern, took at least 4 to 5 weeks and sometimes up to 3 months time. This was a difficult situation for clinicians in which especially the history and in a lesser degree the physical examination of the patient were very important to make an "Educated Guess" about the treatment regimen of drugs. The history and physical examination will always stay important issues.

Meanwhile there have been major changes in resistance development of tuberculosis strains. MDR and XDR are real-life problems with which we nowadays have to cope frequently. There's also a lot more information about the NTM's and there is rising evidence that they are of growing clinical importance. There are much more immunocompromised patients *e.g.* because of HIV and the use of drugs like TNF-a blocking agents. This makes the need for fast diagnostic tools even more important. The faster we know if we are dealing with TB or NTM's and the faster we know whether there is any form of drug resistance, the better we can treat the patient and prevent extended drug resistance or side effects of drugs which are given unnecessary.

In the last decade there have been made big achievements in mycobacteriological diagnostic techniques. The new ways to culture the mycobacteria, but even more the molecular techniques have given us diagnostic information in a much faster way. This helps clinicians a lot in making their decisions. But even with the new information it can still be difficult to make the correct diagnosis or there can be conflicting results from tests.

In the presentation there will be given examples of situations in which the information from the mycobacterial laboratory gives problems with the interpretation and decision making for the clinician.

GL-6

PRACTICAL EXPERIENCE WITH TREATMENT OF MDR/XDR TB

Manfred Danilovits

University Lung Hospital Tartu, Estonian NTP, Riia St 167, Tartu S 1014, Estonia
Contact: manfred.danilovits@kliinikum.ee

Tuberculosis (TB) is a leading cause of mortality from infectious disease, second only to human immunodeficiency virus (HIV)/AIDS. In 2007, there were 9.27 million new cases of TB and 1.77 million deaths. Of the 9.27 million incident TB cases an estimated 1.37 million (15%) were HIV-positive. To date, WHO estimates at least 500,000 new multidrug-resistant TB (MDR-TB) cases annually.

Inadequate and incomplete treatment and poor treatment adherence has led to a newer form of drug resistance known as extensively drug resistant tuberculosis (XDR-TB). XDR-TB is defined as TB caused by *Mycobacterium tuberculosis* strain, which is resistant to at least rifampicin and isoniazid among the first line anti tubercular drugs in addition to resistance to any fluroquinolones and at least one of three injectable second line anti TB drugs *i.e.* amikacin, kanamycin and/or capreomycin. Mismanagement of TB paves the way to drug resistant TB. Reported prevalence rates of XDR-TB of total MDR cases are 6.6% worldwide. The emergence of XDR-TB is one of the threats to global TB control, and there are lots of data documenting greater mortality and treatment failure rates compared with other MDR-TB cases. The development of XDR-TB during MDR-TB treatment is related to two potentially modifiable factors: baseline chronic cavitary disease and nonadherence to MDR-TB therapy.

In Estonia (1.34 million people) the incidence of TB started to increase in 1997, when 51 new TB cases were diagnosed per 100,000 population. In 2000, Estonia was identified as one of the MDR TB „hot spots" in the world. Since 2001 country started the WHO recommended management for treatment of DR-TB. During next years TB notification rate decreased 8% per year and reached to level of 28.4 per 100,000 population. However, the proportion of MDR/XDR-TB has remained still high. In 2009, MDR-TB accounted for 16.4% of all new TB cases.

TB control in Estonia is further complicated by the synergy between TB and HIV/AIDS. During 2009, 411 new HIV cases were detected and the total prevalence number of HIV-positive people reached 7320. In 2008-2009 approximately 10% of all TB cases were HIV-infected.

Treatment of MDR TB patients is complicated due to the difficulties in accessing and handling SLDs which are weaker, cause more adverse-effects, and are much more expensive. For treatment of MDR-TB it is often necessary to use

five or six drugs for an extended period of time, lasting up to 2 years. In most cases, an injectable agent and a fluoroquinolone form the core of the regimen. Different options for treatment strategies of suspected MDR cases are in use; standardised combinations of second-line drugs are recommended, but this choice requires representative DRS data on specific treatment categories. An alternative approach is to design a regimen on the basis of the individual history of previous anti-TB therapy and individual DST. This approach requires a high degree of laboratory capacity necessary to perform accurate DST on most SLDs. In addition, MDR-TB treatment programs must address potential barriers to treatment adherence (*e.g.* patient side effects, socioeconomic factors).

Treatment outcome data showing cure rates of 60–75% are available from DOTS-Plus projects in Estonia, Latvia, Peru, the Philippines and the Russian Federation. Significantly worse treatment outcomes and higher death rates have been demonstrated in XDR-TB. New drugs are needed to address the current global MDR-TB epidemic, drugs with both an improved safety profile and new mechanisms of action that would not be affected by cross-resistance of *M. tuberculosis* strains already resistant to currently used TB drugs.

In recent years novel classes of drugs have entered into multicentered Phase II clinical trials, Estonia is one of these countries where these trials take place.

GL-7

THE ROLE OF A LABORATORY FOR MYCOBACTERIA IN VETERINARY MEDICINE

Aranaz A[1], Bezos J[1], Romero B[1], Castellanos E[1], Rodríguez S[1], Casal C[1], Fernández de Mera Isabel G[1], Lozano F[1], Moya N[1], de Juan L[1], Mateos A[1], Domínguez L[1]

[1]VISAVET Animal Health Surveillance Centre
[2]Dept. Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Av Puerta de Hierro s/n, 28040 Madrid, Spain
Contact: alaranaz@vet.ucm.es

Introduction. The species of the genus *Mycobacterium* are responsible of infections of veterinary interest that cause serious sanitary and economic impact. Several of these infections are zoonoses.

Objective. The tasks of a laboratory involve the diagnosis, identification and molecular characterisation of mycobacterial infections in livestock, pets, wildlife or exotic animals. This commitment contributes to the control of the infections.

Material and Methods. The protocol for diagnosis depends on the disease and animal species. It may involve the evaluation of reagents for the intra-dermal tuberculin tests, the γ-interferon assay, ELISA tests, and bacteriological culture and identification using molecular methods (complex or species-specific PCRs, 16S rRNA and *hsp65* sequencing, probes, etc). Studies on epidemiology also vary according to the pathogen, thus isolates of the *M. tuberculosis* complex are currently fingerprinted using DVR-*spoligotyping* and *variable number tandem repeats* (VNTRs), and isolates within the *M. avium* complex are characterised by PFGE, RFLP, and VNTRs.

Results. Main etiological agents of tuberculosis in animals are *M. bovis* and *M. caprae*, which affect domestic animals (cattle, goats) and wildlife (badger, deer, wild boar, lynx). *M. tuberculosis* complex organisms may also infect pets (dogs, cats) and zoo animals (elephants, primates, pinnipedae). Molecular characterisation allows identification of outbreaks and knowledge of reservoirs. Other relevant mycobacteria include *M. avium paratuberculosis* in ruminant species, *M. a. avium* in birds and raptors, *M. a. hominissuis* in swine, and atypical mycobacteria such as *M. genavense* in birds, *M. nonchoromogenicum, M. fortuitum* and *M. chelonae* in reptiles and turtles, or *M. peregrinum and M. marinum* in fishes.

Discussion. A complete and accurate identification of these mycobacteria in human patients is needed to assess the presence of the zoonoses. In this way, a close collaboration with human health laboratories is essential.

COMPREHENSIVE TB LABORATORY SERVICES IN 2010 AND BEYOND

Max Salfinger

Bureau of Laboratories, Florida Department of Health, Tallahassee, Florida
Contact: max_salfinger@doh.state.fl.us

TB status. Eliminating TB, defined as 1 case per 1 million, would mean that the US would have reported just 305 new TB cases last year – instead, CDC reported 11,540 new TB cases, a figure almost 40 times larger than our goal of eliminating TB in US. The increasing threat of multi-drug resistant (MDR) and extensively drug-resistant (XDR) TB does not only have a human price (more patients are dying of drug-resistant tuberculosis versus patients with drug-susceptible TB), but also an economic impact on health care. It is estimated that preventing a single case of MDR TB would save the US health care system more than $250,000 and the average estimated hospitalization cost for treating a patient with XDR TB is $600,000, not including costs of outpatient care and related health interventions.

Role of the laboratory. The diagnosis and management of TB disease rely on accurate laboratory tests both for the benefit of individual patients and for control of TB in the community through public health services. Therefore, laboratory services are an essential component of effective TB control at the local, state, national and global levels.

In the United States, up to 80% of all initial TB-related laboratory work (*e.g.* acid fast bacilli smear and culture) is performed in hospitals, clinics, and independent laboratories outside the public health system, whereas more than 50% of mycobacterial species identification and drug susceptibility testing is performed in public health laboratories. Thus, effective TB control requires a network of public and private laboratories to optimize laboratory testing and the flow of information. Public health laboratory scientists, as a component of the public health sector with a mandate for TB control, should take a leadership role in developing laboratory networks and in facilitating communication among laboratory scientists, clinicians, and TB Controllers.

Elements. Seven types of tests for the diagnosis of TB disease and detection of drug resistance performed within the tuberculosis laboratory system are recommended for optimal TB control services. These laboratory tests (listed below with their ideal turnaround times) should be available to every clinician involved in TB diagnosis and management and to jurisdictional public health agencies charged with TB control:
I) Nucleic acid amplification test, detection of TB (1 day); II) Nucleic acid amplification test, TB drug resistance markers (1 to 2 days); III) AFB

microscopy (1 day); IV) Growth detection - average 10-14 days (up to 6-8 weeks); V) Identification of *Mycobacterium tuberculosis* complex (1 day* [* after detection of growth]); VI) First-line drug susceptibility testing - liquid medium (1 to 2 weeks*); VII) Second-line and novel compound drug susceptibility testing – liquid medium (1 to 2 weeks*) or agar/egg-based medium (3 to 4 weeks*).

Take home message. Clinicians, public health officials and laboratory scientists (clinical and public health) must work together to develop an integrated system that ensures optimal selection of assays, timely laboratory testing and seamless flow of information among clinicians, TB Controllers and laboratory scientists.

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ECOLOGY OF MYCOBACTERIA

Ivo Pavlik

Veterinary Research Institute, Brno, Czech Republic
Contact: pavlik@vri.cz

The incidence of the disease in men and animals caused by potentially pathogenic or environmentally saprophytic mycobacteria is worldwide increasing. In human population especially people with impaired immunity are affected. In farm animals infections caused by non-tuberculous mycobacteria may often result in complications in intravital and post mortal diagnosis of bovine and/or avian tuberculosis. Those infections are then often incorrectly diagnosed which could have a great negative impact on health, economy and animal breeding. In human patients mycobacterioses are considered as "undiagnosed" infections and in these cases is the treatment long and difficult. Therefore the objective of this presentation is to summarize data from literature and our own experience concerning the occurrence of tuberculous and non-tuberculous mycobacteria in environment. All the presented information are available in the book "The Ecology of Mycobacteria: Impact on Animal's and Human's Health" which is divided into 10 chapters, supplemented with more than 100 Tables, 5 figures and 462 photographs in the Chapter 10.

http://www.springer.com/public+health/book/978-1-4020-9412-5
Comprehensive contents:
http://www.vri.cz/userfiles/file/CONTENTS.pdf
Book review:
http://www.vri.cz/userfiles/file/hide/ruzne/book_review_skovgaard.pdf

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GL-10

PARATUBERCULOSIS: AN OVERVIEW

Norma Arrigoni

National Reference Centre for Paratuberculosis, Istituto Zooprofilattico Sperimentale Lombardia and Emilia Romagna, Strada Faggiola 1, Gariga, 29027 Podenzano (PC), Italy
Contact: norma.arrigoni@izsler.it

Paratuberculosis is a chronic intestinal infection, which typically affects domestic and wild ruminants. The agent, *Mycobacterium avium* subsp. *paratuberculosis* (Map) is an extremely slow-growing, acid-fast and mycobactin dependent bacterium. Paratuberculosis was described for the first time in 1895: since then it has spread in most European countries through unruly animal trading, reaching prevalence values above 50% of infected herds. There are several reasons why we should take into account the problem "Paratuberculosis", starting from the significant economic impact on the infected herds ending in the potential zoonotic role of Map, which has been an object of interest by the European Community.

One of the main problems in controlling the disease is the lack of diagnostic tools that match an early response with high sensitivity and specificity. At the moment, none of the available tests can warrantee all these requirements; in fact a diagnostic response isn't reached until at least 3-5 years after infection, which usually happens in the first weeks of life. The use of vaccines is controversial and not allowed in most European countries for its interference with tuberculosis diagnosis. Moreover the control of the infection requires years of work and is based, besides culling the infected animals, on a series of strict hygienic measures that can prevent calf infection. The high resistance of Map allows its persistence in the environment for years and this characteristic can contribute to complicating control of the infection.

Milk and meat can be contaminated, both by dissemination of Map through blood/lymphatic systems and faecal contamination. Due to the resistance of Map to technological treatments (pasteurisation, ripening) and disinfectants, the food chain could be contaminated. The way to reduce this contamination is to act on the only identifiable critical control point, the bovine herd, limiting the prevalence of infected animals. With this objective, several countries have implemented measures that include control, surveillance and certification.

GL-11

MYCOBACTERIUM AVIUM SUBSPECIES *PARATUBERCULOSIS* AND CROHN'S DISEASE

Tim Bull

George's Hospital Medical School, Department of Cellular and Molecular Medicine, Cranmer Terrace, London SW17 ORE, UK
Contact: tim.bull@sgul.ac.uk

Crohn's Disease (CD) is an important chronic disabilitating inflammatory disease that is increasing in incidence across the world. It is characterised by lesions that extend throughout the gastrointestinal tract with a majority involving the ileum or upper colon. Recent scientific evidence has shown that CD involves both an underlying genetic susceptibility and an infectious aetiology. The similarities of CD to Johne's Disease (JD) in animals caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has led to the hypothesis that this proven animal pathogen could also be involved in CD. JD is widespread with a prevalence of MAP infection ranges from 26%-70% in Europe and 63%-95% in North America depending on herd size. Extensive reservoirs of MAP in wildlife are also present including deer, bison, elk, rabbits and their predators. Infected animals excrete huge numbers of MAP onto pastures and MAP can survive for extended periods in the environment and water reservoirs thereby increasing the associated risk of human exposure. MAP can survive pasteurization and has been cultured from retail milk and other human foodstuffs. It has been detected by PCR in up to 47% of normal human intestinal samples, up to 90% of samples from patients with CD and cultured from both human blood and intestinal biopsies. As ethical restrictions forbid human challenge studies, conclusive proof that MAP is causal has previously been considered an unsolvable dilemma. However recent advances in technology, a more complete understanding of immunological mechanisms in CD and mycobacterial mechanisms of pathogenesis have enabled study designs that are confronting this dogma.

This presentation will summarize the crucial developments and insights now being revealed in this field. It will show that consistent with the increased exposure of humans to MAP from animals and the environment, MAP is present, unequivocally viable and persistent in both Normal human controls and with a significantly increased strength of association in CD patients. It will show that chronic persistence of MAP in humans is associated with a specific phenotype and that MAP from humans are virulent in animals. It will show that CD patients have MAP specific immunological reactivities that are fully consistent with our knowledge of immune dysregulations underlying inflammatory bowel disease. It will show that JD and CD have related genetic susceptibilities that are indicative of defective resistance to mycobacteria and that these defects can preferentially favour MAP pathogenesis. It will also show

that therapy which includes anti-MAP activity can lead to improvement of CD. In summary, this presentation will demonstrate that current works suggest MAP to be a credible candidate as an emerging human pathogen. However, any acceptance of MAP as a true zoonotic agent should not be taken up lightly as it will have wide reaching consequences in many fields including domestic and wild animal welfare, sustainable agriculture, economic planning of infection control policies, food and environmental safety, clinical approaches to CD therapy and possible legal complications.

GL-12

BIOSAFETY IN TB LABORATORIES

Véronique Vincent

UNICEF, UNDP, World Bank, WHO Special Programme for Research and Training in Tropical Diseases, Geneva
Contact: vincentv@who.int

While TB laboratories have suffered years of neglect, biosafety has even more so. Because of the increased incidence of MDR-TB and the emergence of XDR-TB, laboratories have to handle many dangerous strains. Biosafety conditions should therefore be optimal in laboratories to prevent occupationally acquired TB among laboratory staff. Laboratory acquired TB infections are poorly documented. A retrospective study led in Korea (IJTLD 2007; 11: 138-142) showed that the relative risk of TB compared to the general population was found to be 1.4 in microscopists and 21.5 among technicians carrying out drug-susceptibility testing.

Biosafety in laboratories includes three components: 1) facility layout; 2) adequate equipment regarding specifications and maintenance, and 3) work practice. It is generally admitted that good laboratory practice in laboratories associated with good microbiological techniques are essential in minimizing the generation of infectious aerosols. The WHO laboratory safety manual provides guidance on the basic concepts prevailing in biosafety for the development of national codes of practice. However, recommendations should be detailed and tailored to specific laboratory activities for a more efficient support to countries for the implementation or renovation of laboratories adequate for TB activities. As an example, most BSL3 have been implemented in countries (Northern America, Europe) where norms are well beyond the WHO minimal standards. The present situation contributes to create confusion on the really needed requirements for the design of BSL2 or BSL3. Some experts refer to BSL2 plus or BSL3 plus, whereas these terms are not documented; consultant recommendations may widely differ for a same setting.

Following a CDC/WHO technical consultation held in Atlanta in September 2008 on strategies, approaches, and partnerships for improving global laboratory biosafety, an expert meeting convened in WHO, Geneva in April 2009 to elaborate guidance on biosafety related to TB laboratory diagnostic procedures. The purpose of the meeting was to define consensus on interim guidance on basic principles of laboratory design necessary to assure minimum standards for biosafety capability for TB microscopy, culture, drug susceptibility testing (DST), and molecular testing in different countries and epidemiological settings. Experts decided to specify the minimum standards for TB facilities without assigning a biosafety level 1, 2 or 3, according to the risk-based approach recommended in the CWA 15793:2008 laboratory biorisk management standard. The objective is to define the minimum requirements for laboratories ensuring aerosol protection for each technique used for TB diagnosis.

GL-13

UNIVERSAL GENOTYPING OF *M. TUBERCULOSIS* IN THE UNITED STATES

Cowan Lauren S

Division of TB Elimination, Centers for Disease Control and Prevention, MSF08, 1600 Clifton Road, Atlanta, GA 30333, U.S.A.
Contact: LCowan@cdc.gov

The National TB Genotyping Service was established in October 2003 to provide universal genotyping services to TB control programs in the United States. The service allows for the genotyping of one isolate from each culture positive TB patient using spoligotyping and 12-locus MIRU-VNTR typing as the primary typing methods and IS*6110*-RFLP as a secondary typing method as requested. In April 2009, the service was upgraded to include 24-locus MIRU-VNTR. Two laboratories located at the California Department of Health and the Michigan Department of Community Health are each contracted by the CDC to genotype up to 5,000 isolates each year, and to date, 56,757 isolates have been genotyped. Each state TB control program independently determines their utilization of the service; currently all 50 states, the District of Columbia, Puerto Rico, and U.S. affiliated areas in the Pacific Islands submit isolates (53 programs). For cases reported in 2008, 30 programs linked >85% of their surveillance records to a genotype result.

In response to needs of local TB control programs, the TB Genotyping Information Management System (TB-GIMS) was introduced in March, 2010. TB-GIMS is an on-line database that links genotyping data to information reported to the National TB Surveillance System. The database contains 43,605 patient records linked to genotype results. Surveillance data includes sex, race, ethnicity, age, geographical data (zip code), country of origin, length of time in the U.S., results of drug susceptibility tests, type of TB, and risk factors such as homelessness, incarceration history and reported substance abuse along with many other variables. TB-GIMS provides reports that allow local users to view the distribution of a genotype across a county, state or national map; to compare the incidence of a genotype in their jurisdiction to the rest of the nation, and to compare the demographic characteristics of patients in a genotype cluster in their jurisdiction with the rest of the nation. Programs are being provided with tools and training on how to prioritize clusters for further investigation.

Our database provides insight into strains circulating in the U.S. as well as other countries. 25,795 of the records in TB-GIMS represent foreign born patients from 190 countries. To facilitate studies of the diversity of *M. tuberculosis* complex strains from TB patients in the U.S., genotypes are conservatively assigned to lineages based on both genotype results.

GL-14

IGRA TESTS: WHERE ARE WE TODAY?

Jean-Pierre Zellweger

Swiss Lung Association, Berne, Switzerland
Contact: zellvegerjp@swissonline.ch

The Interferon Gamma Release Assays (IGRAs) are *in vitro* tests detecting the presence of latent tuberculosis infection (LTBI) in asymptomatic persons who may have been infected by *M. tbc* in a recent or remote past and who may benefit from a preventive treatment to decrease the risk of later reactivation of tuberculosis[1,2].

Basically, the IGRA tests rely on the same immunological phenomenon as the tuberculin skin tests, namely the detection of Interferon-Gamma Release from T lymphocytes sensitized by a prior contact with antigens from *M. tbc* but they do it in a much more specific way, because the tests are not influenced by a prior vaccination with BGC or by an infection with most of the non-tuberculous mycobacteria present in the environment. Therefore, the indications and the use of the IGRA tests are fundamentally the same as for the tuberculin skin tests[3]:

1. Detection of LTBI in persons in contact with an index case of tuberculosis.
2. Detection of LTBI in persons with a high risk of tuberculosis, if infected (immunosuppressed patients, patients receiveing or due to receive immunosuppressive therapy, small children).
3. Surveillance of exposed health care workers (as the test can be repeated without risk of inducing a booster effect).
4. Aid to the diagnosis of tuberculosis in cases where a bacteriological examination is not feasible or not reliable (severe extrapulmonary TB, TB in children).

The level of IGRA response seems to be proportional to the intensity of exposure tu tuberculosis, and may change under preventive or curative treatment, possibly indicating that the number of living mycobacteria has also decreased. It is therefore possible that the change in the level of IGRA reflects the effect of treatment, but there is unfortunately up to now no solid proof that a decrease in the level is clearly correlated with the eradication of mycobacteria and could be used as a documentation of cure[4]. Furthermore, there is no proof that a positive test result always documents the persistence of living mycobacteria. It may be that the test only detects a lasting immune response to a prior contact with mycobacteria that have disappeared in between[5].

IGRAs may contribute to the diagnosis of difficult TB cases. In smear-negative pulmonary tuberculosis and in extrapulmonary tuberculosis, it may be very

difficult to obtain the bacteriological documentation of the presence of mycobacteria. The release of interferon-gamma from lymphocytes isolated from the organs potentially involved in cases of suspect tuberculosis (BAL or induced sputum in smear-negative pulmonary tuberculosis, CSF in tuberculous meningitis, peritoneal fluid in abdominal tuberculosis) can be measured and is elevated in cases with a final diagnosis of tuberculosis. In such cases, the determination of the level of Interferon-Gamma release can be used as an aid to the diagnosis of tuberculosis[6].

IGRAs may predict the future development of TB disease. Not all cases in contact with a case of tuberculosis will be infected and not all infected contacts will develop tuberculosis. The risk of reactivation of tuberculosis is estimated to be 10% for contacts with a positive tuberculin skin test. Recent studies have demonstrated that the risk of future reactivation is higher in contacts with a positive IGRA test than in negative contacts, independently from the result of the tuberculin skin test[7]. Therefore, a positive IGRA test result may have a higher predictive value for the risk of future reactivation than the tuberculin skin test. As the proportion of contacts with a positive IGRA test is lower than the proportion of contacts with a positive tuberculin skin test, the number of contacts considered as infected and who may benefit from a preventive treatment is lower if IGRA are used as a definition. Using IGRA for the detection of infected contacts and selection of contacts in need of a preventive treatment has therefore a sparing effect[8].

In spite of their superiority, the IGRAs are not totally devoid of problems in practice and the best use of them is still a matter of debate. The intra-observer and inter-observer variability are low, but the level of response may vary if the test is repeated and there are spontaneous conversions and reversions in the absence of exposure or treatment[9-11]. Most of the changes are observed for cases with borderline responses. Therefore, proposals have been made for an increase in the cut-off for positivity and for the definition of conversion in repeated testing[12].

Some Guidelines recommend the use of IGRAs only for the confirmation of positive TST among contacts (the so-called two-step testing procedure) whereas others recommend the routine replacement of the TST by IGRAs. Performing only one test is easier, and avoids a possible influence of a prior TST on the IGRA response.

There is no doubt that the IGRAs are a very useful tool for the detection of tuberculosis infection. More time and studies are needed for defining the precise indication and limits of the available tests, remembering that we have used the imprecise tuberculin skin test for over 100 years and have taken number of sound decisions based on it, and the IGRA are far better than the tuberculin skin test. The art of medicine is somehow the art of taking correct decisions based on a unperfect test.

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ABSTRACTS OF ORAL PRESENTATIONS (OP)

TUBERCULOSIS OF BONES AND JOINTS

Ludvik Travnik

Clinical Center Ljubljana, Orthopedic Clinic, Department of Orthopedic Surgery, Zaloška 9, SI-1000 Ljubljana, Slovenia
Contact: ludvik.travnik@kclj.si

Tuberculosis has been reported in all bones of the body. Bone and joint tuberculosis may account for up to 35 percent of cases of extrapulmonary tuberculosis. Tuberculosis of bones and joints often presents as gradually worsening arthritis. Skeletal tuberculosis most often involves the spine, followed by tuberculous arthritis in weight-bearing joints and extraspinal tuberculous osteomyelitis.

Spinal tuberculosis most commonly involves the thoracic spine. Infection begins in the anteroinferior aspect of the vertebral body with destruction of the intervertebral disc and adjacent vertebrae. The resulting anterior wedging and angulation of adjacent vertebral bodies with disc space obliteration are responsible for the palpable spinal prominence (gibbus) and a classic radiographic appearance. Paraspinal and psoas abscesses can develop, with extensions to the surface or adjacent tissues. Patients present local pain, constitutional symptoms or paraplegia secondary to cord compression.

Articular tuberculosis is a slowly progressive mono-arthritis of the hip or knee. Presentation is indolent with pain, joint swelling, and decreased range of motion. Draining sinuses and abscesses are seen in chronic cases. Systemic symptoms usually are absent. Radiographic changes are nonspecific and include soft tissue swelling, juxta-articular osteopenia, joint space narrowing, and subchondral erosions. Extraspinal tuberculous osteomyelitis often presents with local pain and can involve any bone. Involvement of adjacent structures may result in complications such as carpal tunnel syndrome, tendosynovitis, and facial palsy.

Chest radiography shows pulmonary disease in one half of patients with osteoarticular tuberculosis, but active pulmonary disease is uncommon. Magnetic resonance imaging is very helpful to assess the degree of bony destruction and to identify soft tissue extension and encroachment on adjacent structures such as the spinal cord.

In the period 2000 – 2008 48 patients with skeletal tuberculosis were detected in Slovenia representing 1.86% of all tuberculosis patients registered at Slovenian Registry for Tuberculosis Golnik. Twenty-four of them (50%) had skeletal and pulmonary tuberculosis as well.

Different cases of Slovenian patients with tuberculosis of bones and joints will be exposed during our presentation.

OP-2

AUTOMATED REP-PCR BASED TYPING OF NONTUBERCULOUS MYCOBACTERIA

Jakko van Ingen[1,2], Rina de Zwaan[1], Dick van Soolingen[1]

[1]National Mycobacteria Reference Laboratory, National Institute for Public Health and the Environment (RIVM;LIS, pb22), PO Box 1, 3720BA Bilthoven, the Netherlands
[2]Dept. of Pulmonary Diseases, Radboud University Nijmegen Medical Center, PO Box 9101, 6500HB Nijmegen, the Netherlands
Contact: J. van Ingen, RIVM;LIS(pb22), PO Box 1, 3720 Bilthoven, the Netherlands, Jakko.van.ingen@rivm.nl

Nontuberculous mycobacteria are omnipresent in our environment. Hence, before diagnosing nontuberculous mycobacterial disease in patients, environmental or laboratory contamination needs to be ruled out. If laboratory contamination is likely, however, few typing tools are available for nontuberculous mycobacteria. A recent addition to this field is an automated repetitive sequence (rep)-PCR based system, DiversiLab™ (Bacterial Barcodes, USA) with a specific assay for nontuberculous mycobacteria. To test this system, we have typed isolates from several outbreak and contamination events.

We selected all strains from five separate outbreak investigations. Outbreak 1 was a likely laboratory cross-contamination with *Mycobacterium avium*. Outbreak 2 was a similar cross-contamination event caused by an *M. simiae* strain. Outbreak 3, 4 and 5 were small outbreaks of *M. avium* lymphadenitis in slaughter pigs on three separate farms. From all strains, DNA was isolated by the CTAB method and subjected to rep-PCR according to the manufacturer's prescriptions. For the *M. avium* isolates, typing results were compared to available IS*1245* Restriction Fragment Length Polymorphism (RFLP) typing results.

Outbreak 1 proved to consist of two distinct groups of *M. avium* isolates and thus two contamination events; these results were identical by rep-PCR and IS*1245* RFLP. In outbreak 2, the outbreak strains all revealed identical rep-PCR patterns; unrelated strains from the same laboratory revealed distinct patterns. The inclusion of epidemiologically unrelated *M. simiae* isolates from other regions confused results, as part of these "negative controls" in fact yielded rep-PCR patterns identical to the outbreak strain. Outbreaks 3 to 5 were all *M. avium* strains; most had unique rep-PCR and IS*1245* pattern, although small clusters were noted by both methods. Outbreak 5 was caused by "bird-type" *M. avium* strains, which could be further typed by rep-PCR but not IS*1245* RFLP.

The automated rep-PCR system provides an interesting tool, especially for clinical and veterinary laboratories. For *M. avium* typing, the resolution equals that of IS*1245* RFLP typing and exceeds it in "bird-type" *M. avium*. For its interpretation, an assessment of variability within species is needed and epidemiological data should be considered leading. Until variability data is available, the power is mainly in excluding strains from outbreaks, *i.e.* its specificity.

OP-3

REPRODUCTIVE FITNESS OF DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* TRANSMITTED AMONG MEMBERS OF DIFFERENT FAMILIES

Nora Morcillo[1], Belen Imperiale[1,2]

[1]Reference Laboratory of Tuberculosis Control Program of Buenos Aires Province, Dr. Cetrangolo Hospital, Italia 1750 (1602) Vicente López, Buenos Aires
[2]National Council of Scientific and Technical Research, Rivadavia 1917, CABA, Argentina
Contact: nora_morcillo@yahoo.com.ar

Physiological fitness has been defined as the ability of a microorganism to survive, reproduce and be transmitted. Mutations in *M. tuberculosis* genome leading to a drug-resistant profile compatible with resistance to isoniazid and rifampicin (MDR) may influence the fitness of mycobacteria and are associated to tuberculosis (TB) treatment failure. *In vitro* growth can be used as a measure of fitness. Variations in fitness can be also used as indicators to study the transmission capability of MDR-TB among close contacts. The aims of this study were to determine the difference in growth velocity of several clinical isolates transmitted among members of different families; to establish the relationship between mutations related to drug-resistance and genetic patterns with the *in vitro* fitness of these strains and with elements given by conventional epidemiology. A total of 101 clinical isolates from 68 members of 24 different families were included in the study. TB episodes occurred in a 12 years long period. For these cases, clinical and epidemiological data as well as drug-susceptibility and genetic patterns of the isolates were obtained. MDR was also analyzed by molecular methods. For fitness experiments, previously stored strains were cultured on fresh Middlebrook 7H9 media enriched by OADC (M7H9). A volume of 0.5 ml of a dilution 1:500 from N°1 McFarland suspension, was added to a tube of the BACTEC MGIT 960 system (BD, Argentina), incubated and continuously monitored for increasing of fluorescence. The software Epicenter with modifications was used to detect the changes in fluorescence registered as growth units (GU). The length of lag phase (t_0), the exponential growth time, and the relative fitness (RF) compared to H37Rv reference strain were calculated. MDR was found in 27 patients from 15 families; 17 patients generated 32 secondary cases (range 1-4) with identical genetic patterns. Generally, t_0 was longer in MDR isolates; RF was decreasing as secondary cases appeared. Mutations in positions 531 and 526 of the *rpoB* gene were found in 6 and 16 patients, 2 and 8 of the secondary cases generated respectively from the index cases. Drug-resistant strains had diminished RF compared to their ancestors. Mutations in *katG* 315 leading to isoniazid resistance were not associated to cost-fitness.

OP-4

CLINICAL AND MICROBIOLOGICAL CHARACTERISTICS OF *NOCARDIA* ISOLATES IN SLOVENIA IN 2004-2009

Katarina Osolnik, Viktorija Tomič, Manca Žolnir-Dovč

University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia
Contact: katarina.osolnik@klinika-golnik.si

Introduction. *Nocardia* species is an uncommon pathogen that affects both immunosuppressed and immunocompetent patients. Nocardiosis is a rare disease that affects mainly patients with deficient cell-mediated immunity, patients with underlying chronic debilitating disease or immunodeficiency. *Nocardia* spp. is responsible for cutaneous, pulmonary and disseminated human infections.
We report the epidemiologic characteristics of *Nocardia* spp. observed in Slovenia and discuss the risk factors, clinical features, diagnosis, and management of the disease.

Materials and methods. We retrospectively reviewed the laboratory records of the laboratories of University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia, from January 2004 to December 2009 to identify patients with nocardiosis. We reviewed the clinical records of all patients with *Nocardia* spp. isolated from clinical specimens.

Results. During the study period, 38 isolates (36 from sputum, 1 from faeces, 1 from tracheal aspirate) of *Nocardia* spp. were identified from 30 patients (18M, 12F), but only 4 (2M, 2F) patients had *Nocardia* infection, all the others were considered to be colonized. The species identified were: *N. asteroides* in 70% pts, *N. nova* in 16.6% pts, *N. brevicatena* in 6.6% pts and *Nocardia* spp. in 6.6% pts.

In University Clinic of Respiratory and Allergic Diseases Golnik we treated 21 of 30 patients (12 M, 9F), av. age (70.7 y). The most common underlying condition in our institution was chronic obstructive pulmonary disease (COPD) (13 pts 61.9 %), followed by diabetes melitus II (6 patients 28.5 %), post tuberculosis changes in the lung (5 patients 23.8%), bronchiectases (5 patients 23.8%), autoimmune diseases (4 patients 19 %) and cancer (2 patients 9.5 %).
According to patients' medical history before the isolation of *Nocardia* spp., 10 of 21 patients (47.6%) were treated with corticosteroids: 7/10 patients (70%) with inhaled corticosteroids. Their main symptome was cough.
Fatigue, fever, cough, dyspnoea and loss of weight were main symptoms in 4 patients with nocardia infection who were treated successfully with trimetoprim-sulphametoxasol for a 6 month to one year.
Chest X-ray or CT imaging of the lungs showed masses in 3, infiltrates in 4 and nodular lesions in 1 case. They were all treated previously with inhaled corticosteroids.

Conclusions. In our hospital most patients with *Nocardia* spp. isolated from sputum had pre-existing pulmonary disease and a substantial part of them was treated with corticosteroids. Recognition of nocardia infection is important for the choice of appropriate antibiotic treatment. Due to its nontypical clinical manifestations especially in immunocompromised patients it can easily be missed.

OP-5

PERFORMANCE OF THE GENOTYPE® MTBDR*sl* ASSAY IN A HIGH TB DRUG RESISTANCE SETTING

Nikolayevskyy V[1], Balabanova Y[1,2], Kontsevaya I[2], Mironova S[2], Mitchell S[1], Chinkova Y[2], Ignatyeva O[2], Fedorin I[2], Drobniewski F[1]

[1]National *Mycobacterium* Reference Laboratory, Institute of Cell and Molecular Sciences, Queen Mary College, Barts and the London School of Medicine, University of London, 2 Newark street, E1 2AT, London, UK
[2]Samara Oblast Tuberculosis Dispensary, 154 Novo-Sadovaya street, 443068, Samara, Russia
Contact: v.nikolayevskyy@qmul.ac.uk

The Russian Federation is a high tuberculosis (TB) burden country with high rates of TB drug resistance and dominance of Beijing strains reported to be associated with multidrug resistant TB (MDRTB). Increasing MDRTB and XDRTB rates require development and implementation of rapid diagnostic systems for detection of resistance to second-line drugs which is essential for the administration of effective drug regimens and implementation of appropriate infection control measures limiting the spread of TB.

A total of 51 individual MDR *M. tuberculosis* strains isolated from new (N=23) and previously treated (N=28) patients in Samara, Russian Federation was tested for resistance to fluoroquinolones (FQ) using phenotypical culture (BACTEC MGIT960) methods and a set of molecular methods, including HAIN GenoType® MTBDR*sl*, pyrosequencing and conventional sequencing of quinolone-resistance determining regions (QRDR) of *gyrA* and *gyrB* genes.

Phenotypical resistance to moxifloxacin and ofloxacin was detected in 32 (62.7%) strains; 28 of these (87.5%) had mutations in *gyrA* QRDR correctly identified by GenoType® MTBDR*sl* and other molecular methods and one more strain had a mutation (1453C→T) in the *gyrB* gene detected by sequencing only. The most prevalent mutations in the *gyrA* gene included D94G (52.6%) followed by A90V (17.9%) and D94N (14.3%). Four strains displayed "mixed" genotype on sequencing and GenoType® MTBDR*sl* methods (simultaneous presence of bands indicating both wild type and mutations on the membrane) probably indicating mixed cultures.

GenoType® MTBDR*sl* assay is a useful tool in rapid detection of resistance to FQ in high TB drug resistance settings with the sensitivity and specificity values of 87.5% and 100.0% respectively. Inclusion of probes capable of detection of less common mutations in the *gyrB* QRDR region could improve the sensitivity of the assay.

OP-6

COMBINATION OF A CASSETTE BASED FULLY AUTOMATED DNA-ISOLATION DEVICE WITH PCR BASED DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX AND FIRST AND SECOND LINE DRUG SUSCEPTIBILITY

Michael Weizenegger[1], Vera Allerheiligen[2], Roger Lovlie[3]

[1]Laboratory Group Limbach, Dept. of Microbiology and Molecular Genetic, Im Breitspiel 15. D-69126 Heidelberg, Germany
[2]Hain Lifescience GmbH, Hardwiesenstrasse 1, D-72147 Nehren, Germany
[3]NorDiag ASA, Frysjaveien 40, N-0884 Oslo
Contact: weizenegger@labor-limbache.de

Introduction. Rapid *Mycobacterium tuberculosis* (MTB) complex detection and drug susceptibility testing (DST) is available by nucleic acid based amplification assays like the GenoQuick MTB, GenoType MTBDR*plus* and GenoType MTBDR*sl* (Hain Lifescience, Nehren, Germany).

Material and methods. We evaluated a prototype of a newly designed version of the GenoType MTBDR*plus* with a higher sensitivity and a PCR-dipstick based MTB detection assay (GenoQuick MTB). The DNA isolation was done with an optimized automated procedure from NALC decontaminated specimens (GenoXtract, Hain Lifescience). Results were compared to culture (Bactec MIGIT 960, Becton Dickinson, Germany, GenoType *Mycobacterium* CM, Hain Lifescience) and to the present version of the MTBDR*plus* and MTBDR*sl*.
51 NALC decontaminated pulmonary (46) and extrapulmonary (5) specimens (35 smear negative, 15 smear positive, 1 no smear result) were investigated. 17 cultures were MTB positive, 2 were positive for *Mycobacterium intracellulare* and 32 were negative.

Results. All four different DNA based assays had congruent results. 17 MTB culture confirmed (all susceptible to antibiotic drugs, so far investigated) samples were positive for MTB in the DNA based assays. In one sample with a *Mycobacterium intracellulare* culture isolate all PCR assays had a MTB result. One smear positive sample with previous MTB results was only positive with the DNA amplification assays.

Conclusion. The new developed fully automated procedure for mycobacterial DNA isolation from decontaminated specimens is highly efficient. The four different Hain assays for detection of MTB and molecular DST resulted in excellent congruence. The newly designed prototype of the GenoType MTBDR*plus* showed better clear cut results in comparison to the present version. Further studies have to be initiated to prove these findings regarding sensitivity values for smear negative culture MTB positive specimens.

OP-7

MUTATIONS IN A TRANSCRIPTIONAL ACTIVATOR OF *MYCOBACTERIUM TUBERCULOSIS* LEAD TO CROSS RESISTANCE OF KANAMYCIN AND STREPTOMYCIN

Zaunbrecher Analise M[1], Campbell Patricia JH[1], Sultana Razvan[2], Murray Megan[2], Posey James E[1]

[1]Division of TB Elimination, Centers for Disease Control and Prevention, 1600 Clifton Rd, M/S F08, Atlanta, GA 30333 USA
[2]Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA
Contact: jposey@cdc.gov

Since the discovery of streptomycin's (SM) bactericidal activity against *Mycobacterium tuberculosis* (MTB) in 1944, aminoglycosides have been utilized to treat TB. Today, the aminoglycosides kanamycin (KAN) and amikacin are used to treat MDR TB, and resistance to one or both of these drugs is a defining characteristic of extensively drug-resistant (XDR) TB. Resistance to both SM and KAN occurs at two phenotypic levels. Mutations in either the *rrs* or *rpsL* loci lead to high level SM resistance (MIC>10 µg/ml), while low level resistance (2µg/ml<MIC<10µg/ml) is conferred by mutations in *gidB*. High level KAN resistance (MIC>80µg/ml) has been attributed to mutations in the 16S rRNA gene, *rrs*, while low level resistance (MIC<80µg/mL) is due to promoter mutations in the aminoglycoside acetyltransferase, *eis*.

By utilizing whole genome sequencing of the cross resistant spontaneous mutant, K301, we mapped the mutation in this strain to the promoter region of the transcriptional activator, *whiB7*, whose regulon is involved in intrinsic antibiotic resistance of MTB. We have identified six unique point mutations in the *whiB7* promoter that lead to a 70 to 100 fold increase in *whiB7* transcript. Using qRT-PCR we have shown that this increase in *whiB7* transcripts leads to the upregulation of both *eis* and the *tap*(Rv1258c) efflux pump.

Although resistance to KAN and SM is typically thought to be due to separate mechanisms, we have isolated single-step spontaneous mutants that demonstrate low level cross-resistance to both aminoglycosides. Our data concludes that the upregulation of eis confers KAN resistance in these mutants, and the increased expression of the *tap* efflux pump is a previously uncharacterized mechanism of SM resistance. As drug resistant TB cases continue to develop and spread, understanding the mechanisms and molecular basis of antibiotic resistance will be valuable for rapid diagnosis of drug resistant strains and ultimately how treatment is designed for MDR- and XDR-TB cases.

OP-8

MUTATIONAL AND PHYSIOLOGICAL DYNAMIC OF DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

Machado D[1,2], Couto I[1,3], Rodrigues L[1,2], Amaral L[1,2,4], Viveiros M[1,4]

[1]Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal
[2]UPMM (IHMT, UNL)
[3]Centro de Recursos Microbiológicos (CREM), UNL
[4]COST ACTION BM0701 (ATENS)
Contact: dianamachado@ihmt.unl.pt

Multidrug resistant tuberculosis (MDRTB) is caused by *M. tuberculosis* strains resistant to isoniazid (INH) and rifampicin (RIF), the main antibacillary used in TB therapy. In this work we intended to understand the mechanism by which MDR develops using four *M. tuberculosis* strains, two strains fully susceptible including the H37Rv reference strain and two clinical, RIF monoresistant strains, exposed to INH and RIF. The cultures were phenotypically characterized by standard antibiotic susceptibility testing and MICs determination for INH and RIF, in the presence/absence of the efflux pump inhibitors verapamil (VP) and thioridazine (TZ), by the BACTEC™ MGIT™ 960 system, equipped with the Epicenter V5.53A software and the TB eXIST module. Genotypic characterization included analysis of specific regions of the *katG*, *inhA* and *rpoB* genes by PCR and reverse hybridization protocols and evaluation of expression level of genes coding for five efflux pumps (*mmpL7*, *mmr*, *efpA*, *tap* and P55) by qRT-PCR. For the antibiotic exposure process, these strains were constantly and independently subjected to the critical concentrations of INH and RIF. Comparison of the cultures exposed to INH showed that after 3 weeks of exposition, all the cultures became resistant to INH, a phenotype reversed by VP to levels below the critical concentration. qRT-PCR of these cultures detected over-expression of all efflux pump genes tested. The H37Rv culture obtained at the end of the INH exposure process presented a deletion in the *katG* gene. When the RIFR strains were exposed to INH, they became MDR. Again, the INH resistance was reverted by VP. qRT-PCR analysis of these cultures detected over-expression of all efflux pump genes tested, with no alterations detected in the other genotypic targets screened. No significant alterations were detected when the strains were exposed to RIF. The calculation of the mutation rates for INH showed a higher mutation rate for the RIFR strains, when compared with the reference strain, showing that the presence of a mutated RNA polymerase may increase the frequency of mutations in other genes, possibly leading to resistance to other antibacillary drugs. Overall, these results illustrate different strategies by which *M. tuberculosis* strains respond when exposed to clinically relevant concentrations of the same antibiotic, all of which may ultimately result in the emergence of MDRTB.

TOWARDS EVIDENCE-BASED BREAKPOINTS FOR DRUG SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* USING WILD-TYPE MINIMUM INHIBITORY CONCENTRATION (MIC) DISTRIBUTIONS

Ängeby KA[1], Jureen P[2], Giske CG[1], Chryssanthou E[1], Sturegård E[3], Nordvall M[4], Johansson AG[4], Werngren J[2], Kahlmeter G[5,6], Hoffner SE[2], Schön T[7]

[1]Clinical Microbiology, MTC – Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden
[2]Department of Bacteriology, Swedish Institute for Infectious Disease Control, Stockholm, Sweden
[3]Department of Clinical Microbiology, Malmö University Hospital, Malmö, Sweden
[4]Department of Clinical and Experimental Medicine, Clinical Microbiology, Linköping University Hospital, Linköping, Sweden
[5]Department of Clinical Microbiology, Växjö Hospital, Växjö, Sweden
[6]Department of Medical Sciences, Uppsala University, Uppsala, Sweden
[7]Department of Clinical Microbiology, Kalmar County Hospital, Kalmar, Sweden
Contact: kristian.angeby@karolinska.se

The currently recommended breakpoints (so-called critical concentrations) used for drug susceptibility testing of *Mycobacterium tuberculosis* (Mtb) are to a large extent based on experience and consensus rather than scientific evidence and for many second-line drugs, no recommended breakpoints have been defined. For other bacterial pathogens it has been shown that the minimum inhibitory concentrations (MIC) of a large number of consecutive strains form a normal distribution (the wild-type MIC distribution) and that strains with MICs above the wild-type cutoff, usually called the epidemiological cutoff (ECOFF), generally have resistance mechanisms.

Using a 96-stick replicator and Middlebrook 7H10 plates with 2-fold serial dilutions (0.002-256 mg/L) of 19 anti-tuberculosis drugs we could define the MICs of 90 consecutive clinical *M. tuberculosis* strains and 21 additional strains with known drug resistance.

M. tuberculosis MICs formed normal distributions (as do the MICs of other bacteria), and the wild-type MICs mostly were clearly separated from the MICs of resistant strains. The obtained ECOFFs in mg/L are presented with the currently by the WHO recommended critical concentrations on Middlebrook 7H10 agar in parenthesis: isoniazid 0.125 (0.2), rifampicin 0.5 (1.0), rifabutin 0.064 (not defined=ND), ethambutol 4.0 (5.0), ciprofloxacin 1.0 (2.0), ofloxacin 1.0 (2.0), moxifloxacin 0.5 (ND), amikacin 1.0 (ND), kanamycin 4.0 (5.0) streptomycin 2.0 (2.0), capreomycin 4.0 (10.0), viomycin 4.0 (ND), ethionamide 2.0 (5.0), protionamide 1.0 (ND), thioacetazone 2 (ND), cykloserine 32 (ND), clofazimin 0.25 (ND), linezolid 0.5 (ND).

The MICs of 12 strains have also been obtained with the MGIT system for rifampicin, isoniazid, ethambutol, amikacin, ofloxacin. These MICs in general did not differ more than one 2-fold dilution step from the corresponding MICs achieved in Middlebrook 7H10 medium.

In conclusion we suggest tentative epidemiological cutoffs for 19 first and second line drugs. These ECOFFs may be used as clinical susceptibility breakpoints provided other investigators can confirm our findings. This approach is likely to improve the reproducibility and accuracy of drug susceptibility testing and thereby the management of patients infected with drug resistant *M. tuberculosis* strains.

OP-10

WILD-TYPE DISTRIBUTIONS OF MICs FOR ANTI-TUBERCULOSIS DRUGS, DNA SEQUENCE AND CROSS-RESISTANCE

Juréen P[1], Ängeby K[2], Engström A[1,8], Sturegård E[3], Chryssanthou E[2], Giske CG[2], Werngren J[1], Nordvall M[4], Johansson A[4], Kahlmeter G[5,6], Hoffner SE[1], Schön T[7]

[1]Swedish Institute for Infectious Disease Control (SMI), Stockholm, Sweden
[2]Clinical Microbiology, MTC – Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden
[3]Medical Microbiology, Department of Laboratory Medicine, Malmö, Lund University, Sweden
[4]Department of Clinical and Experimental Medicine, Clinical Microbiology, Linköping University Hospital, Sweden
[5]Department of Clinical Microbiology, Växjö Hospital, Sweden
[6]Department of Infectious Diseases, Uppsala University, Sweden
[7]Department of Clinical Microbiology, Kalmar County Hospital, Sweden
[8]Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Sweden
Contact: pontus.jureen@smi.se

Minimal inhibitory concentration (MIC) determinations generating wild-type distributions and epidemiological cut offs (ECOFF) have been shown to be a useful tool to detect resistance in several bacterial pathogens. We have used DNA sequencing in combination with the concept of wild-type distributions to establish tentative breakpoints and to clarify cross resistance patterns in *Mycobacterium tuberculosis*. MICs were performed on Middlebrook 7H10 agar using two-fold dilutions of each drug tested (0.002 to 512 mg/l). Sequencing was performed for the mutational hotspot regions of *rpoB*, *katG* and gyrA and the full genes of *rrs* and *tlyA* as well as the *mabA-inhA* region. Sequence correlated well to the wild-type distribution where the majority of strains harboring mutations were either clustered outside or in close proximity of the ECOFF of the wild-type population. For instance, the ofloxacin wild-type strains did not contain any mutations correlated to resistance while all strains harboring the A90V or any mutations at codon 94, were belonging to the non wild-type population. Regarding capreomycin, the wild type distribution (ECOFF 4 mg/l) did not correlate well with the present critical concentration (10mg/l). Presence of mutations at position 1401 in the *rrs* gene correlated well with having a MIC above the tentative ECOFF of 4 mg/l whereas if the presently used critical concentration (10 mg/l) was applied, the majority of strains with the 1401 mutation would be classified as susceptible to capreomycin. MIC distributions correlated well among capreomycin, amikacin and kanamycin, indicating of at least partial cross resistance. In contrast MIC distributions did not show any cross resistance between viomycin and kanamycin/amikacin.

In conclusion, the use of wild type distributions and the adjoined ECOFFs probably is an appropriate way to set susceptibility breakpoints and the usefulness is even more emphasized when comparing MIC distributions to sequence data. Using ECOFFs may clarify variations in standard DST but also inconsistencies between sequencing and DST.

OP-12

AN INTEGRATED APPROACH TO RAPID DIAGNOSIS TUBERCULOSIS AND MULTIDRUG RESISTANCE IN A MIDDLE INCOME COUNTRY

Balabanova Y[1,2], Drobniewski F[1], Nikolayevskyy V[1], Kruuner A[1], Malomanova N[2], Simak T[2], Ilyina N[2], Zakharova S[3], Lebedeva N[3], Alexander H[4,5], O'Brien R[4], Sohn H[4], Shakhmistova A[2], Fedorin I[2]

[1]National *Mycobacterium* Reference Unit, Institute of Cell and Molecular Sciences, Queen Mary College, Barts and the London School of Medicine, University of London, 2 Newark street, E1 2AT, London, UK
[2]Samara Oblast Tuberculosis Dispensary, 154 Novo-Sadovaya street, 443068, Samara, Russia
[3]Samara City Tuberculosis Service, 76 Volskaya str, 443008, Samara, Russia
[4]Foundation for Innovative New Diagnostic, 71, av. Louis-Casaï Case Postale 93, 1216 Cointrin/Geneva, Switzerland
[5]US Centers for Disease Control and Prevention, Division of TB Elimination, 1600 Clifton Road, Mailstop E-10, Atlanta, Georgia, USA, 30333
Contact: y.balabanova@qmul.ac.uk (Balabanova Yanina)

Timely diagnosis and prompt treatment of tuberculosis (TB) and drug resistance are the key elements of the international effort to combat this illness. Although the highest rates of multi-drug resistant tuberculosis (MDR TB) have been reported from the countries of the former Soviet Union, the use of available rapid diagnostics systems in these settings have been limited due to concerns of increased cost and contamination rates relative to conventional methods.

The objective of the study was analysis of the feasibility, cost and performance of rapid tuberculosis molecular and culture systems, in a high MDR TB middle-income region (Samara, Russia) and development of evidence for WHO policy change.

Performance and cost evaluation was conducted to compare the BACTEC™ MGIT™ 960 system for culture and drug susceptibility testing (DST) and molecular systems for TB diagnosis, resistance to isoniazid and rifampin, and MDR TB identification compared to conventional Lowenstein-Jensen cultureassays.

In total 698 consecutive patients (2487 sputum samples) with risk factors for drug-resistant tuberculosis were recruited. Overall *M. tuberculosis* complex culture positivity rates were 31.6% (787/2487) in MGIT and 27.1% (675/2487) in LJ (90.5% and 83.2% for smear-positive specimens). In total, 809 cultures of *M. tuberculosis* complex were isolated by any method. Median time to detection was 14 days for MGIT and 36 days for LJ (10 and 33 days for smear positive specimens) and indirect DST in MGIT took 9 days compared to 21 days on LJ. There was good concordance between DST on LJ and MGIT (96.8% for rifampin and 95.6% for isoniazid). Both molecular hybridization assay results correlated well with MGIT DST results, although molecular assays generally yielded higher rates of resistance (by approximately 3% for both isoniazid and rifampin).

OP-13

MYCOBACTERIAL MICROCOLONY DETECTION AND DRUG SUSCEPTIBILITY TESTING BY AUTOMATED MONITORING OF GROWTH ON SOLID POROUS SUPPORTS

den Hertog AL[1], Visser DW[2], Ingham CJ[3], Fey FHAG[4], Klatser PR[1], Anthony RM[1]

[1]KIT Biomedical Research, Royal Tropical Institute, Meibergdreef 39 1105 AZ Amsterdam
[2]No affiliation; dvisser@tbcol.nl
[3]Microdish BV, Hazenakker 18, 3994 EJ, Houten, The Netherlands
[4]CCM, De Pinckart 24, 3670 AA Nuenen, The Netherlands
Contact: a.d.hertog@kit.nl

Even with the advent of nucleic acid (NA) amplification technologies the culture of mycobacteria for diagnostic and other applications remains of critical importance. Notably microscopic observed drug susceptibility testing (MODS), as opposed to traditional culture on solid media or automated liquid culture, has the potential to both speed up and increase the provision of mycobacterial culture in high burden settings.

We explored the growth of *Mycobacterium tuberculosis* microcolonies on solid porous aluminium oxide supports, imaged by automated digital microscopy. Our microscopy system (Mu-scan, CCM) allows repetitive imaging of a predefined set of fields of each sample, thus enabling monitoring the growth of large numbers of individual microcolonies over time. Critically, this approach greatly simplifies automated image analysis as well as allowing automated presumptive identification of colonies based on their growth rate. In addition, as we culture on solid supports, we can change the media during the growth phase without disrupting the microcolonies.

Thus, colonies can be transferred at any time onto selective media, allowing direct drug susceptibility testing of individual microcolonies within a few bacterial generations. This eliminates the need to either inoculate all samples directly in parallel onto selective and nonselective media, or to re-culture positives on selective media and wait for new microcolonies to form.

We were able to detect microcolonies derived from laboratory cultures of TB within 4-7 days and, after transfer to selective media, susceptibility to RIF was apparent within 1-2 additional days. In a mixed population of RIF susceptible and resistant strains, the phenotype of individual microcolonies could also readily be determined on the basis of their response to RIF exposure within 2 days.

Thus this method, in which the phenotype of individual microcolonies is monitored as they grow, has considerable potential for research, screening, and *M. tuberculosis* diagnostic applications.

OP-14

DEFINING *M. TUBERCULOSIS* RESISTANCE MECHANISMS BY COMPARATIVE GENOMICS – PRACTICAL CONSIDERATIONS AND NEW POSSIBILITIES

Silke Feuerriegel[1], Claudio Köser[2], Susanne Homolka[1], Stefan Niemann[1]

[1]Molecular Mycobacteriolgy Group, Research Center Borstel, Borstel Germany
[2]Department of Genetics, University of Cambridge, Cambridge, UK
Contact: stniemann@yahoo.de

Resistance associated single nucleotide polymorphisms (SNPs) represent powerful markers to detect existing multi-drug resistance (MDR) in clinical *Mycobacterium tuberculosis* complex (MTBC) strains. However, for several drugs resistance mechanisms are not well defined. Here, comparative genomics of susceptible and resistant strains is the classical tool to gain further insights that has become even more powerful following the recent progress in sequencing technologies. However, a valid study design is of outmost importance as the association between particular mutations and phenotypic resistance is not always clear-cut and phylogenetic SNPs represent potential confounders especially when large amounts of sequence data are obtained.

In this work, we have carried out an in-depth investigation of resistance mechanisms for first line (rifampin, ethambutol) and second line drugs (*para*-aminosalicylic). The results obtained, clearly demonstrate that phylogenetic markers are present in confirmed resistance genes and refute candidate genes. In several genes, a high number of SNPs were identified that are likely to be involved in the development of drug resistance. On the other hand, we also found SNPs that are specific for particular phylogenetic lineages such as Beijing or Haarlem. All of these were confirmed by sequence analysis of a reference collection comprising the major phylogenetic lineages of the MTBC.

In conclusion, comparative genomics is a powerful tool to confirm suggested resistance mechanisms and to discover new once. However, phylogenetic markers represent potential confounders and MTBC population structure should be considered when defining new resistance mechanisms by comparative sequencing.

OP-15

APPLIED NANOTECHNOLOGY FOR THE DETECTION OF MYCOBACTERIAL NUCLEIC AND ANTIGENIC TARGETS

Ikonomopoulos J[1], Liandris E[1], Andreadou M[1], Tachtsidis I[2], Gazouli M[3]

[1]Faculty of Animal Science, Laboratory of Anatomy-Physiology, Agricultural University of Athens, Athens, Greece
[2]Department of Medical Physics and Bioengineering, University College London, London, UK
[3]Laboratory of Biology, University of Athens School of Medicine, Athens, Greece
Contact: ikonomop@aua.gr

Despite the considerable progress recorded to this day, undiagnosed tuberculosis continues to be regarded a potent public health threat. The need for sensitive, low-cost diagnostic tests is stressed by many international organizations. To this direction we have recently developed a set of assays incorporating gold nanoparticle (AuNPs) and cadmium selenite (CdSe) quantum dots (QDs) for the detection of non-amplified mycobacterial cells and DNA. AuNPs 20 nm in size were functionalized with oligonucleotide probes designed on the 16S-23S ITS DNA region of *Mycobacterium* spp. The solution containing the functionalized conjugates exhibits a pink color because of surface plasmon resonance at an absorption peak of ~520 nm. The addition of HCl in the absence of target sequence causes absorption peak shift towards a longer wavelength that generates a color change from pink to purple. Notably in the case of positive samples no precipitation is recorded. The minimum detection limit (MDL) of the assay was defined to 18.75 ng of DNA diluted in a sample volume of 10 μl.

The second of the assays that were developed incorporates two biotinylated oligonucleotide probes that were used to recognize and detect specific complementary mycobacterial target DNA through a sandwich hybridization reaction. CDSe QDs conjugated with streptavidin and species specific probes were used to produce fluorescent signal. Magnetic beads (MBs) conjugated with streptavidin and a genus specific probe were used to isolate and concentrate DNA targets. The MDL of the assay was defined at 12.5 ng of DNA diluted in a sample volume of 20 μl.

The method incorporating CdSe was extended to the detection of mycobacterial cells. MB conjugated to polyclonal anti-*Mycobacterium tuberculosis* antibodies were used for the separation and the concentration of BCG cells from different artificially contaminated liquid matrices including PBS, blood and sputum. For the detection of mycobacterial cells, CdSe conjugated with anti-*hsp* 65 *Mycobacterium tuberculosis* monoclonal antibodies was added followed by a secondary anti-mouse QDs conjugated antibody. The presence of bacterial cells in the solution leads to the formation of MB antibody/cell/primary antibody/secondary QDs antibody that fluoresces under UV excitation. Preliminary data indicate that as little as 102 mycobacteria can

be specifically detected in PBS solution through direct observation of the sample on a transilluminator.

The methodology presented here provides the substrate for the development of a technology platform targeting detection of mycobacterial pathogens in a low-cost, highly specific and sensitive manner. The application of these methods can be easily adapted for use at point of care with minimum dependency on trained personnel or dedicated equipment. Most significantly, the combination of the CdSe probes mentioned above allows multi-labelling and multi-target detection directly on clinical samples through a single assay that can be completed within just a few minutes.

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OP-16

SPANISH DATABASE OF ANIMAL MYCOBACTERIOSIS (MYCODB): APPLICATION IN EPIDEMIOLOGICAL STUDIES

de Juan L[1,2], Rodríguez S[1,2], Romero B[1,2], Aranaz A[1,2], Bezos J[1,2], Castellanos E[1,2], González S[1], Sáez JL[3], Mateos A[1,2], Domínguez L[1,2]

[1]VISAVET Health Surveillance Centre. Complutense University of Madrid. Avenida Puerta de Hierro, s/n. 28040 Madrid. Spain
[2]Department of Animal Health. Faculty of Veterinary Medicine. Complutense University of Madrid. Avenida Puerta de Hierro, s/n. 28040 Madrid. Spain
[3]Subdirección General de Sanidad de la Producción Primaria, Dirección General de Recursos Agrícolas y Ganaderos, Ministerio de Medio Ambiente, y Medio Rural y Marino. 28071 Madrid. Spain
Contact: dejuan@visavet.ucm.es

Epidemiological studies based on molecular characterization have allowed a better understanding of several factors as for example transmission between domestic and wildlife animals, animal movement, outbreaks, etc. The Direct Variable Repeat Spacer Oligonucleotide Typing technique or DVR-spoligotyping is based on polymorphism of the chromosomal DR locus, which consists of a variable number of direct repeats (DR) interspersed with nonrepetitive spacers. This technique is specific for bacterial species included in the *M. tuberculosis* complex and nowadays, this technique is the method of choice for epidemiological studies.

Due to the importance of epidemiological studies in tuberculosis together with the Ministry of the Environment and Rural and Marine Affairs (MARM) we designed a Spanish Database of Animal Mycobacterosis (mycoDB) which includes the national isolates of *M. bovis* and *M. caprae* from 1996. The access is restricted to Veterinary Services and Laboratories involved in the National Eradication Program. The access is available at the Veterinary Health Alert Network website (RASVE). The database includes information regarding year of isolation of the strain, animal species and geographical location. All the information is detailed in tables and maps. The database offers three different searches by spoligotype, isolate and maps.

Currently, the database contains 13,731 *M. bovis* strains grouped in 319 spoligotypes, and 919 *M. caprae* strains classified in 15 spoligotypes. During the presentation examples of the application of the database in epidemiological studies will be shown at three levels: 1) identification of outbreaks; 2) transmission between domestic and wildlife animals; and 3) impact of bovine tuberculosis in Public Health.

The Spanish Database of Animal Mycobacterosis (mycoDB) is a useful tool for epidemiological studies at a national and international level since a standardized protocol and nomenclature is used. We encourage all countries to centralize all the typing information in a database for surveillance and epidemiological studies purposes.

OP-17

MOLECULAR ANALYSIS OF *MYCOBACTERIUM BOVIS* AND *MYCOBACTERIUM CAPRAE* ISOLATES FROM PORTUGAL: A SIX-YEAR SURVEY OF BOVINE TUBERCULOSIS

Matos Filipa, Cunha Mónica V, Amado Alice, Botelho Ana

INRB, I.P./LNIV- Laboratório Nacional de Investigação Veterinária
Laboratório de Bacteriologia, Estrada de Benfica 701, 1549-011, Lisboa, Portugal
Contact: monica.cunha@lniv.min-agricultura.pt

Mycobacterium bovis is the main causative agent of bovine tuberculosis (bTB). This zoonotic disease produces important economic losses in livestock farming and is regarded as a threat to endangered animal species and public health. In Portugal, a compulsory National Eradication Plan based on the intradermal tuberculin testing of cattle and the slaughter of reactor animals was introduced in 1992, leading to a substantial reduction of TB herd incidence (0.14 in 2007). However, sporadic outbreaks are still registered, involving a considerable percentage of positive animals, and difficulties subsist in the final eradication steps, persisting indefinitely the low prevalence condition, possibly due to transmission and maintenance in wildlife. One opportunity to reduce this threat is to enhance epidemiological surveillance on animal mycobacteriosis. Therefore, in the present study, we performed the systematic molecular analysis of 482 Portuguese *M. bovis* and *M. caprae* isolates obtained in the last 6 years from 5 animal species: cattle, goat, sheep, wild boar and red deer. Genotyping was based on DVR-spoligotyping and MIRU-VNTR using a panel of 8 previously validated loci. The isolates clustered into 49 spoligotypes that varied largely in frequency, geographical distribution and prevalence in host species; 262 isolates representing 22 spoligotypes could be further discriminated into 118 MIRU types. The most frequent spoligotypes (SB0119 and SB0121) were found all over the country and in different animal species. Spoligotype SB0120 (BCG-like), the third more frequent, is widespread in mainland European countries, suggesting a common ancestor strain. The transmission of TB between wild and domestic animals within restricted geographical areas was also confirmed.

Results from this study emphasize the high genotype diversity of Portuguese isolates compared with other countries and are a contribution to the understanding of *M. bovis* population structure within a European context. In addition, it demonstrates how different strains vary in their epidemiology and transmission routes across diverse geographical regions and host species.

OP-18

DIAGNOSIS OF *MYCOBACTERIUM BOVIS* INFECTION IN BOVINE AND NON-BOVINE SPECIES IN GREAT BRITAIN

Jacky Brewer

Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB United Kingdom
Contact: j.i.brewer@vla.defra.gsi.gov.uk

Mycobacterium bovis (*M. bovis*) is responsible for causing bovine tuberculosis in cattle and other mammals, including humans. During the 1930s a large proportion of dairy cows were infected with *M. bovis* and in 1950 the Government introduced a compulsory test and slaughter policy as well as pasteurisation of milk in order to control the disease. By 1985, this policy had reduced the national incidence of bovine TB (bTB) to a very low level despite a reservoir of infection being identified in badgers, but over the last 15 years the incidence has increased. The test and slaughter policy for cattle is the current control method employed to prevent the spread of disease, together with movement restrictions, pre-movement testing, interferon gamma testing and biosecurity measures.

Tissues from slaughtered cattle are submitted to the Veterinary Laboratories Agency (VLA) at Weybridge for confirmation of the disease. These tissues are processed and inoculated onto and into solid and liquid media. Following incubation the cultures are examined for the presence of *M. bovis*. During 2009, 13,392 bovine tissue samples were examined at VLA, 5119 of which yielded *M. bovis* (37%). Examination of samples from non bovine species for bTB is also carried out at VLA. Samples have been received mainly from alpacas, pigs, deer, sheep, cats, and dogs.

Continued efforts are being made by the British Government to control bTB and to prevent the spread of the disease.

OP-19

A SIMPLE AND INEXPENSIVE LIQUID CULTURE METHOD FOR THE DIAGNOSIS OF BOVINE PARATUBERCULOSIS

Elisabetta Stefani[1], Jacek Gwozdz[2], Michele Gastaldelli[1], Katia Capello[1], Annalucia Tondo[1], Anna Valente[1], Nicola Pozzato[1]

[1]Istituto Zooprofilattico Sperimentale delle Venezie, Verona, Italy
[2]Department of Primary Industries, Attwood, Australia
Contact: npozzato@izsvenezie.it

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiologic agent of paratuberculosis, a chronic, wasting disease of ruminants. MAP infection is characterized by granulomatous enteritis and excretion of large numbers of the organism in faeces. Automated liquid systems for culture of MAP are more sensitive and rapid than culture on solid media, but they are expensive and require specialised equipment. In this study an inexpensive, non-automated culture method using a modified Middlebrook 7H9 liquid medium (7H9+) was compared with the conventional method on Herrold's solid medium (HEYM) and direct Realtime-PCR on feaces for MAP detection in dairy cattle manure. MAP growth in 7H9+ was analyzed weekly by Realtime-PCR until the 12th week post-inoculation. Analytical sensitivity of the three methods was evaluated using faecal samples from a healthy cow spiked with ten fold dilutions of MAP organisms (106-101) and naturally MAP-infected faeces serially diluted 1 to 10 into negative faecal samples. All dilutions were tested in triplicate. The limit of detection (LoD) of the solid culture was 102 MAP/g for spiked samples and 103 MAP/g for naturally infected samples, while the one of direct Realtime-PCR from faeces was 103 MAP/g, both in spiked or naturally infected samples. On the other hand, analytical sensitivity of 7H9+ was increased by almost two logs, revealing as low as 1 MAP/g in both spiked and naturally infected specimens.

In conclusion, the use of 7H9+ for MAP-detecion from cattle feaces resulted far more sensitive than culture on HEYM and could replace solid medium, maximizing diagnostic sensitivity. In addition, we propose a two-step semiquantitative protocol for the assessment of MAP faecal excretion based on: 1) direct Realtime-PCR on all samples; 2) inoculation of negative samples into 7H9+ and analysis after 4 and 8 weeks by Realtime-PCR.

OP-20

EXPLORING THE AETIOLOGY OF CROHN'S DISEASE THROUGH COMPARATIVE GENOMICS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

Wynne James W[1], Seemann Torsten[2], Bulach Dieter M[1], Wagner Josef[3], Kirkwood Carl D[3], Michalski Wojtek P[1]

[1]CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia
[2]Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria, Australia
[3]Enteric Virus Group, Murdoch Childrens Research Institute, Parkville, Victoria, Australia
Contact: james.wynne@csiro.au

Crohn's disease (CD) is classified as an autoimmune disease with unknown, but most likely multifactorial, aetiology. A role for varying microbial and environmental factors, including exposure to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been proposed for many years because of clinical and pathological similarities of CD with Johne's disease (JD), a chronic granulomatous enteritis of ruminants, of which MAP is clearly the causative agent. Although previous studies have demonstrated that extensive genomic variation exists between MAP isolates derived from different animal species, the genomic variation of MAP isolates derived from CD patients remains unknown. To this end we have examined and compared the genomic variation of MAP derived from human CD patients with MAP derived from JD infected animals. Using GAIIx pair-end sequencing we have sequenced the genomes of seven human and four animal derived MAP isolates. Extensive variation observed as single nucleotide polymorphisms, indels and genetic duplication were observed between animal isolates derived from bovine and ovine hosts. Less variation was seen between the human MAP isolates which more closely resembled one bovine isolate type. The ovine isolate appears to show greater similarity, particularly within indels, to *Mycobacterium avium* subsp. *avium* compared to the human or bovine isolates. The biological relevance of these polymorphisms will be presented and their functional consequences on CD discussed.

OP-21

PRACTICAL TRAINING ON GROWTH DETECTION AND DRUG SUSCEPTIBILITY TESTING OF TB IN RESOURCE POOR SETTINGS TO ABSORB NEW DIAGNOSTIC TESTS

Somoskovi Akos[1], Gutierrez Cristina M[1], Paramasivan CN[1], Parsons Linda[2]

[1]Foundation for Innovative New Diagnostics, Geneva, Switzerland
[2]Centers for Disease Control and Prevention, Atlanta GA, USA
Contact: akos.somoskovi@finddiagnostics.org

Background. In 2008, FIND has participated in a review of the WHO TB culture and DST draft training materials. Other invited reviewers included scientists from the Italian WHO TB SRL, India, Indonesia and Thailand, WHO staff and CDC. Following the review, WHO agreed that the draft materials could be used as a basis for developing a more hands-on oriented two week practical course for the African Center for Integrated Laboratory Training (ACILT) in Johannesburg, focusing on methods for resource-poor countries with high burdens of TB and HIV. This two week course has been presented three times, so far, at ACILT for 35 participants from 8 African countries. In January 2010, FIND, together with CDC and the NTRL at Bangkok organized a 10 day training on detection, identification and DST of TB. The training, using ACILT was completed with added modules on DST developed by FIND and CDC. The objective of this workshop was to train TB laboratorians from resource limited settings to perform and interpret quality assured liquid culture, species identification, and DST for the detection of TB and MDR TB. The training curriculum consisted of state of the art lectures in the mornings followed by extensive hands-on practice sessions and lessons on several topics concerning quality assured laboratory practices. Presenting this course as a practical, hands-on workshop is extremely labor-intensive because of biosafety issues, the number of varied supplies required and the equipment, infrastructure and faculty guidance that is needed to provide the participants ample opportunity to safely practice the procedures. The laboratory sessions were organized on a one-on-one basis to enable proper understanding and technical proficiency of the laboratory tests. Participants also engaged in exercises on the basics of biosafety and adequate BSL3 laboratory layout, sputum processing, polymerase chain reaction and DNA hybridization, and how to handle spills in the laboratory. The eleven invited participants were from India, Myanmar, Vietnam and Thailand. Compared to the pre-training KAP (knowledge, attitude and practice) level of the participants, the post-training level showed a significant increase.

This presentation is intending to share the experiences on how to prepare laboratories to conduct and evaluate such a complex field training that is serving the implementation of novel TB diagnostics in resource poor settings.

OP-22

QUALITY CONTROL AND BIOSAFETY IN A TB FACILITY

Danica Šprajcar

University Clinic for Respiratory and Allergic Diseases Golnik
Contact: danica.sprajcar@klinika-golnik.si

Quality control is a set of activities intended to ensure that quality requirements are actually being met. In case of a biosafety emergency, quality control should ensure that with the facilities, equipment, practices and procedures in use the risk of exposure of workers and environment to dangerous pathogens is reduced or prevented.

Quality control is a part of quality management. Quality management includes all activities that an organisation uses to direct, control and coordinate quality. Quality management system enables the organisation to direct and control the implementation of quality policies, and to observe how quality objectives are being achieved.

The ISO 9001 and ISO 15189 standards are process standards. Their use can help every TB facility, including TB laboratory, to provide quality services to patients and ensure health and safety for empoyees and the entire environment.

The following is an example of use of standard demands in practice:
The ISO 15189 standard requires, among other things, that a laboratory have a suitable environment, infrastructure (facilities, laboratory equipment …) and suitable primary samples. It also requires that the laboratory be designed for the efficiency of its operations. These requirements also apply for the primary sample collection.
At the University Clinic Golnik, cough is induced in order to obtain a sample and to confirm or rule out the suspected pulmonary tuberculosis infection. The induction of cough can only be performed in a negative pressure room because this intervention is dangerous for the personnel and other patients.
In order for the negative pressure room to serve its purpose, we have to ensure that it functions without fault. The procedures and responsibilities for regular maintenance of the negative pressure room were determined in order to ensure the optimal protection of patients, visitors and the personnel from possible infection with *M. tuberculosis*.

More examples of practical implementation of the ISO 9001 and ISO 15189 standards' demands will be presented.

OP-23

AEROSOL PRODUCTION AND DISSEMINATION AND IMPLICATIONS FOR PUBLIC HEALTH

Ruth McNerney[1], Ka-Man Lai[2]

[1]Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, WC1E 7HT, UK
[2]Healthy Infrastructure Research Centre, Department of Civil, Environmental & Geomatic Engineering, University College London, London, WC1E 6BT, UK
Contact: Ruth.Mcnerney@lshtm.ac.uk

Transmission of tuberculosis occurs when small droplet nuclei containing viable *M. tuberculosis* are inhaled. The size of droplet carrying the bacteria is crucial in determining transmission events. Larger particles are likely to remain in the upper airways but smaller particles of less than 5 microns can penetrate the fine branches of the lung and deposit bacteria in the alveoli. To prevent the spread of TB it is important to avoid the formation of these fine aerosols.

We have investigated aerosols produced by the vuvuzela, a plastic blowing horn used by soccer fans. The horn requires sustained forceful blowing and the average airflow measured was 3 litres per second. Using a six channel laser counter (Lighthouse Handheld 5016) particles were classified into size categories ranging from 0.5 to 10 microns. Our results indicate that the vuvuzela is an extremely efficient instrument for the production and dissemination of aerosols. The number of particles less than 5 micron in diameter ranged from 177,781 to over 1 million per litre of air expelled. We attribute the creation of fine aerosols to the mode of blowing the horn where air is forced though the lips and into the instrument. Interestingly on average men produced significantly more particles than women when playing the vuvuzela but not during other activities such as shouting. Dissemination of aerosols in high density populations with a high prevalence of both TB and HIV is contrary to international infection control policies.

Our findings suggest that if blown by an infected person vuvuzelas may be deleterious to public health in some settings, including events such as the FIFA World Cup.

OP-24

THE MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN EUROPE

Dick van Soolingen, Anita Schürch, Jessica de Beer, Lilly Verhagen, Jakko van Ingen, Kristin Kremer

National Institute for Public Health and the Environment (RIVM), P.O. BOX 1, 3720 BA Bilthoven, The Netherlands
Contact: Dick.van.soolingen@rivm.nl

In 2009, a new ECDC/RIVM project was started to investigate the international transmission of MDR-TB in Europe. In the previous EU project 85% of the MDR-TB transmissions in the EU were caused by Beijing strains. Moreover, one Beijing cluster was identified comprising 174 cases. Retyping of 136 cases of latter cluster revealed identical (n=124) and highly similar (n=12) VNTR profiles.

To further investigate the phylogeny of the Beijing genotype of *M. tuberculosis*, in total six Beijing strains from China, Vietnam and South Africa were subjected to genome sequencing. High-confidence single nucleotide polymorphisms in non-repetitive regions of the genome were used to construct a phylogeny. The Typical Beijing strains clustered very tightly in the established phylogenetic tree, suggesting they resulted from recent clonal expansion. Typing of 150 Beijing strains from a wide spread area with a subset of the SNPs revealed that 79.6% of all Beijing strains belong to the Typical Beijing sub-lineage. The genomic changes that characterize the Typical Beijing strains were for a large part found in regulatory genes.

Although VNTR typing is gaining recognition as the gold standard for typing of *M. tuberculosis*, international quality control is not well developed. Therefore, 30 DNA samples of *M. tuberculosis* complex strains, of which 10 were duplicates, were sent to 33 international laboratories all over the world. The average inter-laboratory reproducibility amounted 62% and the average intra-laboratory reproducibility was only 72%. These errors visualize a problem in standardization and control.

In the molecular epidemiology, rates of transmission are often attributed to patient risk factors such as age, a smear-positive status, or living in dense settings. However, DNA fingerprint analysis has provided anecdotal evidence suggesting a role for bacteriological factors in TB transmission. To examine this, we investigated the number of tuberculin skin test positive (TST>=10 mm) contacts in relation to the size of the RFLP cluster the source case represented. Larger clusters at the time of diagnosis of the index case were indeed associated with an increased number of TST positive contacts. The mean number of positive contacts ranged from 3.8 for cases in clusters of 2 cases, 4.7 for clusters of 3-10 cases, to 6.0 for cases in clusters of >10 cases.
These results suggest that spread of tuberculosis strongly depends on bacteriological factors.

OP-25

DECIPHERING THE BIOLOGICAL MEANING OF THE IS*6110* INSERTION ELEMENT

Sandoval A[1,7], Reyes A[1,2], Cubillos A[1,7], Ritacco V[4], López B[4], Robledo J[3,7], Samper S[5], Martin C[5], García MJ[6], Zambrano MM[1], Mitra R[2], Del Portillo P[1,7]

1Corpogen, Cra 5 No. 66A-34, Bogotá, Colombia
[2]Washington University, St.Louis, 4444 Forest Pk Av St Louis, USA
[3]CIB, Universidad Pontificia Bolivariana, Cra 72a No. 78B-141, Medellín, Colombia
[4]Instituto Nacional de Enfermedades Infecciosas ANLIS "Carlos G. Malbrán", Velez Sarsfield 563, BA, Argentina
[5]Universidad de Zaragoza, Pedro Cerbuna, 50009 Zaragoza, España
[6]Universidad Autónoma de Madrid, Arzobispo Morcillo 28029, Madrid, España
[7]Consorcio Colombiano de Investigación en Tuberculosis
Contact: pdelportillo@corpogen.org

In addition to the invaluable contribution of the insertion element IS*6110* to the molecular epidemiology of tuberculosis, growing evidence confirms its role in the phenotypic characteristics observed in *M. tuberculosis* strains. IS*6110* can have different phenotypic effects depending on their integration site. When the insertion occurs in intergenic regions it may either increase or decrease expression of adjacent genes. Insertion in the middle of an open reading frame could silence the gene. Moreover, IS*6110* mediates chromosomal deletions and might influence deeply the epidemiological behavior of a strain. Trying to decipher the biological meaning of IS*6110*, we took advantage of the capacity offered by next-generation sequencing technologies to identify IS*6110* insertion sites in more than 500 *M. tuberculosis* genomes, with IS*6110* element copy number ranging between 2 to >25, from representative collections of Colombia, Argentina and Spain. DNA was processed to generate ~200 bp fragments harbouring sequencing primers and sample-specific barcodes. Multiplex PCR was performed and massive parallel sequencing was carried out on the amplified products, using the Illumina platform. Sequence reads were sorted according to barcodes, assembled, and mapped against annotated sequenced genomes. Using this high throughput sequencing approach, we were able to identify more than 7,000 IS*6110* insertion sites that were found to interrupt over 400 genes that represent ~10% of *M. tuberculosis* genome. Thus, in a single experimental setup we substantially increased the survey of naturally occurring mutations and defined novel *in vivo* genetic requirements for *M. tuberculosis*.

Work supported by Colciencias grant 4312004; EC FAST-XDR TB 201690 and EC StopLATENT-TB 200999.

OP-26

THE STUDY OF TRANSMISSION OF MDR-TB IN EUROPE AS INDICATOR OF WHAT HAPPENS IN TB HIGH-ENDEMIC COUNTRIES

Gavín P[1], Iglesias MJ[2], Lezcano MA[1], Jiménez MS[3], Rodríguez-Valín E[4], Martin C, Samper S[1], the Spanish Working Group on MDR-TB

[1]Instituto Aragonés de Ciencias de la Salud, Gómez Laguna 25, Zaragoza, Spain
[2]University of Zaragoza, Medicine Faculty, Department of Microbiology, Preventive Medicine and Public Health, Domingo Miral s/n, Zaragoza, Spain
[3]Mycobacterial Reference Laboratory, National Centre for Microbiology, Majadahonda-Pozuelo km. 2,200, Madrid, Spain
[4]Carlos III Health Institute, Sinesio Delgado 6, 28029 Madrid, Spain
Contact: ssamper.iacs@aragon.es

MDR-TB in low-incidence countries is mainly an imported disease, also in Spain where immigrant population has increased substantially in the last few years. Our research group has conducted a molecular epidemiological surveillance of this severe form of TB since 1998. We aimed to analyse the characteristics of particular clusters related to imported MDR strains.

From a total of 567 MDR isolates studied, we detected 12 clusters suggesting recent transmission of MDR-TB that comprised foreign-born or mixed population. Epidemiological, demographic and molecular data allowed us to distinguish three different patterns:

1. Cases geographically and temporally dispersed carrying a strain that most probably acquired drug resistance and was transmitted in the patients' countries of origin. Tb27 (n=10) and Tb13 (n=4) comprised patients from Equatorial Guinea and Romania.
2. Imported strains that were transmitted as MDR in Spain, the recipient country. This hypothesis was supported by the fact that the strains were isolated in the autochthonous population. Tb22 (n=11) and Tb25 (n=8) grouped patients born in Spain and Morocco.
3. Endemic strains from poor-resource settings that have been described previously in other studies and are still prevalent. Tb23 consisted of two patients from Ethiopia.

The extent and features of MDR-TB in many resource-poor settings still remain widely unknown. Immigration in Spain has changed the population structure of MDR *M. tuberculosis* and, in particular cases, our findings might reflect the situation in countries where very little information is available on recent transmission. The collaborative project carried out in the EU on the "Management of the molecular typing activities of MDR-TB strains at EU level" also has brought valuable information on clusters affecting European countries and other incoming countries.

EVALUATION OF SEQUENCE DATA FOR THE PHYLOGENETIC CLASSIFICATION OF THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

Michaela Projahn[1], Susanne Homolka[1], Sabine Rüsch-Gerdes[2], Roland Diel[3], Stefan Niemann[1]

[1]Molecular Mycobacteriology, Research Center Borstel, Parkallee 1, Borstel, Germany
[2]National Reference Center for Mycobacteria, Borstel, Germany
[3]Department of Pneumology, Medical School Hannover (MHH), Carl-Neuberg-Str.1, Hannover, Germany
Contact: mprojahn@fz-borstel.de

Pathogens of the *Mycobacterium tuberculosis* complex (MTBC) can be classified into several phylogenetic lineages *e.g.* based on MIRU-VNTR (Mycobacterial Interspersed Repetitive Units–Variable Number of Tandem Repeats) typing. Recent studies show that sequence-based tools like single nucleotide polymorphisms (SNP) can also be used for phylogenetic classification of MTBC strains with very high specificity and sufficient discriminatory power. However, an evaluation of SNP markers in a larger set of clinical isolates has not been done so far.

A population based collection of 105 MTBC strains obtained in year 2007 from tuberculosis patients living in Hamburg, Germany, was analyzed to detect and verify phylogenetic informative SNPs in ten genes. In addition, we established and evaluated a high throughput SNP assay based on the ABI SNPlexTM Genotyping System.

Sequence analysis revealed at least one specific SNP for each phylogenetic lineage enclosed in this study. Assignment to genotypes based on SNPs was in full concordance with previous classification based on MIRU-VNTR typing. In addition to known variants, our results reveal further SNPs that can be used for identification of previously not described lineages. To facilitate more effective SNP typing, a high-throughput SNPlexTM genotyping assay comprising 37 phylogenetic SNPs was established. Evaluation of the assay, performed in 340 strains, showed that 29 SNPs were called correctly with high specificity and low failure rates (~1%). The final SNP set used in the assay allows the classification of MTBC strains into 19 phylogenetic lineages.

This study clearly demonstrates the suitability of SNPs for a high resolution and accurate phylogenetic classification of MTBC isolates. The established high-throughput assay can be used for future phylogenetic grouping of MTBC strains *e.g.* for population genetic studies.

OP-28

A NOVEL GENETIC MARKER REVEALS HUMAN TUBERCULOSIS CAUSED BY "ANTELOPE CLADE" BACTERIA

Jakko van Ingen[1,2], Arnout Mulder[1], Roland Brosch[3], Dick van Soolingen[1]

[1]National Mycobacteria Reference Laboratory, National Institute for Public Health and the Environment (RIVM; LIS, pb22), PO Box 1, 3720 BA Bilthoven, the Netherlands
[2]Dept. of Pulmonary Diseases, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB Nijmegen, the Netherlands
[3]Departement Pathogenomique Mycobacterienne Intégrée, Institut Pasteur, Paris, France
Contact: Jakko.van.ingen@rivm.nl

The "antelope clade" *Mycobacterium tuberculosis* complex bacteria (sometimes labelled "*Oryx* bacilli") represent a distinct taxon of the animal-adapted lineage, with an intermediate position between *M. africanum* and *M. bovis*. During a study of *pncA* sequencing, we noted a T112G (aa38 Ser=>Ala) mutation in the Rv2042c gene, immediately upstream from *pncA*, in two of our previously described "antelope clade" isolates. Since we hypothesized that "antelope clade" bacteria could affect humans as a zoonotic disease, we screened our laboratory databases for potential cases of human tuberculosis caused by "antelope clade" bacteria.

We screened our database for strains with IS*6110* RFLP patterns >90% similar to those of our "antelope clade" strains. For these strains, we performed additional spoligotyping, 24 loci MIRU-VNTR typing, Region of Difference (RD) typing and *pncA*/Rv2042c sequencing and retrieved clinical and epidemiological information.

On basis of RFLP patterns, we found 9 clinical strains of human origin, as well as 4 animal strains, not previously recognized as "antelope clade" bacteria. All strains harboured the T112G mutation in Rv2042c. Most strains yielded an ST587 (labelled *M. africanum* in SpolDB4) spoligotype, some showed variations; this spoligotype was also observed in cow and monkey isolates from Bangladesh. The 24 loci MIRU-VNTR pattern of these strains is highly distinct and shares features of both *M. africanum* and *M. bovis*. In RD analysis, the typical pattern of a partial lack of RD5 and complete absence of RD7-RD10. A partial RD12 deletion was noted in all isolates; its borders are under investigation. The human patients were all of South Asian origin and had mostly pulmonary and lymph node tuberculosis. The animal strains were cultured from waterbucks, oryx and gazelles.

The "antelope clade" bacteria can be readily identified by the T112G mutation in the Rv2042c gene, an ST587 or similar spoligotype and its specific RD pattern; the RD12 partial deletion had not been previously recognized. Its host range is broader than previously thought; it includes oryxes, gazelles and waterbucks in Africa as well as cows and monkeys on the Subcontinent. Here, transmission to humans seems to occur from these animal sources; humans likely represent dead-end hosts.

OP-29

MOLECULAR EPIDEMIOLOGY UNDERLINES THE IMPORTANCE OF *MYCOBACTERIUM CAPRAE* IN LIVESTOCK AND WILDLIFE

Rodríguez S[1,2], Bezos J[1,2], de Juan L[1,2], Romero B[1,2], Álvarez J[1], Castellanos E[1,2], González S[1], Sáez JL[3], Mateos A[1,2], Domínguez L[1,2], Aranaz A[1,2]

[1]VISAVET Health Surveillance Centre, Complutense University of Madrid, Avenida Puerta de Hierro s/n, 28040 Madrid, Spain
[2]Departamento de Sanidad Animal, Facultad de Veterinaria, Complutense University of Madrid, Av. Puerta de Hierro s/n, 28040 Madrid, Spain
[3]Subdirección General de Sanidad de la Producción Primaria, Dirección General de Recursos Agrícolas y Ganaderos, Ministerio de Medio Ambiente, y Medio Rural y Marino, 28071 Madrid, Spain
Contact: sabrina.rodriguez@visavet.ucm.es

Mycobacterium caprae (*M. caprae*) is a member of the *M. tuberculosis* complex which was first described as the main etiological agent of caprine tuberculosis in Spain. However, this pathogen can also infect other animal species and human beings.

In the present study we have characterised *M. caprae* isolates from 791 animals by DVR-spoligotyping. The diversity of spoligotypes based on the number of patterns (n=15) and discrimination index (D=0.58) is lower compared to *M. bovis*. Additionally, we used Variable Number Tandem Repeat (VNTR) typing when more than one spoligotype was observed in the same herd. *M. caprae* infection was widespread in Spain and although the majority of the strains (n=536) were identified in goats, we also observed *M. caprae* in other domestic and wild animal species [cows (n=235), sheep (n=2), pigs (n=2), wild boars (n=14), red deer (n=1), and fox (n=1)]. Proportion of *M. caprae* isolates from bovine samples and *M. caprae*-infected cattle herds increased consistently in the period 2004-2009, highlighting an emergence of this pathogen in cattle.

Unlike results found in reports from other European countries, we observed that the epidemiology in Spain is driven by caprine infections. Considering the damage that *M. caprae* causes in caprine flocks, the possibility of its transmission to other animal species, and its zoonotic potential, we suggest that relevant legislation should be adapted to address the infection as it is done with *M. bovis*.

OP-30

VALIDATION OF MOLECULAR TYPING FOR *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP)

Isabel Fritsch, Heike Köhler, Petra Möbius

Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Naumburger Str. 96a, 07743 Jena, Germany
Contact: Isabel.Fritsch@fli.bund.de

Some molecular epidemiological studies about MAP showed the need of a combination of different typing techniques because of limited genetic diversity of MAP.

The aim of the study was the validation of such a combined protocol. The typing technique should provide a high discriminatory power to characterize the isolates most comprehensively for epidemiological studies as reliable as possible.

About 200 bovine isolates from 56 herds of nine Federal States of Germany, and 28 isolates of other hosts were typed by three established techniques: IS*900*-RFLP (*Bst*EII and *Pst*I), MIRU-VNTR (8 markers), and MLSSR analysis (4 loci).

Application of combined typing revealed a relatively large heterogeneity of MAP in Germany (49 profiles). The results of individual typing methods did not correlate with each other. Some of the MIRU-VNTR and MLSSR markers showed a very low discriminatory power, characterized the same target in the genome or were suggested to be not stable enough for epidemiological studies. Removal of these markers could reduce the effort of the method.
Two MAP genotypes were more prevalent than others in Germany.
It was possible to validate the combined typing protocol by typing of isolates from well characterized herd and population origin of one federal state. Identical profiles of some cattle and red deer isolates around the Eifel national park suggested a transmission between these species. The same was found for one sheep, one goat and one cattle isolate or between cattle isolates from different herds of another area. When patterns of one technique (RFLP, MIRU-VNTR or MLSSR) were omitted, the resulting apparent epidemiological links changed completely.

The different kinds of typing techniques (fingerprint-, PCR- und sequence based methods) describe different targets in the genome. Therefore, at present, considering the validity of epidemiological investigations, it is not possible to omit one of the detected typing results randomly.

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OP-31

RESOLVING LINEAGE ASSIGNATION ON *MYCOBACTERIUM TUBERCULOSIS* CLINICAL ISOLATES CLASSIFIED BY SPOLIGOTYPING WITH A NEW HIGH THROUGHPUT SNPs-BASED METHOD

Edgar Abadia[1], Jian Zhang[1], Tiago Dos Vultos[2], Viviana Ritacco[3], Kristin Kremer[4], Elif Aktas[5], Tomoshige Matsumoto[6], Guislaine Refregier[1], Dick Van Soolingen[4], Brigitte Gicquel[2], Christophe Sola[1,2]

[1]Institute of Genetics and Microbiology, UMR8621, IGEPE Team, Universud, CNRS Université Paris-Sud 11
[2]Unité de Génétique Mycobactérienne, Institut Pasteur, Paris, France
[3]Instituto Nacional de Enfermedades Infecciosas ANLIS Carlos Malbrán, Vélez Sarsfield 563, 1281 Buenos Aires, Argentina
[4]National Institute for Public Health and the Environment, Bilthoven, the Netherlands
[5]Department of clinical microbiology, faculty of medicine, Zonguldak Karaelmas University, Turkey
[6]Department of Clinical Research and Development, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Japan
Contact: christophe.sola@pasteur.fr

We developed a new multiplexed PCR-assay to accurately classify *Mycobacterium tuberculosis* complex (MTC) isolates at the sublineage level by single nucleotide polymorphisms (SNPs). This new method determines 7 SNPs located in the following genes: *recC, rec0, recR, ligB, ligC, alkA,* and *mgtC*. Most of these genes are involved in replication, repair and recombination (3R) functions of *M. tuberculosis* strains, and four of the mutations are synonymous, and thus neutral. Genes were chosen as a first empirical approach to assess the congruence between spoligotyping-based phylogeographical classification and SNP-typing.

Although the method in its current form could not yet efficiently classify all phylogeographical groups of the MTC, it allowed to: (1) confirming and identifying new sublineage-specific SNPs, (2) unraveling phylogenetical relationships between MTC spoligotyping-defined sublineages, (3) appropriately assigning sublineages to some spoligotype groups and reassign sublineages to others mis-labelled spoligotype signatures. This study opens the way to a more meaningfull taxonomic, evolutionary and epidemiologic classification. It also allows evaluation of the significance of "spacer signatures" and improved understanding of the evolutionary mechanisms of the clustered regularly interspaced short palindromic repeats (CRISPR) locus in MTC.

OP-32

TRANSLATING INTO PRACTICE LINE PROBE ASSAY TRAINING FOR MDR-TB SCREENING IN HIGH BURDEN COUNTRIES: LESSONS FROM THE FIELD

Gutierrez Cristina[1], Barnard Marinus[2], Paramasivan CN[1], Somoskovi Akos[1]

[1]Foundation for Innovative New Diagnostics, Geneva, Switzerland
[2]National Health Laboratory Services (NHLS), Cape Town, South Africa
Contact: cristina.gutierrez@finddiagnostics.org

TB laboratory staff education in high burden countries is experiencing a paradigm shift, from overseas purely service-based systems to *in situ* curriculum-driven models. International partner organizations are developing training packages in TB microscopy, culture and DST to support countries in scaling-up their TB laboratory services by providing standard materials.

Recently, WHO recommended the adoption of line probe assays (LPA) for rapid detection of MDR-TB, within the context of national country plans for appropriate management of MDR-TB patients. The implementation of LPA in MDR-TB screening algorithms may significantly reduce the demand on conventional culture and DST laboratory capacity. The success to implement these assays in high-volume laboratories is heavily reliant on the quality and training of personnel and on their adherence to strict working practices to minimize the risk of contamination leading to false-positive results. FIND has developed a comprehensive training package in both English and French to adequately prepare TB laboratory staff to perform day-to-day routine quality assured LPA procedures for screening of MDR-TB and to interpret results, following rigorous laboratory protocols, standard operating procedures, and internal quality controls. So far, this 5-days LPA training has been conducted at the new facilities of four National TB Reference Laboratories in high burden countries, under supervision of senior scientists with extensive experience in training, clinical mycobacteriology and use of molecular technology applied to TB diagnostics. The LPA training package was piloted in Ethiopia and Lesotho in 2009, and then conducted in Myanmar and Ivory Coast in 2010. The modules cover topics on essential biosafety related to molecular testing, assay related instrument operation, calibration and maintenance, molecular laboratory layout and set-up, specimen and reagent preparation, hybridization of amplified products, interpretation of line probe patterns, contamination control and trouble shooting, and quality control of LPA. The standardized training curriculum consists of state of the art lectures, demonstrations of the assay and extensive hands-on practical sessions on a one-on-one basis. The participants have shown a good competence on conducting the method and properly interpreting the results. A main challenge to roll out the trainings has been to properly organize a minimally acceptable work environment to conduct the hands-on practical sessions. While constructions were finished at the new facilities, elementary non-specific tools and items were missing, not assembled or not installed, precluding a prompt post-training use of the laboratories for routine testing.

OP-33

IDENTIFICATION OF NON-TUBERCULOUS MYCOBACTERIA BY SEQUENCING A FRAGMENT OF THE 16S rRNA GENE: WHICH INTERNET DATABASE TO BE USED TO ANALYSE THE SEQUENCE?

Vanessa Mathys, Brigitte Vanfleteren, Maryse Fauville-Dufaux

Scientific Institute of Public Health, Communicable and Infectious Diseases,
NRL Tuberculosis & Mycobacteria, Engelandstreet 642, B-1180 Brussels, Blegium
Contact: mfauville@iph.fgov.be

Introduction. Correct species identification of growing mycobacteria from clinical specimens is important because only some of them are pathogenic. Moreover, the treatment must be adapted according to the mycobacterial species. Sequencing of the gene coding for the 16S rRNA is a molecular method largely used for bacterial species identification. After sequencing, the succession of nucleic acids is compared with sequences from mycobacterial databases freely available on Internet. Our purpose is to determine which database is the most reliable or if the different databases are equivalent. To answer this question, the sequences of the 16S rRNA gene of 233 non-tuberculous mycobacterial isolates were analysed by comparison with the sequences given in the BEN, RDP-II and RIDOM databases.

Materials and Methods. 233 mycobacterial strains collected in our national reference laboratory (Scientific Institute of Public Health, Brussels) were analysed in this study. Amplification of a 200 bp fragment of the 16S rRNA gene was performed on the genomic DNA of all isolates. The sequencing of the purified amplicons was performed on an ABI PRISM 3100 Genetic Analyzer (Apllied Biosystem). The sequencing outputs were analysed by BLAST (Basic Local Alignment Search Tool) analysis with 3 different public databases containing mycobacterial 16S rRNA gene sequences: (1) the Wemboss/Ben program (www.be.embnet.org), (2) the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu) and (3) the RIDOM web server (Ribosomal Differentiation of Medical Microorganisms) (www.ridom-rdna.de).

Results. The analysis shows 3 categories of BLAST results: (i) easy identification because similar results were obtained whatever the database used (85.4%, n=199), (ii) possible identification in spite of small identification differences from one database to another one (12.01%, n=28) and (iii) difficult identification due to the obtaining of different species identification results according to the database used (2.57%, n=6).

Conclusion. For 97% of the strains, the identification is stable whatever the database used. Only ~3% of the sequences present a problem of identification. The detailed analysis of these problematic 16S rRNA sequences (completed by sequencing of other genomic regions or use of commercial hybridization kits) will be discussed. The contribution of analyzing longer fragments of the 16S rRNA gene will be evaluated.

OP-34

DRUG RESISTANCE PATTERNS OF TREATMENT FAILURE HOSPITALIZED TB PATIENTS OF BANGLADESH

Rahim Zeaur[1], Khan Sharmin Shaila[1], Nakajima C[2], van der Zanden AGM[3], Suzuki Y[2]

[1]International Centre for Diarrhoeal Disease Research, Bangladesh, 68 Shaheed Tajuddin Ahmed Sarani, Mohakhali, Dhaka 1212
[2]Hokkaido University,Sapporo, Japan
[3]Laboratory for Medical Microbiology and Public Health, Enschede, The Netherlands
Contact: Zeaur@icddrb.org (Zeaur Rahim)

Introduction. Tuberculosis (TB) is a treatable disease. Treatment failure patients develop multidrug resistance (MDR). In many developing countries like Bangladesh, MDR is emerging, but the exact situation is still unknown. Cost of TB treatment increases with the emergence of resistance. This study was aimed to find out the drug resistant patterns of treatment failure hospitalized TB patients of Bangladesh.

Materials and Method. *M. tuberculosis* was isolated on Lowenstein-Jensen slants from sputum samples of treatment failure hospitalized TB patients. Strains were identified *M. tuberculosis* using an multiplex PCR. Strains were tested for sensitivity to first line anti-TB drugs using proportion susceptibility testing (PST) method. Multi drug resistant (MDR) stains were tested for sensitivity to O-floxacin and amikacin using PST method. MDR strains, which were resistant to O-floxacin and amikacin were designated as extensively drug resistant (XDR). Sequencing was performed to confirm XDR strains for the detection of mutation at *gyr*A and *rrs* genes to confer resistance to quinolone and to amicasin respectively. *rpo*B, *kat*G and *inh*A genes were sequenced to detect mutation for the confirmation of resistance. Standard spoligo typing technique was practiced to genotype the MDR strains.

Results. During a period from February 2007 through December 2008, 270 sputum samples were cultured from hospitalized TB patients. Their age ranged from 12 to 90 years and male to female ration was 2.9:1. *M. tuberculosis* grew from 73% sputum samples. All the strains were identified *M. tuberculosis*. Resistance to all first line anti-TB drugs was detected in 70% strains and 5.6% strains were sensitive to all these drugs. Five of 270 (2%) strains were XDR. Mutation was detected at *gyr*A and *rrs* genes confirmed XDR. Different proportions of mutations were detected at *rpo*B, *kat*G and *inh*A genes of MDR strains. Spoligo patterns of 80 strains were matched with SpolDB4 to ascertain phylogenetic clades. Of them, unique spoligo patterns were detected in 14 isolates. The remaining 64 patterns were grouped into East African Indian (n=5), Central Asian (n=10), Beijing (n=23) and principal genetic group 2 and 3.

Conclusion. In Bangladesh TB is caused by *M. tuberculosis*. Prevalence of MDR is more among hospitalized treatment failure TB patients. MDR strains are heterogeneous in genotypes. XDR is an emerging threat to National TB Control Program.

Key words: *Mycobacterium tuberculosis*, MDR TB, XDR TB.

OP-35

MANAGEMENT OF TUBERCULOSIS OUTBREAKS

Miranda A[1], Alves A[1], Tavares Magalhães A[1], Duarte R[2], Correia A[3]

[1]National Institute of Health Dr. Ricardo Jorge, National *Mycobacterium* Reference Laboratory, Rua Alexandre Herculano 321, 4000-055 Porto, Portugal
[2]Hospital of Vila Nova de Gaia/Espinho, Pneumology Service, Rua Conceição Fernandes, 4434-502 Vila Nova de Gaia, Portugal
[3]Public Health Department of the North Regional Health Administration, Rua Anselmo Braancamp 144, 4000-078 Porto, Portugal
Contact: amiranda@ibmc.up.pt

Classic epidemiology plays a valuable role in understanding disease dynamics although it has certain limitations. Molecular epidemiology complements classical epidemiology enhancing the accuracy and resolution of the epidemiological picture. The goal of the present study was to demonstrate the decisive value of the molecular epidemiological approach in confirming (or not) outbreaks of tuberculosis identified by conventional contact tracing.

Between 2005 and 2009, 3 tuberculosis outbreaks were investigated. In 2005, 5 cases of active tuberculosis occurred in a shopping centre. 4 *M. tuberculosis* strains were typed. 352 contacts were screened. In 2007, 7 cases of active tuberculosis occurred in a high school in the city of Alijó. 6 *M. tuberculosis* strains were typed. 1360 contacts were screened. In 2009, 10 cases of active tuberculosis occurred in the city of Resende. 7 *M. tuberculosis* strains were typed. 183 contacts were screened. All clinical isolates were fingerprinted using 15 loci MIRU-VNTR.

Classic epidemiology established weak links among the 5 patients of the shopping centre even though they worked in different shops. Yet genotyping of 4 *M. tuberculosis* strains of this "outbreak" showed that there was no connection between the cases, since all strains were different. In the Alijó's high school outbreak, classic epidemiology established links between 5 of the 7 patients. Genotyping revealed that all strains were identical. Furthermore, 2 strains isolated 3 years before from family members of one of the students had the same fingerprint, which enabled the identification of the primary case. Finally, classic epidemiology failed to yield solid links between patients in the outbreak of Resende. However, genotyping uncovered not one but two distinct outbreaks.

This work shows the usefulness of molecular epidemiology with regards to the investigation of tuberculosis outbreaks. Without *M. tuberculosis* genotyping proper management of the outbreaks described in this work would have not been possible.

ABSTRACTS OF POSTER PRESENTATIONS (PP)

USING GENOTYPE® MTBC FOR DIAGNOSTICS OF BCG VACCINATION COMPLICATIONS

Zalutskaya AM[1], Sahalchyk ER[1], Nikolenko AN[1], Skrahina AM[1], Dziusmikeyeva MI[1], Kryvasheyeva ZI[2], Morozkina NS[2], Emeljanova NA[2], Babchenok IV[1]

[1]Republican Scientific and Practical Centre for Pulmonology and Phthisiology, Department of Laboratory Diagnostic and Treatment of Tuberculosis, Dolginovsky tr. 157, 220053, Minsk, Belarus
[2]Belorussian State Medical University, Department of Phthisiopulmonology, Dolginovsky tr. 157, 220053, Minsk, Belarus
Contact: akasana@inbox.ru

Last years, the tendency towards an increase of the number of BCG vaccination with complications was registered in Belarus. In 2009, 77 BCG postvaccinal complications were registered. The every second infant with BCG vaccination complications was exposed to operative treatment because of severe complications coursing including caseous necrosis formation. Identification of vaccine strain BCG is very important for diagnostics of postvaccinal complications in infants, but it is impossible to differentiate BCG and *M. bovis* using biochemical tests.

We performed cultural examination of operative material of 21 infants at the age of under a year. Patients were operated in the clinic of the Centre in 2008-2009 in connection with BCG vaccination complications. Among these infants, 8 had a left axillary adenitis, 7 had a left shoulder cold abscess, 5 had osteoarticular lesions and 1 had a left shoulder granuloma. Identification of isolated *Mycobacterium* strains was performed with the use of GenoType® MTBC (Hain Lifescience). Vaccine strain *M. bovis* BCG was used as the control. The results of the study enabled us to identify all 21 *Mycobacterium* strains isolated from operative material as *M. bovis* BCG.

Thus, the results of our study with the use of GenoType® MTBC reliably confirmed the diagnosis of BCG vaccination complication.

NONTUBERCULOUS MYCOBACTERIA (NTM): MICROBIOLOGY AND CLINICAL RELEVANCE

Kontos F, Bobola M, Tarpatzi A, Siveri T, Zerva L

Clinical Microbiology Laboratory, Attikon Hospital, University of Athens, Rimini Street, Athens 12462, Greece
Contact: fankon68@hotmail.com

Introduction. NTM are considered emerging pathogens implicated in lung, lymphnode, skin/soft tissue or disseminated infection. This retrospective study assessed the microbiological characteristics and clinical relevance of NTM isolates recovered during routine testing.

Materials and Methods. Clinical samples submitted for mycobacterial culture (12/2006 to 02/2010) were processed by standard methodology and inoculated into Loewenstein-Jensen slants, BACTEC MGIT 960 or BACTEC 9000 bottles (Becton-Dickinson). NTM were identified with Genotype *Mycobacterium* CM and AS (Hain-Lifescience), while *hsp65* gene PCR-RFLP analysis and 16S rRNA gene sequencing were applied when necessary. Established bacteriological criteria for NTM lung disease by the American Thoracic Society (ATS) were used to determine the clinical relevance of isolates.

Results. During the study period, 6224 specimens were cultured and 103 NTM isolates (1.65%) were recovered, which belonged to 18 species. *M. lentiflavum* (23), *M. avium* (9), *M. intracellulare* (8) *M. fortuitum* (6) and *M. gordonae* (3) represented the most frequent species. Isolates originated most commonly from respiratory (90 strains, 52 patients) than other sites samples (13 strains, 7 patients). The latter included two rare cases of disseminated disease by *M. marinum* and *M. bolletii*, respectively. Among pulmonary isolates, only for 12 out of 52 patients the ATS diagnostic criteria were met. In this subgroup (9/12 with acid fast positive smears; 9/12 men; 5/12 immunosuppressed; median age 62 years, range 36-82 years) *M. avium* was isolated most frequently (6 patients). For 32 out of 52 patients (61.5%) sampling was inadequate (less than 3 samples/patient), hence the clinical significance of rare or novel species isolates (*M. arupense*, *M. monacense*, *M. conceptionense*, *M. massiliense*) could not be determined. The frequent isolation of *M. lentiflavum* most probably represented contamination of hospital water system. MGIT 960 was more sensitive and rapid (98.9%, mean time to detection 16.4 days) than solid medium cultures (47.2% and 26.6 days, respectively), while only in-house molecular methods enabled the identification of rare/novel species.

Conclusions. Only a minority of patients with pulmonary NTM isolates met the ATS criteria, mainly because of inadequate sampling of a large number of individuals. The application of MGIT 960 and molecular methods enabled improved isolation rates, timely diagnosis and informative species identification.

PP-3

NON-TUBERCULOUS MYCOBACTERIA ISOLATED FROM CLINICAL SPECIMENS IN CROATIA 2000-2008

Obrovac Mihaela, Zmak Ljiljana, Katalinic-Jankovic Vera

Croatian National Institute of Public Health, Rockefellerova 7, HRV-10000 Zagreb, Croatia
Contact: Tel. +385 1 4683 010, Fax. +385 1 4683 388, mihaela.obrovac@hzjz.hr

The genus *Mycobacterium* currently has more than 140 species, including *M. tuberculosis* complex, *M. leprae* and other organisms referred to as nontuberculous mycobacteria (NTM). In recent years, there has been a marked increase in the number of cases of human disease due NTM that seem to be related to the geographic distribution of these species in the environment. A wide range of diseases is caused by NTM, including lung disease, lymphadenitis, skin and soft tissue infections, osteoarticular disease and disseminated disease.

In Croatia, TB diagnostics is conducted in 14 laboratories that use Löwenstein-Jensen and MGIT media for the recovery of mycobacteria from clinical specimens. Full-grown AFB cultures, negative for *M. tuberculosis*, are sent to Croatian National Institute of Public Health where the identification is performed by means of GenoType *Mycobacterium* CM/AS kits (Hain Lifescience) and/or by phenotypical methods.

Of total of 39,580 clinical isolates isolated in Croatia from 2000 to 2008, 37,426 (94.55%) were identified as belonging to *M. tuberculosis* complex and 2,154 (5.45%) as NTM. During that period, NTM prevalence increased steadily, starting with 4.2% in 2000 and rising to 9.9% in 2008. Total of 95 patients with NTM isolates met microbiologic criteria for mycobacteriosis. After *M. xenopi* (n=36; 37.89%) and MAC (n=31; 32.63%), most patients had *M. kansasii* (n=15; 15.79%) isolated. *M. fortuitum* was isolated in 5 patients (5.26%), *M. abscessus* in 3 (3.16%), while *M. chelonae*, *M. celatum*, *M. scrofulaceum*, *M. gordonae*, *M. peregrinum* and *M. marinum* were isolated in one patient each (1.05%).

With TB notification rate of 19/100.000, Croatia is a middle-incidence country and TB is still a public health problem. Nevertheless, the incidence of NTM, as well as number of mycobacteriosis patients, is steadily rising and the key role of the laboratory is increasing the awareness of NTM as potential pathogens. The correct and rapid identification of NTM is at the basis of the proper therapeutic modality.

PP-4

SIX YEARS OF NONTUBERCULOUS MYCOBACTERIA ISOLATES IN HOSPITAL S. JOÃO, PORTO, PORTUGAL

Carvalho T, Gonçalves H, Pinheiro MD

Laboratório de Microbiologia do Serviço de Patologia Clínica, Hospital de S. João, Alameda Hernâni Monteiro, 4200 319 Porto, Portugal
Contact: m.dolores.pinheiro@gmail.com

Mycobacterium genus has more than 130 species. Although *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*, the etiologic agents of human tuberculosis and Hansen disease respectively, are the main causes of mycobacteriosis in humans, other species, known as Nontuberculous Mycobacteria (NTM), may underlie the condition. An increased incidence of mycobacteriosis induced by these NTM is recognized worldwide and, as they are opportunistic pathogens, their infection is probably acquired from the environment. The purpose of our work is to present the diversity of NTM isolated from biological samples, over 2004-2009, in patients attended in Hospital S. João.

A prospective six years study was done to include all patients in whom NTM was isolated and the underlying pathology affecting them. Recovery of NTM was accomplished using Löwenstein-Jensen and Middlebrook broth 7H9 (Mgit®, Bactec) media. The identification to species level was obtained with Gen-probe® Accuprobe (bioMérieux) and GenoType® Mycobateria CM/AS (Hain, Lifescience GmbH) methodologies.

Over the period of this study, 330 patients (51 in 2004, 42 in 2005, 47 in 2006, 69 in 2007, 62 in 2008 and 59 in 2009) had isolates of NTM; they were aged 55.5 years-old on average, the range was 7-92 years and included 211 males (63.9%). A total of 339 were isolated from 335 biological samples of which, 269 (80.3%) belonged to respiratory tract. Their distribution was 145 *Mycobacterium avium* complex, 57 *M. gordonae*, 26 *M. intracellulare*, 26 *M. kansasii*, 26 *M. peregrinum*, 23 *M. chelonae*, 14 *M. fortuitum*, 6 *M. avium*, 6 *M. scrofulaceum*, 5 *M. xenopi* and 1 each of *M. abscessus*, *M. simiae*, *M. mucogenicum*, *M. marinum* and *M. genavense*/*triplex*. In what affecting pathology is concerned, 67 (20.3%), were HIV positive; 166 (50.3%) had respiratory diseases, mainly COPD, asthma, cystic fibrosis, pulmonary fibrosis and emphysema, bronchiectasis and neoplasic diseases; the remaining 67 patients exhibited a variety of pathologies, all of them with some degree of immunosuppression.

The results evidence the common perception of the increase of NTM mycobacteriosis in recent years. They also show a considerable diversity of strains, a point not uncommon when the international literature is surveyed. Another interesting feature to be emphasized is the high incidence of respiratory entities and an unexpectedly low incidence of HIV patients.

PP-5

NONTUBERCULOUS MYCOBACTERIAL REVIEW OF 36 CASES

Nuak J[2], Vilde A[2], Carvalho T[1], Gonçalves H[1], Gomes MH[2], Sarmento A[2]

[1]Infectious Diseases Service and Clinical Phatology Service
[2]Hospital São João, Alameda Prof. Hernani Monteiro, Porto – Portugal
Contact: jotanuak@gmail.com

Nontuberculous mycobacterial (NTM) is widely distributed and person to person transmission is rare. Advanced age, immunosuppression or chronic lung disease predispose for these infections.

Retrospective review of clinical records of patients (pts) with NTM admitted from Jan/07 to Dec/09.
Thirty-six cases of NTM were diagnosed, 27 (75%) male; age ranged from 27-82 (x=42.9 ± 12.3) y. Thirty (83%) pts were HIV positive (CD4 1-796/mm^3 (x=141±217)), 4 had prophylaxis with azithromycin; other diagnosis: Chronic lymphocytic leukemia, diabetes mellitus type 2, old age (82y), previous tuberculosis, drug addiction one pt each.
Thirty one pts had constitutional symptoms, 6 diarrhea, 6 chest pain and in 11 lymphadenopathies were found. Increased C reactive protein was found in 29, hypoalbuminemia in 28, hyponatremia in 20, anemia in 18, increased adenosine deaminase in 16, abnormal liver function in 15, thrombocytopenia in 14, leukopenia in 9, leukocytosis in 4. Abnormal chest X-ray was found in 20 pts.
NTM was isolated from culture of one specimen in 24 pts; 3 pts had more than 1 NTM species and 5 were associated with *M. tuberculosis*. Sputum culture was positive in 19, BAL/BL in 7, gastric fluid in 7, blood in 4, urine in 4, feces in 3, and CSF in 1.
MAC was identificated in 16, *M. gordonae* in 8, *M. xenopi* in 4, *M. peregrinum* in 4, *M. kansasii* in 3, *M. cheloneae* in 2 and *M. fortuitum* in 2 pts.
Treatment was started in 15 pts: 12 with INH, PZA, RMP/RFB, EMB and in 4 therapy was changed after the identification of NTM; in 3 patients treatment was started after diagnosis of NTM with EMB, RFB, azytromicin/claritromicin and a quinolone. Three other had post-mortem diagnosis.
The remaining 18 pts were not treated because they were considered colonized, or were lost to follow-up or non-compliant.
Four pts are still on treatment; 4 are cured; 13 remain in observation; 6 died; 8 are lost to follow-up.
The time from admission until treatment ranged from 0-44 days (x=16 ±16). The time until confirmation of diagnosis ranged from 14-160 days (x=57±35), in 3 cases diagnosis was confirmed post-mortem.

Conclusion: 1) MAC was the most common isolation 2) Most pts had pulmonary involvement and were HIV infected. 3) The decision to treat was based mostly on clinical signs and the isolation of NTM was evaluated according to sample and risk of pts as most isolations correspond to colonization of respiratory tree.

PP-6

USE OF GENOTYPE *MYCOBATERIUM* FOR RAPID DETECTION OF *MYCOBACTERIUM SZULGAI* SINOVITIS IN AN IMMUNOCOMPETENT PATIENT

Molinari GL[1], Ceffa R[2], Camaggi A[1], Losi M[3], Mantovani M[1], Kroumova V[1], Andreoni S[1], Fortina G[1]

[1]Department of Microbiology and Virology, "Maggiore della Carità" University Hospital, C.so Mazzini 18, 28100, Novara, Italy
[2]Trauma and Orthopedic Unit, Hand Surgery Unit,"Maggiore della Carità" University Hospital, C.so Mazzini 18, 28100, Novara, Italy
[3]Section of Respiratory Diseases, Department of Oncology, Hematology and Respiratory Disease, University of Modena and Reggio Emilia, Via del Pozzo 71, 41100, Modena, Italy
Contact: gianlorenzo.molinari@maggioreosp.novara.it

Mycobacterium szulgai (*M. szulgai*) is a slow growing nontuberculous mycobacterium (NTM), isolated and described by Marks *et al.* in 1972. Previous data in the literature suggested that pulmonary disease caused by *M. szulgai* commonly occur in patients with chronic lung disease and/or concomitant lung infections; while non-pulmonary infections, such as osteomyelitis, were more frequent in patients with a severe immunocompromission due to comorbidities or immunosuppressive treatment.

We reported our first case of *Mycobacterium szulgai* isolated in a biopsia of a 54 years-old man's hand, without any causes of immunosuppression, such as a rheumatic disease, trauma or infective events in the last recent years. Electromyography (EMG) showed swelling fly wrist paralysis and sensory and motor median and ulnar nerve of the hand. Ultrasonography (US) and Magnetic resonance imaging (MRI), indicated synovitis of the wrist flexors. Granulomatous tissue carried off during surgery was sent to Microbiology Laboratory for standard microbiological analysis to isolate pathogens, included bacteria and mycobacteria.

The microbiological investigation showed the growth of *Staphylococcus epidermidis*. Direct examination of speciment was negative for acid-fast bacilli, but after fifteen days a pigmentate colony were isolated on Löwenstein-Jensen solid medium (Becton Dickinson, USA). No bacteria were isolated by MGIT 960 tubes (Becton Dickinson, USA) after standard incubation time (42 days). Colony were positive with Ziehl-Neelsen staining. The Genotyping (GenoType *Mycobacterium* CM and AS assays, Hain, Lifescience, Nehren, Germany) of the bacterial colonies identified a *Mycobacterium szulgai*.

This result show that the GenoType *Mycobacterium* system might be a useful tool to identify enlarge number of mycobacteria that can be reliably isolated in routine clinical laboratories. Moreover, this clinical report suggest that the role of NTM is probably underestimated because the difficulty of isolating and identifying these organisms.

A CASE OF PULMONARY DISEASE DUE TO *MYCOBACTERIUM SZULGAI* IN A PATIENT WITH ADENOCARCINOMA OF THE LUNG

Triller N, Svetina Šorli P, Žolnir–Dovč M

University Clinic of Respiratory and allergic Diseases Golnik, Golnik 36, SI – 4204 Golnik, Slovenia
Contact: nadja.triller@klinika-golnik.si

Mycobacterium szulgai (*M. szulgai*) is slow growing nontuberculous mycobacterium which is rarely recovered from the environment and it has been reported from a small number of cases as a human pathogen. The first case of clinical important *M. szulgai* disease in Slovenia is reported in this presentation.

In a 60-year-old female, ex-smoker in the right upper lobe adenocarcinoma was diagnosed in 2006. The treatment with right upper lobe resection (pT2N2MO) was completed with four cycles of adjuvant chemotherapy. One year later resection of solitary metastasis from liver was performed and a few months later, the patient was irradiated due to bone metastasis (L5). Systemic therapy with Erlotinib began soon after irradiation.

At the beginning of the 2009, the patient complained about severe abdominal pain and dry coughing. Computed tomography (CT) scan revealed a nodular lesion in the right upper lobe and enlarged lymph nodes in the upper abdomen. Bronchial lung biopsy found granulomas with necrotic tissue. Acid-fast bacilli were isolated from bronchial washing and identified by as *M. szulgai*. EUS biopsy of enlarged lymph nodes found metastases of adenocarcinoma. A 12-month regimen of rifampicin, ethambutol and clarithromycin resulted in a complete eradication of *M. szulgai;* the patient was also treated with pemetrexed as monotherapy.

In March 2010 she is still alive with the regression of enlarged lymph nodes in the abdomen and a complete eradication of *M. szulgai.*

PP-8

NON TUBERCULOUS *MYCOBACTERIUM* INFECTION WHICH NEEDED TO BE DISTINGUISHED FROM LUNG CANCER ON CT FINDINGS

Eisuke Matsuda, Kazunori Okabe, Hiromasa Yamamoto, Hiroyuki Tao, Seiki Kobayashi, Katsutoshi Hirazawa, Kazuro Sugi

Yamaguchi-Ube Medical Center, Department of Chest Surgery, Higashi kiwa 685 Ube City, Yamaguchi, Japan
Contact: malphajg@yahoo.co.jp

Introduction. Recently, the number of patients with non tuberculous *Mycobacterium* infection has been increasing, and about 80% of them are *Mycobacterium avium* complex (a following MAC), and among the remainder, *Mycobacterium kansasii* is most common in Japan. In MAC cases, there are many cases with the diffuse lesion at the time of diagnosis. In cases of *Mycobacterium kansasii*, the majority of cases showed a thin walled cavity lesion. The case with the isolated nodular shadow is comparatively rare in non tuberculous *Mycobacterium* infection.

Material. Case 1 was a 56-years-old woman. Her Chest X-ray and chest CT showed 12mm nodule in left S4. Case 2 was a 42-years-old woman. Her Chest X-ray and chest CT showed 15mm nodule in left S10. Case 3 was a 24-year-old man. His chest X-ray and chest CT showed 13mm solitary nodule in the right S2. Case 4 was a 65-year-old man. His chest X-ray and chest CT showed 15mm solitary nodule in the left S1+2. They were asymptomatic. Chest CT revealed irregular form shadow in all cases, and pleural indentation in case 1 and 3. All of them needed to be distinguished from lung cancer.

Method. Bronchofiberscopy could not make a pathological diagnosis. Thoracoscopic resection was performed to obtain pathological diagnosis.

Result. Thoracoscopic partial resection was performed, intraoperative pathology revealed that all of them were granuloma. Postoperative pathology revealed that solitary nodules were *Mycobacterium avium* infection nodules in case1 and 2, *Mycobacterium kansasii* infection nodules in case 3 and 4. Four years later, all of them are asymptomatic, and disease free on chest CT.

Conclusions. The case with the isolated nodular shadow is comparatively rare in non tuberculous *Mycobacterium* infection. Surgical resection will take not only pathological diagnosis but also complete cure. If lesion of non tuberculous *Mycobacterium* spread over another lobe, complete resection is difficult. It is necessary for indeterminate solitary nodule to be resected as soon as possible. Thoracoscopic surgery is low invasive and useful method for indeterminate lung nodule.

AN ADULT CASE OF MEDIASTINAL TUBERCULOUS LYMPHADENITIS WITHOUT PRIMARY LESION IN LUNG FIELD

Eisuke Matsuda, Kazunori Okabe, Hiromasa Yamamoto, Hiroyuki Tao, Seiki Kobayashi, Katsutoshi Hirazawa, Kazuro Sugi

Yamaguchi-Ube Medical Center, Department of Chest Surgery, Higashi kiwa 685 Ube City, Yamaguchi, Japan
Contact: malphajg@yahoo.co.jp

Introduction. Mediastinal tuberculous lymphadenitis is found in childhood usually, it is rare in adult. If there is no lesion in lung field, it is difficult to be distinguished from other disease. Recently, adult initial infection of tuberculosis is increased, and adult case of mediastinal tuberculous lymphadenitis may increase. We report a case of mediastinal tuberculous lymphadenitis of the adult onset that did not show primary lesion in lung field and other lymph nodes.

Material and Method. A 22-year-old man admitted to our hospital due to swallowing disturbance October 2005. He had no history of tuberculosis. Chest CT scan showed mediastinal lymph node swelling. There is no abnormal shadow in lung field. Esophagography revealed passage disturbance caused by lymph node swelling. The tuberculin skin test was positive, but *Mycobacterium tuberculosis* bacilli were not found in the sputum. We performed thoracoscopy to make pathological diagnosis.

Results. Mediastinal tuberculous lymphadenitis was diagnosed histologically and bacteriologically from the specimen obtained by thoracoscopy. Chemotherapy with isoniazid, rifampicin, streptomycin sulfate and pyrazinamide was performed. Chest CT scan 2 months after from the start of chemotherapy revealed that the mediastinal lymph nodes were decreased in size. Chemothrapy was continued for six months. Four years later, he is doing well without disease.

Conclusions. Mediastinal tuberculous lymphadenitis in adults is rare, but the number of reports has been increased. Adult acquired immunity prevalence decreases recently, and an adult initial infection onset increases. An adult case of Mediastinal tuberculous lymphadenitis may increase. Mediastinal tuberculous lymphadenitis has risk of tracheal perforation, SVC syndrome. Some author reported the case which needed for emergency operation due to dyspnea caused by the tracheal stenosis. Mediastinal tuberculous lymphadenitis should be diagnosed immediately, must be distinguished from other causes of mediastinal lymph node swelling. Thoracoscopy and mediastinoscopy are low invasive, useful method for undetermined mediastinal lymph node swelling.

PP-10

PREVALENCE OF NON-TUBERCULOUS MYCOBACTERIA IN PATIENTS WITH CYSTIC FIBROSIS

Satana D, Genc GE, Erturan Z, Uzun M

Istanbul University, Istanbul Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Istanbul 34390, Turkey
Contact: dileksatana@yahoo.com

Non-tuberculous Mycobacteria (NTM) have emerged as significant pathogens in cystic fibrosis (CF) patients. The aim of this study is to describe the prevalence of NTM isolates in pediatric CF patients from April 2003 to November 2008 in a hospital of Istanbul Faculty of Medicine.

In this period 376 sputum samples were collected from 130 pediatric patients. Twenty eight (7.44 %) out of 376 samples were positive for *Mycobacterium* species and 5 of the species were identified as *Mycobacterium tuberculosis* complex (MTBC) (17.9 %), 14 as *M. abscessus* (50 %) and 9 as *M. lentiflavum* (32.1 %) by GenoType *Mycobacterium* CM/AS (Hain Lifescience GmbH, Nehren, Germany).
All of the MTBC isolates were susceptible to streptomycin, isoniazid, rifampin and ethambutol by Bactec TB 460 system (Becton Dickinson, Sparks, MD, USA).
Susceptibilities of clarithromycin (CH), tigecycline (TGC), linezolid (LZ), amikacin (AK), trimethoprim-sulfamethoxazole (TS), doxycycline (Dox), cefoxitin (FX), ciprofloxacin (CL), imipenem (IP) and tobramisin (TM) against *M. abscessus* and *M. lentiflavum* strains were determined by E test method (BioMérieux, France).
All of the *M. abscessus* isolates were susceptible to CH (MICs 0.125-1 µg/ml) and TGC (MICs 0.19 -0.5 µg/ml). Eleven of 14 strains were determined as susceptible to LZ (MICs 4 µg/ml), while the remaining 3 were resistant (MICs >256 µg/ml). One strain was found to be resistant to AK (MIC > 256 µg/ml) and 13 strains showed intermediate MICs (32 -48 µg/ml). All of *M. abscessus* isolates were resistant to TS (MICs > 32 µg/ml), Dox (MICs >256 µg/ml), FX (MICs >256 µg/ml), CL (MICs >32 µg/ml), IP (MICs >32 µg/ml) and TM (MICs >256-64 µg/ml).
All of the *M. lentiflavum* isolates were susceptible to CH (MICs 0.25 µg/ml), AK (MICs 12 µg/ml) and CL (MICs 0.032 µg/ml), while they were fully resistant to IP (MICs >32 µg/ml), TGC (MICs > 256 µg/ml), FX (MICs >256 µg/ml), Dox (MICs 128 µg/ml), and TM (MICs 64 µg/ml).

Although there are few studies related to the prevalence of NTM in pediatric CF populations, mycobacterial infections appear to be increasing. This study showed that *M. abscessus* was the most prevalent *Mycobacterium* species in pediatric CF patients in Istanbul Faculty of Medicine.

PP-11

A CASE-REPORT OF *MYCOBACTERIUM ABSCESSUS* FROM THE PLEURAL FLUID OF AN IMMUNOCOMPROMISED PATIENT

Kontos Fanourios[1], Kosmadakis Georgios[2], Bazigos Stavros[2], Gitti Zoe[2]

[1]Clinical Microbiology Laboratory, Medical School of Athens, Attikon University Hospital, Athens, Greece
[2]Microbiology Laboratory, University Hospital of Heraklion, Greece
Contact: fankon68@hotmail.com

The introduction of molecular techniques has facilitated the detection and identification of new NTM species, the role of which is under constant evaluation. *Mycobacterium abscessus* is a rapidly growing NTM that has attracted global interest due to its increased pathogenety.

Mycobacterium abscessus, a rapid-growing mycobacterium was isolated from the pleural fluid of a 56-y old female patient with fever, dyspnea and history of lung cancer.

Acid-fast staining of the pleural fluid sample was performed and was negative. The liquid culture (BacT/Alert 3D; bioMérieux, Durham, NC) turned positive after 5 days, followed by a positive solid culture (Lowenstein-Jensen; bioMérieux, Marcy l'Etoile, France) two days later. The isolate identified by the combined use of GenoType Common Mycobacteria (GenoType CM) and GenoType Additional Species assay (GenoType AS, Hain Lifescience, Nehren, Germany) as *M. abscessus*. Drug susceptibilities were determined by E-test (AB Biodisk, Solna, Sweden). This isolate was resistant to doxycyline, and trimethoprim/sulfamethoxazole, whereas it was susceptible to clarithromycin and ethionamide. The less active molecules were ethambutol, amikacin and ciprofloxacin. The isolate was considered to be a pathogen.

This report should increase the awareness for the ubiquity of this species and raise the index of suspicion for the detection of the pathogen, particularly, in immunocompromised patients. Molecular techniques are essential for its identification because biochemical identification is time-consuming and can only provide identification at *Mycobacterium fortuitum-chelonae* complex level.

PP-12

MYCOBACTERIUM AVIUM SUBSP. *HOMINISSUIS* DETECTION BY TRIPLEX QUANTITATIVE REAL TIME PCR IN CHILDREN WITH NECK LYMPHADENITIS AND IN THEIR ENVIRONMENT

Kaevska M, Slana I, Kralik P, Pavlik I

Veterinary Research Institute, Brno, Czech Republic
Contact: kaevska@vri.cz

Mycobacterium avium subsp. *hominissuis* (MAH) is the most prevalent causative organism of lymphadenitis in children of 1 to 5 years old in developed countries where the incidence of tuberculosis is low. The source of infection is the environment as human-to-human transmission has not been demonstrated. Due to the difficulty in isolation of mycobacteria by culture method caused by their slow growth, identification of MAH by molecular methods can be advantageous.

Two patients with enlarged neck lymph node were tested in our laboratory for confirming the diagnosis of mycobacterial lymphadenitis. We examined tissue samples from the enlarged area and tried to find the possible source of infection in the environment.

A method for DNA isolation from environmental samples was based on a protocol from commercially available kit with several modifications. Our developed qPCR is based on triplex semi-competitive real time qPCR reaction for the detection of specific insertion sequences IS*1245* (specific for *Mycobacterium avium* subsp. *avium* and MAH) and IS*901* (specific for MAA), as well as plasmid internal amplification control. This system allows quantification of both targets according to the plasmid gradients.

From the 21 samples tested from the environment of the first patient, ten samples (43.4%) were positive for presence of MAH DNA, and nine (39.1%) contained MAA DNA. The MAH DNA positive samples consisted mostly of pot soil from the patient's house. From the environment of the second patient, seven samples were examined by triplex qPCR. There were three positive samples for MAH DNA, and there was one sample positive for MAA DNA.
The analysis of the environmental samples by triplex real time qPCR offers two main advantages in comparison with traditional cultivation methods: faster detection with improved sensitivity. By the examination of the samples in the closest environment to the patients we proved that possible and most likely sources of MAH infection in both cases were present in their immediate surroundings.

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PP-13

TWENTY YEARS OF NTM IN MICROBIOLOGICAL LABORATORIES IN PUBLIC HEALTH ORGANIZATION OF BROD-POSAVINA COUNTY, SLAVONSKI BROD, CROATIA

Krištof Ž, Petanović M, Tomić Paradžik M

Department of Microbiology, Health Organization of Brod-Posavina County, V. Nazora 2a, 35000 Slavonski Brod, Croatia
Contact: zeljka.kristof@yahoo.com

Mycobacterium genus consists of approximately 100 species and they are, according to clinical relevance, divided in three groups: species pathogenic for people and animals (*Mycobacterium tuberculosis* complex, *M. leprae* and *M. lepraemurium*) that cannot be found in environment, second group is conditionally pathogenic for people and animals and most of the species come from the environment (*M. avium* complex – MAC) and third group are saprophyte mycobacteria, which are only exceptionally pathogenic, while the most of them are only accidental, unimportant findings or contamination of samples. Second and third group are often called "atypical mycobacteria", or non-tuberculous bacteria" (NTM).

According to the time of growth on selective media all NTM are divided in "fast-growing" and "slow growing", *i.e.* mycobacteria of fast and mycobacteria of slow growth. First works on NTM date as far back as early 1900, and only two decades ago they were recognized as possible cause of infections in humans.

Clinical samples submitted for tuberculosis test (79200) over a period of 20 years (1990–2009) were cultivated according to protocol on classic solid medium Lőwenstein-Jensen (L-J) and in liquid medium Mycobacteria Growth Indicator Tube (MGIT). Final identification of grown NTM was done in Croatian Institute for Public Health in Zagreb, in Department for bacteriological diagnostic of tuberculosis.

During tested period from various clinical samples we isolated totally 5062 mycobacteria and 448 (8,8%) were from NTM species. Totally in the period from 1990 to 2009 out of 79200 samples tested on tuberculosis and 448 (0.56%) NTM were isolated in Slavonski Brod.

Mycobacterium gordonae is the most frequent NTM, so 217 isolates (48.4%) of total NTM number is *Mycobacterium gordonae* from clinical samples.

Number of isolating NTM from clinical samples in Microbiological laboratory in Public Health Organization of Brod-posavina County is higher every year. The most often isolated NTM is *Mycobacterium gordonae*. Possible reason for increasing number of isolated NTM is cultivation of samples in liquid medium (MGIT) or increasing number of immunocompromised persons (HIV infections, malignant diseases, corticosteroid therapy). Taking into consideration that NTM are ubiquitous environmental bacterias and could be found in potable water, should be also considered about possible contamination of samples during processing in microbiological laboratory.

PP-14

PULMONARY NOCARDIOSIS IN AN MDR-TB PATIENT – CASE STUDY

Muntean Ionela[1], Ticau Oana[1], Rosales Senia[2], Hoffner S[2]

[1]Pneumophtisiology Hospital Brasov, Sitei street 17, 500260 Romania
[2]Swedish Institute for Infectious Disease Control (SMI), Nobels väg 18, SE17182 Solna Sweden
Contact: ionelasorina40@yahoo.com

Background. Romania is a country with a high incidence of TB infection and considerable MDR percentage. In our country, the incidence of *Nocardia* infections is unknown, being an unusual differential diagnostic with pulmonary TB.

Material and methods. Study of a prominent case treated in our hospital for pulmonary TB between 2002 and 2009.
A 35 years old patient, male, with low treatment compliance because of a limited intellectual level, but good physical health state, was diagnosed and treated for TB, based on clinical and radiological findings and confirmed by positive culture on Lowenstein-Jensen media.
Treated, was lost from the survey for 2 years and he came back, smear and culture positive for TB. The symptoms became severe and the radiological findings revealed extensive lesions in both lungs. A new cycle of treatment was applied till 2005, when the patient was declared MDR-TB.
After 3 years of intensive treatment, including the treatment for all the adverse reactions, in 2008, due to smear and culture negative results, the DOTS was stopped.
Next year, the patient was admitted again in the hospital with a serious clinical and radiological deterioration. Laboratory tests showed positive AFB smear microscopy and macroscopic atypical positive culture.
The *rpoB* sequencing was performed in SMI and was identified as *Nocardia asteroides*. After specific treatment for nocardiosis, the patient was completed healed.

Results. The patient was successfully treated for MDR-TB, despite the poor compliance. The next infectious episode, thought to be a relapse was demonstrated to be a pulmonary nocardiosis. The moment of the infection with *Nocardia* should be concomitant or successively with MDR-TB, due to immunosuppresed state of the patient.

Conclusions. On clinical and standard laboratory basis, pulmonary nocardiosis can be easily diagnosed as pulmonary TB, especially in patients previously treated. Here comes the incontestable role of the molecular tests. We strongly recommend higher degree of clinical suspicion regarding the possibility of nocardiosis in long term treated pulmonary patients.

PP-15

THE INCIDENCE OF POSITIVE SMEARS RELATED TO THE MACROSCOPIC ASPECT OF SPUTUM SAMPLES IN A REGIONAL REFERENCE LABORATORY FROM ROMANIA

Ionela Muntean, Oana Ticau

Pneumophtisiology Hospital Brasov, Sitei street 17, 500 260 Brasov, Romania
Contact: ionelasorina40@yahoo.com

Background. In Romania, in 2009, the incidence of TB cases was 99.9%. Compared with whole country, the central county Brasov has a half-incidence (50.9%). The Pneumophtisiology Hospital Brasov is performing medical services for 600,000 inhabitants.

Objective. Study the correlation between the macroscopic aspect of the sputum samples and the positivity rate in AFB smear microscopy in TB diagnostic.

Material and methods. Study of 868 positive cultures registered in the regional reference laboratory Brasov during one year (1.08.2008 – 31.07.2009).
From all the sputum samples, 576 (66.4%) were positive in the microscopic examination. Regarding the macroscopic aspect of the positive sputum samples, 502 (57.8%) were mucous-purulent, 320 (36.9%) were serous, 19 (2.2%) were hemorrhagic and 27 (3.1%) were saliva-like samples.
Results:
The positivity rate between the four macroscopic categories was: 66.8% in mucous-purulent sputum, 28.5% in serous sputum, 1.7% in hemorrhagic ones and 3% in saliva-like samples.
Speaking about AFB quantification, a considerably percentage (30%) from the saliva-like positive samples was "3+" smears.

Conclusions. Based on theoretical knowledge, it was expected that saliva-like sputum, usually excluded from bacteriological testing, to have the lowest percentage of positivity. According to these findings, we recommend that the first macroscopic selection made in the labs to be reconsidered. This step could lead us to delay the positive diagnostic or, more dramatic, to miss positive patients.

BRONCHOSCOPY AS PROCEDURE IN DIAGNOSIS OF LUNG TUBERCULOSIS

Prnjavorac B, Mehić J, Ajanović E, Sejdinović R, Irejz N, Halvadžić E

Ul. Braće Pobrić 17, 74260 Tešanj, Bosnia and Herzegovina
Contact: pbesim@bih.net.ba

Background. Bronchoscopy is not directed for first lines of of diagnostic procedures of tuberculosis. But, in everyday medical praxis it could be seen a lot of signs of previous tuberculosis (TBC) infection on bronchial walls during endoscopy. The most frequent sign of bronchial tuberculosis is antracosis.

Material and methods. We analyzed results of bronchoscopic procedure in last 12 month, during 2008. year performed in General Hospital Tešanj. The bronchoscopies were performed according to standard protocol. Among other, we analyzed signs of actual or previous tuberculosis, caseose necrosis or antracotic plaques and any signs of mucosa inflammation. We collected aspiration material for bacterial culture, direct microscopy for tuberculosis bacillus (BK) and culture of BK.

Results. During examined period we performed 414 broncnosopies. Among them there were 73 cases with antracotic plaques, 35 women and 38 men. In 36 cases antracosis was found on both sides of the lungs, in 19 cases only right, and in 17 cases only left. Mean aging was 69,23 (SD 9,3), rage from 35 to 85 years. Positive BK culture were in two cases, as well as BK was shown in direct microscopy. In two cases we found caseose necrosis. In seven cases we found suppuration. Others types of materials were analyzed according to schedules, for patohystology, cytology, and other examinations if something else was suspected.

Conclusion. Bronchoscopy could be one of diagnostic procedures if others failed to establish TB diagnosis. Many cases with antracosis were found as the sign of previous contact with BK. It is to be mentioned, bronchoscopy is not first choice for TB diagnosis.

Key Words. Bronchoscopy, tuberculosis, BK culture, antracosis.

PP-17

STUDY OF SPUTUM AND BRONCHOSCOPIC LAVAGE FOR ACID FAST BACILLI IN PATIENTS

Mehdinejad M, Hashemzadeh M, Jorfi M

Dept. of Microbiology, School of Medicine, Ahvaz Jondi Shapour University of Medical Sciences
Contact: Mehdinejad20@yahoo.com

The diagnosis of tuberculosis is based on the detection of *Mycobacterium tuberculosis* on clinical specimens with different methods. There are many techniques, such as molecular methods and direct examination of Acid fast stain and cultures. The aims of this study were determination of the reliability of acid-fast stain in diagnosis of suspected patients of pulmonary infections.

In the present study, 2872 specimens (sputum & bronchoscopic lavage) for laboratory diagnosis collected, specimens submitted for smear were stained with Ziehi Neelsen stain and examined under the light microscope for smear examination.

1726 (60%) specimens were isolated from male patients and 1146 (40%) were from female. There were 2758 sputum and 114 bronchoscopic lavage. One hundred eighty three (6.4%) of total specimens were positive for acid-fast bacilli which 18.6% were lavage and 81.4% sputum. Also, in specimens positive 60.7% were male and the female were 39.3%.

The results of the present study indicated that acid-fast stain (Ziehl Neelsen stain) is the best for all suspected tuberculosis cases. Specimens (sputum and lavage) were more in male patients than female.

PP-18

PLEURAL EFFUSION AND CLOSED PLEURAL BIOPSY

Tina Jerič, Aleš Rozman, Petra Svetina Šorli

University Clinic of Respiratory and Allergic Diseases Golnik, Golnik 36, 4204 Golnik, Slovenia
Contact: tina.jeric@klinika-golnik.si

Background. In Slovenia about 200 people are diagnosed with tuberculosis every year. Although the lungs are the major site for *Mycobacterium tuberculosis* infection tuberculous pleurisy is not so rare. Several methods are used to confirm the diagnosis and pleural biopsy is one of them. Closed pleural biopsy is used in pleural effusion of undetermined etiology (particularly lymphocytic) and useful in conditions where pleura is diffusely involved.

Aims. To evaluate the role of pleural biopsy in TB diagnostics in the University Clinic of Respiratory and Allergic Diseases Golnik. Although the efficacy of the procedure is operator-dependent closed pleural biopsy is reported to have a very high specificity.

Methods. Between 2004 and 2008 323 patients with unexplained lymphocytic pleural effusion had undergone closed pleural biopsy. 35 (10.8%) patients (23 (66%) men and 12 (34%) women) had been diagnosed with tuberculous pleurisy. In this retrospective study samples of sputum, diagnostic thoracocentesis, pleural effusion and tissue obtained during closed pleural biopsy were assessed.

Results. In all 35 cases all samples were microscopically negative. Cultures of sputa were rarely positive – only in 8 (23%) patients. Cultures of pleural effusion obtained at diagnostic thoracocentesis were positive in 20 (57%) patients and in 13 (37%) pleural effusions obtained during pleural biopsy. Samples of parietal pleura added significantly to the diagnostic yield while 25 (71%) cultures of pleural tissue were positive.

The biopsies were done by five operators (all men). There was only 1 major complication (hemorrhage), other 10 were minor (syncope, cough, one partial pneumothorax). All 35 patients had completed the standard regime of TB treatment.

Conclusions. When the etiology of lymphocytic pleural effusion is unknown closed pleural biopsy is a useful method. It is also a relatively safe procedure and has a high specificity in the diagnostics of TB pleurisy.

TUBERCULOSIS IN PATIENTS TREATED WITH BIOLOGICAL THERAPIES

Silva S, Azevedo C, Figueiredo P, Xerinda S, Gomes MH, Sarmento A

Infectious Diseases Service, Hospital S. João. Alameda Prof. Hernâni Monteiro, Porto, Portugal
Contact: cmazevedo@gmail.com

New biological therapies are being used in a more generalized way for the treatment of several diseases with an immunological basis. In consequence, reactivation of latent tuberculosis is being frequently diagnosed in these patients.

We made a retrospective review of clinical records of patients with tuberculosis and biological therapies admitted in our Infeccious Diseases Service since 2003. Diagnosis of tuberculosis was made by identification of *M. tuberculosis* in culture or by Polimerase Chain Reaction (PCR) in the appropriate clinical context. Demographic, clinical and analytical aspects were collected.

Six patients were identified. Four were male; age ranged from 41 to 80 (x=54.5±15.5) years. Four had Crohn disease, and two had psoriatic dermatitis. Five had previous treatment with prednisolone or azathioprine without disease control and had indication for infliximab, adalimumab (each prescribed for 3 to 5 moths before Tb diagnosis) or Mesassalazine (prescribed for more than 1 year). No pt had history of tuberculosis, and Mantoux test was negative in the 3 pts in which it was performed. No data about chest x-ray or sputum examination before therapy was available. At presentation all pts had fever, anorexia, weigh loss, asthenia and night sweats. Abdominal discomfort was registered in all Crohn pts. In the clinical examination lynph node enlargement was observed in three pts and no other clinical evidence of focal disease was detected. Chest x-ray was abnormal in four. Identification of M tuberculosis in culture of biological specimens was positive in 3 Pts, direct smear (Ziehl-Neelsen) was positive in 2 pts and PCR of ascitic fluid in one. Liver or lymph node biopsy was performed in one pt each. All pts began first line 4 anti-tuberculous drugs with slow clinical improvement, and hepatic toxicity was observed in 3 (50%). Four pts cured and two are still in treatment. Immunological treatment was withdrawn, during disease, with reactivation of their primary disease in psoriatic pts.

Tuberculosis has a high prevalence in Portugal and its screening is mandatory in every patient submitted to imunossupression, mainly the new biological therapies. Disseminated disease is frequent and unspecific symptoms may mimic the progression of basal disease and delay diagnosis and impair treatment.

PP-20

CLINICAL PRESENTATION OF DISSEMINATED TUBERCULOSIS AS AN AUTO-IMMUNE DISEASE

Quintas E[1], Silva H[2], Pinto J[3], Gomes MH[1], Sarmento A[1]

[1]Service of Infectious Diseases, [2]Internal Medicine and [3]Rheumatology, Hospital S. João and FMUP, Porto-Portugal
Contact: edna19d@hotmail.com

Extra pulmonary tuberculosis occurs in 10% of immunosuppressed individuals, most commonly in lymphatic nodes and reticuloendothelial system organs. The authors describe the case of a 56-year-old Caucasian man with fever, asthenia, non-pruriginous generalized maculopapular skin rash, lower limbs pain and joint stiffness for 3 months, with worsening in the week before admission. He lost 13.5 kg in 2 years. He had been started on prednisolone without response and was admitted in hospital. Physical exam was irrelevant, besides rash, and chest x-ray was normal. Laboratory tests: Hgb: 9.1g/dL microcytic normochromic, WBC: 11.5x109/L - neutrophils: 89.4%, PCR: 22.9mg/L, ADA: 24,3U/L, AcANA=1/1000; anti-nucleosome, anti-SSa and SSb tests were positive and dsDNA negative. Thyroid function, tumor markers, syphilis, *T. gondii*, B19 parvovirus, HSV, CMV, EBV, HIV, HBV, HCV, *R. conorii*, *Brucellla* and *C. burnetti*, serology were negative. Tuberculin test was negative. The anemia worsened (6g/dL), without blood loss and he needed transfusion, ESR> 100mm/1h; LDH: 468U/L; Coombs test was positive. Eosophago-gastroscopy and colonoscopy were normal. Severe auto-immune hemolytic anemia was considered and corticotherapy and immunoglobulins were prescribed without resolution; a splenectomy was performed after which the hemoglobin level stabilized. Nevertheless he remained febrile and cervical and axilary lymph nodes were detected - the largest 14x6mm; chest x-ray then revealed miliary pattern. Thoracic, abdominal and pelvic CT scan revealed multiple mediastinal retroperitoneal and mesenteric lymph nodes. *M. tuberculosis* was isolated in the gastric fluid and bronchial secretions. Histological examination of lymph nodes and spleen identified chronic granulomatous disease, ZN and culture positive for *M. tuberculosis*. Blood culture, uroculture, bronchial and bronchoalveolar wash fluid culture were negative. The patient was started on first line anti-tuberculous therapy with improvement and complete resolution of clinical and analytical manifestations.

Conclusion. Although the prevalence of tuberculosis is low in Western Europe, it is high in Portugal. We emphasize the unusual presentation of tuberculosis in this patient, with no previous known disease, who had characteristics of an autoimmune disease with anaemia so severe that indicated a splenectomy. The tuberculosis was revealed by the appearance of generalized lymphadenopaties, during hospital stay.

PP-21

COMPARISON OF NESTED-PCR TECHNIQUE AND CULTURE METHOD IN DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* FROM PATIENTS SUSPECTED TO GENITOURINARY TUBERCULOSIS

Khosravi AD[1,2], Hashemzadeh M[2], Seghatoleslami S[2]

[1]Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Dept. of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
Contact: khosraviaz@yahoo.com

Although *Mycobacterium tuberculosis* (MTB) is mainly affecting the lungs, however kidneys are the second target organ for the bacterium. While renal TB is uncommon in developed countries, as many as 15% to 20% of TB patients in developing countries are found with MTB in the urine. The diagnostic criterion for genitourinary tuberculosis (GUTB) is the isolation of MTB from urine. This is not easy to achieve, as the discharge of organisms into the urine is sporadic and, more importantly, involves few organism. The conventional method for diagnosing TB using clinical samples by the acid-fast bacilli (AFB) smear has low sensitivity and specificity and culture for MTB is time consuming. Due to the difficulties associated with diagnosing GUTB, there has been considerable interest in applying PCR methods for the detection of the disease.

The aim of present study was to evaluate the diagnostic value of nested PCR in GUTB compared with acid fast staining and culture method.

In total 200 urine samples from suspected cases of GUTB were collected during the period of study. Urine pellets were used for smear preparation, culture and DNA extraction by ether-chloroform method. Nested PCR was performed according to standard protocol using primers based on IS*6110* gene fragment. The results obtained by PCR were compared with those obtained by standard acid-fast bacilli stain and culture method.

Based on obtained results, the positivity rate of urine samples in this study was 5.0% by using culture and PCR methods and 2.5% for acid fast staining. Four out of total samples showed positive results in all three methods (2%). The sensitivity of PCR in this study was estimated as high as culture equal to 100%, while the sensitivity for direct smear staining was 41.6%.

PP-22

DRUG-INDUCED HEPATOTOXICITY DURING ANTITUBERCULOSIS CHEMOTHERAPY

Dobravc Verbič M[1,2], Toni J[1], Svetina Šorli P[1], Mrhar A[2]

[1]University Clinic of Pulmonary and Allergic Diseases Golnik, Golnik 36, 4204 Golnik, Slovenia
[2]University of Ljubljana, Faculty of pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia
Contact: matejdobravc@yahoo.com, janez.toni@klinika-golnik.si

Hepatotoxicity is one of the most important undesirable effects of isoniazid, rifampin and pyrazinamide, first line agents in antituberculosis chemotherapy. The aim of our study was to determine the incidence of drug-induced hepatotoxicity in patients, treated at the University Clinic Golnik. Risk factors, time for onset of drug-induced hepatotoxicity and treatment period were also evaluated.

We conducted a retrospective study, which included all patients with active tuberculosis treated during years 2006–2008. Hepatotoxicity was defined as the elevation of AST and/or ALT or serum bilirubin levels above the upper limit of normal or above increased starting values, taking into consideration the presence of clinical symptoms of hepatitis. Each parameter was analysed individually. Both univariate (using $\chi 2$ test or Mann-Whitney test) and multivariate analysis (using Binary logistic regression) were used to define potential risk factors.

48 out of 287 included patients (16.7%) developed drug-induced hepatotoxicity. Significantly associated with drug-induced hepatotoxicity in multivariate analysis were old age ($p<0.0005$), extensive tuberculosis disease ($p=0.017$) and high baseline AST ($p=0.009$). Comorbidity, malnutrition, concomitant use of other potentially hepatotoxic drugs, use of pyrazinamide and high baseline total and direct bilirubin showed statistically significant influence only in univariate analysis. Female sex, self-reported daily alcohol consumption, history of liver disease and high baseline ALT were not associated with development of drug-induced hepatotoxicity. The median time for onset of hepatotoxicity from the beginning of the treatment was 7 days (range 0–131). Treatment of tuberculosis was significantly longer in patients who developed hepatotoxicity (7.6 versus 6.1 months, $p<0.0005$).

The first estimation of incidence of drug-induced hepatotoxicity for Slovenian patients during antituberculosis treatment (16.7%) was similar to results of diverse foreign studies (5-33%). Risk factors found to be significantly associated with the development of hepatotoxicity were in accordance with the results of other researches. However, in comparison to other studies, liver tests were analysed individually and only high AST at baseline was associated with hepatotoxicity. Further investigations are needed to possibly confirm some other risk factors (*i.e.* malnutrition, alcohol use).

PP-23

STRAIGHTFORWARD DIFFERENTIATION OF *MYCOBACTERIUM ABSCESSUS*, *M. MASSILIENSE* AND *M. BOLLETII* CLINICAL STRAINS BY THE *ERM41* SEQUENCES AND CLARITHROMYCIN SUSCEPTIBILITY

Sylvaine Bastian[1,2], Edouard Macheras[3,4], Nicolas Veziris[1,2,5], Florence Brossier[1,2,5], Jean-Louis Gaillard[3,4], Vincent Jarlier[1,2,5], Emmanuelle Cambau[1,6,7]

[1]Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux, Paris, F-75013 France
[2]AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Service Bactériologie, Paris, F-75013 France
[3]Université Versailles-Saint Quentin, EA3647
[4]AP-HP, Hôpital Ambroise Paré, Service Bactériologie, Boulogne, F-92100 France
[5]Université Pierre et Marie Curie-Paris6, EA1541 Paris, F-75013 France
[6]Université Paris Diderot, EA3946, Paris, F-75010 France
[7]AP-HP, Hôpital Saint Louis, Service Bactériologie, F-75010, France

Contact: Emmanuelle CAMBAU, CHU Saint Louis, Bactériologie, 1, avenue Claude Vellefaux, 75010 Paris, France
Tel. + 33 1 42 49 93 48, Fax. + 33 1 42 49 92 00, emmanuelle.cambau@sls.ap-hop-paris.fr

Clarithromycin was the drug of choice for *Mycobacterium abscessus* infections until inducible resistance due to *erm41* gene was described in some strains. Since *M. abscessus* is now divided into *M. abscessus*, *M. massiliense* and *M. bolletii*, our objectives were to seek the presence of *erm41* in strains of the three new species and find correlation with clarithromycin resistance. Clinical strains were isolated from cystic fibrosis patients (n=54) and other pulmonary diseases (n=33). There were 46 *M. abscessus*, 27 *M. massiliense* and 14 *M. bolletii* identified on a molecular basis (*rpoB* and *hsp*). All strains were screened for *erm41* by PCR and clarithromycin MIC was determined by the broth microdilution method with extended incubation at day 14. *erm41* was present in all strains, although only 157 bps of the 5' end was found in the *M. massiliense* strains. This correlated with clarithromycin susceptibility (MIC90 of 1 µg/ml at day 14). All *M. bolletii* showed the same intact *erm41* and inducible clarithromycin resistance (MIC90 > 16 µg/ml at day 14). Although all *M. abscessus* strains harbored also an intact *erm41*, they differed from *M. bolletii* by promoter sequences. With regard to clarithromycin susceptibility, they distributed into one group with inducible resistance (MIC90 > 16 µg/ml at day 14) and a T28 in *erm41* and another group with susceptibility (MIC90 of 2 µg/ml at day 14) and a C28 in *erm41*. *M. massiliense* was easily differentiated from *M. abscessus* and *M. bolletii* using a PCR encompassing *erm41*, whereas *M. abscessus* and *M. bolletii* were differentiated by the *erm41* promoter sequences. Among *M. abscessus* isolates, the T28 or C28 *erm41* genotype correlated with clarithromycin resistance and susceptibility, respectively.

PP-24

POPULATION STRUCTURE OF MULTIDRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS FROM GERMANY

Leona Trübe, Sabine Rüsch-Gerdes, Stefan Niemann

National Reference Center for Mycobacteria, Research Centre Borstel, Parkallee 1, 23845 Borstel, Germany
Contact: ltruebe@fz-borstel.de

Resistant and multidrug resistant (MDR, resistance to at least isoniazid and rifampin) *Mycobacterium tuberculosis* complex (MTBC) strains have emerged worldwide and represent a serious challenge for global tuberculosis (TB) control. Even more worrisome is the existence of extensively drug resistant (XDR, defined as MDR *plus* resistance to any fluoroquinolone and injectable drugs) strains that has been documented in nearly all geographical regions surveyed so far. Interestingly, high rates of MDR TB have been associated with particular phylogenetic lineages of the MTBC such as the Beijing lineage and the strong clonal expansion of particular MDR strains.

Resistance testing and molecular fine typing (24 loci MIRU-VNTR-typing and spoligotyping) was performed for 179 MDR strains obtained in the years 1995, 2006 and 2007 from patients living in Germany.

Genotyping revealed that in the total population the majority of the strains belonged to the Beijing lineage (48%), followed by strains of the LAM (12%), Ural (4.5%), and Delhi/CAS (4%) lineages. Interestingly, there is a clear shift in the population structure from 1995 to 2006/2007: The rate of Beijing strains increased from 21 to 62% as well as the rate of Ural strains from 1.6 to 6%. On the contrary, the rate of LAM strains decreased from 21 to 8.5% as well as the rate of "T-type" strains (H37Rv-like) from 26 to 4%. This increase is mainly driven by two major Beijing clones (MLVA types 94-32, 100-32; MIRU-VNTR*plus* nomenclature) which represent approx. 30% of all strains in the years 2006/2007.

The Beijing genotype, which has been shown to be a major cause of resistant TB in several high incidence settings, is also frequent among MDR strains from Germany. The shift in the population structure caused by particular clones and reduced population diversity among Beijing strains argues for a strong clonal expansion of particular MDR strains in countries of the former Soviet Union, where the majority of German MDR TB patients came from.

PP-25

MDR-TB: HIGH-THROUGHPUT SCREENING TO IDENTIFY TRANSMITTING STRAINS VIA POPULATION GENETICS

Refrégier G, Abadia E, Zhang J, Clermont A, Sola C

IGEPE team, Institut de Génétique et Microbiologie, Université Paris-Sud 11, Bât. 400, rue Gregor Mendel, Faculté des Sciences, 91405 Orsay Cedex, France
Contact: guislaine.refregier@u-psud.fr

Introduction. The spread of Multidrug resistant tuberculosis threatens the progresses in TB management. Multi-resistance mainly arises from successive mutations in treated patients initially infected by sensitive strains. However, depending on drug policies, between 0.5 to 15% of the resistant strains are primary, *i.e.* resistant strains managed to transmit.

Methods. Two parameters control this proportion: mutation rate and transmission rate. Mutation rate may vary from one lineage to the other if they have different efficiencies in DNA repair. Transmission rate may vary according to the fitness of the strain which may be both determined by the resistance mutation and by compensatory mutations, so that they may vary among lineages.

Using published data on rifampicin resistant mutants in five different Indian cities and population statistics, we investigated whether some lineages produced resistant mutants 1) at a higher rate, or 2) that were more able to transmit.

Results and conclusions. We will discuss the preliminary results suggesting a higher transmissibility for Beijing strains, and higher mutability for modern strains (T and related).

We propose in addition a high throughput method for identifying most frequent rifampicin resistance mutations using Luminex technology, a method that will help us testing our hypothesis on a wider range of strains in the coming future.

PP-26

SURVEILLANCES, STUDYING EPIDEMIOLOGY OF DRUG RESISTANT TUBERCULOSIS IN SAUDI ARABIA AND THEIR IMPACTS ON THE NATIONAL TUBERCULOSIS PROGRAM

Bright Varghese, Ruba Al-Omari, Mais Al-Hirbawi, Ranyah A Moneim, Sahal Al-Hajoj

Tuberculosis Research Unit, Department of Biological and Medical Research, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia
Contact: hajoj@kfshrc.edu.sa

Despite the global efforts to combat tuberculosis, the disease remains a major public health problem worldwide especially in the developing countries. Saudi Arabia stands with a different population structure of admitting 6 million expatriates and 3 million annual pilgrims mainly from highly TB prevalent countries. The major objective of the study is to conduct a nationwide survey of all positive (proven by culture) cases (both smear positive and negative falling into all age groups) of tuberculosis to determine the drug resistance rate and its impact on the national tuberculosis program. Drug susceptibility to the four first line drugs (SIRE) was carried out for 978 among 1633 isolates collected so far using non-radiometric fully-automated Bactec MGIT-960 system (Becton-Dickinson, Maryland, USA).

The results show the proportion of mono and multidrug resistance as 26%. The amount of MDR-TB reported is 5%. There is 18% of Streptomycin resistance, 13% of Isoniazid resistance, 6% of Rifampicin resistance and 8% of Ethambutol resistance reported among the isolates tested so far.

The current study is the first of its type to be carried out in the country in concordance with the WHO-TB survey guidelines and under supervision from the WHO experts. Despite the fact that the result is preliminary, the findings showed the Tuberculosis drug resistance is on an inclining status in the country and the rate of MDR-TB is really alarming. The results suggest that continuous effort should be directed at the prevention of MDRTB infection and its transmission.

PP-27

PERFORMANCE OF GENOTYPE MTBDR*plus* ASSAY FOR RAPID DETECTION OF RESISTANCE TO RIFAMPIN AND ISONIAZID IN *M. TUBERCULOSIS* STRAINS

Satana D, Uzun M, Erturan Z

Istanbul University, Istanbul Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Istanbul 34390, Turkey
Contact: dileksatana@yahoo.com

Mycobacterium tuberculosis remains one of the most significant causes of death from an infectious agent because of the increase in multi-drug resistant (MDR) strains recently. Rapid drug susceptibility testing and early-targeted therapy may be crucial to inhibit the further spread of MDR-TB. The GenoType MTBDR*plus* assay (Hain Lifesciense GmsbH, Germany) is a novel kit-based method for the detection of the most common mutations in *rpoB*, *katG*, and *inhA* genes.To evaluate the usefulness of the test for rapid detection of resistance to RMP and INH, total of 82 strains were included in this study and the results obtained by the GenoType MTBDR*plus* assay were then compared with the results obtained by phenotypic drug susceptibility testing (by BACTEC TB 460 system, Becton Dickinson, Sparks, MD).

The GenoType MTBDR*plus* assay identified 81 strains (98.7%) with mutations in *rpoB* gene; only 1 strain could not be recognized as RMP resistant by the assay since probably it had a mutation outside of the *rpoB* hot spot region. Codon 531 and 533 were the most frequently affected in 60 of 82 isolates (73%), with 51 (62.2%) isolates showing the amino acid exchange of serine to leucine at the codon 531. The nine other strains (10.97%) had a mutation located in the region from codon 531 to 533.

Of the 82 isolates that were phenotypically resistant to INH, 41 (50%) had Ser315Thr mutation in *katG* codon 315, and 35 (42.68%) had mutation in *inhA* gene. The remaining 6 (7.31%) INH resistant isolates did not contain a mutation in this region.

The GenoType MTBDR*plus* assay was found to be a useful method for rapid screening of *M. tuberculosis* isolates obtained from patients suspected of having resistance to RMP and INH. However, the molecular drug susceptibility testing results should always be confirmed by phenotypic methods.

PP-28

GENOTYPE MTBDR*sl* ASSAY FOR FLUORQUINOLONE, AMIKACIN-CAPREOMYCIN AND ETHAMBUTOL RESISTANCE TESTING OF *MYCOBACTERIUM TUBERCULOSIS*

Ruiz P, Gutierrez J, Causse M, Zerolo J, Casal M

Mycobacteria Reference Center, Mycrobiology Service, Hospital "Reina Sofía", Córdoba, Spain
Contact: mi1rumap@uco.es

The world guide emergence of extensively drug-resistant tuberculosis (XDR-TB) is a serious global health problem. The actual incidence, make necessary the second line drug susceptibility testing (DST) and in a short time. This is possible with genetic methods, to detect mutations that cause resistance. The GenoType *Mycobacterium tuberculosis* drug resistance second line (MTBDR*sl* assay) (Hain Lifescience, Nehren, Germany) is an assay thet was developed with a specific focus on the most prevalent *gyrA*, *rrs* and *embB* gene mutatios to detection fluorquinolones (FQs), Amikacin-Capreomycin (AM-CM) and ethambutol (EMB) resistance associated mutations in culture and directly clinical samples. The aim of the present study was determine the efficacy of this method against conventional methods.

A set of 85 strains of *Mycobacterium tuberculosis* were used. All strains were isolated to discard mixtures and identified by biochemical, HPLC, ACCuprobe or GenoType. First and second-line drug (Amikacin (AK) 1.0 µg/ml, capreomycin (CM) 2.5 µg/ml, kanamycin (K) 1µg/ml, ofloxacin (OF) 2.0 µg/ml, ciprofloxacin (CIP) 2.0 µg/ml, moxifloxacin (MOX) 2.0 µg/ml, levofloxacin (LVX) 4.0 µg/ml, susceptibility testing were made with Bactec MGIT 960 system and GenoType MTBDR*sl* were used to detect mutation to ethambutol (EB), Amikacin (AK) / capreomycin (CP) and fuorquinolones (FQs). Sequencing of specific DNA fragments was performed only if any discrepancy was observed between both systems.

With the phenotypic study, 11 strains were susceptible to first line drugs, 52 strains were resistant to some first line drug and 30 strains were resistant to second-line drugs. With the genotypic study, 68 strains were susceptible and 14 were resistant. The disagrees strains were: 3 strains resistant with MGIT to ofloxacin and sensibles the other quinolones and with MTBDR*sl* the mutation in *gyrA* were detected; 2 strains were resistant with MGIT to capreomycin, 1 resistant to capreomycin and kanamycin and 1 resistant to amikacin, kanamycin and capreomycin and no mutations were detected; 2 strains were resistant to ethambutol but no mutation was detected an other 2 strains were detected mutation and were sensible in the MGIT. The sequencing confirme the MTBDR*sl* results.

Genotype MTBDR*sl* assay represent a reliable tool for the detection of fluorquinolone and amikacin/capreomycin and to a lesser extent also ethambutol resistance.

PP-29

DRUG RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* IN KOSOVA

Gjyle Mulliqi-Osmani[1,2], Xhevat Kurhasani[3], Fatmir Bislimi[1], Ariana Kalaveshi[1,2], Lul Raka[1,2], Bahri Tigani[4], Naser Ramadani[1,2], Rukije Mehmeti[4]

[1]National Institute of Public Health of Kosova, Rrethi i spitalit, p.n. 10000 Prishtina, Kosova
[2]Faculty of Medicine, University of Prishtina, Rrethi i spitalit, p.n. 10000 Prishtina, Kosova
[3]Director of NGO "KeA", Bul.Nena Tereze, p.n., 10 000 Prishtina, Kosova
[4]National Tuberculosis Program, TB Global Fund Project, Program Implementation Unit, Ministry of Health of Kosova, Rruga e Zagrebit, p.n., 10000 Prishtina, Kosova
Contact: gjylemulliqi2001@yahoo.com

Drug resistant tuberculosis is major public health-problem in developing countries and reflects the quality of tuberculosis control. From 2000 to 2009, a total of 12.032 new cases of TB were notified in Kosova, of which 211 had fatal outcome. Incidence rate during same period decreased from 84.3 into 42.8 cases per 100.000 population. The purpose of this study was to determine the resistance pattern of *Mycobacterium tuberculosis* to antituberculosis drugs in Kosova.

A retrospective survey was conducted of all new cases of pulmonary tuberculosis with positive culture for *M. tuberculosis* during time period 2002-2009. All isolated strains were grown in Lowenstein-Jensen media and tested against isoniazid (INH), rifampicin (RIF), streptomycin (SM) and ethambutol (ETH) using the proportion method. Bacteriologic examinations were performed at the Reference Laboratory for Tuberculosis in the Department of Microbiology within the National Institute for Public Health of Kosova.

A total of 22.412 clinical samples were processed in TB laboratory, of which 1.415 positive cases were subjected to *in vitro* drug sensitivity test against the first line drugs. Overall 1.415 positive cases of *M. tuberculosis* (83.4%) were susceptible to all first line antituberculosis drugs. Resistance to at least one drug was found in 232 patients (16.4%). Monoresistance of 14.3% (203 cases) was seen against streptomycin, 6% against ethambutol and only 0.63% to isoniazid. Seventy four patients (5.22%) had dual-resistance, eight patients presented resistance against three drugs and nine of them (0.63%) were resistant to four antituberculotics (SM, ETH, INH and RIF). During the last eight years in Kosova twenty five patients (1.74%) were registered as multi-drug resistant.

Monoresistance pattern to antituberculosis drugs was high in Kosova. Fortunately, multidrug resistance tuberculosis was not common. High incidence of TB in Kosova requires joint efforts to monitor the prevalence of drug resistance tuberculosis.

PP-30

TWO CASES OF MDR TB

Müllerová Maria[1], Vašáková M[2], Kopecká E[2], Homolka J[2], Pavlík E[1]

[1]Department of Microbiology and Molecular Biology TB Klinlab Ltd.; Clinical Laboratories Střešovice, U vojenské nemocnice 1200, 16900 Prague 6, Czech Republic
[2]Pneumologická klinika, I. LF UK and FTNsP, Vídeňská 800, 140 00 Prague 4, Czech Republic
Contact: Tel. +420 620 253 941, maria.mullerova@klinlab.cz

Tuberculosis is an infectious disease, which is a worldwide problem even in the 21st century. In the Czech Republic (CZ), which has a low incidence of TB (around 10) for a long time, recently the MDR TB becomes a problem. What is also causing this, is the location of the CZ, which is the cross-road of Europe both for migration and asylum.

We tested strains of M.TB patients coming from approximately 20% of the CZ population. From January 1st, 2003 to December 31st, 2009 1,514 strains were tested, of which 514 were resistant to AT and 91 of them where resistant on INH+RIF (MDR TB). MDR TB strains: men - 30 domiciled, 41 foreigners; women - 11 domiciled, 9 foreigners.
Drug susceptibility tests were performed by conventional methods and by BACTEC MGIT 960 (S.I.R.E.+PZA).

1st case history: Z.A. (M) from Belarus, age 33 years
In 2005 we diagnosed TBC of respiratory system. Repeatedly we confirmed positivity, both Mi+Cult from sputum. We found resistance to INH+RIF. Combined treatment by AT was applied according to extended susceptibility both inpatient and outpatient for 3 years. After negativisation of sputum Mi+Cult the cavern persisted. Before the planned excision the pos of sputum was found, both Mi+Cult. The treatment lasts until today (both inpatient and outpatient), with the exception of the short period of negativisation.

2nd case history: K.H. (F) from CZ, age 31 years
In 2008 CT of chest suggested suspect TB/aspargillosis.
From bioptic material: The histology pointed to a specific pulmonary process therefore treatment with first line AT was introduced. BK was Mi neg, Cult pos.
The result of identification and susceptibility tests: M.TB resistant to INH+RIF. The result from lavage was performed giving positive results in all test systems *i.e.* MTD - T2, BACTEC MGIT 960, Cult, Quanti Feron TB Gold in Tube. The strain was again identified as M.TB with primary MDR. Therefore, the treatment with second and third line AT was introduced. The histology confirmed specific pulmonary process with caseous powder neucrosis. Sputum and laryngeal swabs on BK were always neg (both Mi+Cult). The patient was hospitalised and treated for 8 months on MDR special unit and afterwards was successfully treated outpatient after 12 months.

The MDR TB disease deserves long-lasting and financially demanding treatment, which affects the patient's health and economics of the medical facility. Modern laboratory methods help to rapidly identify MDR TB and lower the risk of contact.

PP-31

EVALUATION OF THE GENOTYPE MTBDR*plus* ASSAY FOR RIFAMPIN AND ISONIAZID SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* STRAINS IN SERBIA

Živanović I[1], Stefanović G[2], Dakić I[3], Savić B[3], Rüsch-Gerdes S[4]

[1]School of Medicine, University of Belgrade, Dr Subotica 1, 11000 Belgrade, Serbia
[2]Institute of Lung Diseases and Tuberculosis, Clinical Centre of Serbia, Dr Koste Todorovica 2, 11000 Belgrade, Serbia
[3]Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Dr Subotica 1, 11000 Belgrade, Serbia
[4]National Reference Centre for Mycobacteria, Parkallee 1-40, D-23845 Borstel, Germany
Contact: irence@gmail.com

Multidrug-resistant (MDR) tuberculosis (TB) is a major health problem worldwide. Conventional drug susceptibility testing (DST) is a long-term process and, thus, molecular methods for rapid recognition of MDR *Mycobacterium tuberculosis* strains have become essential for the early administration of appropriate therapy and prevention of MDR TB spread. The aim of the present study was to evaluate GenoType MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany), as a diagnostic tool for detection of resistance to rifampin (RMP) and isoniazid (INH) in MDR *M. tuberculosis* strains isolated in Republic of Serbia during 2008 and 2009.

The total number of MDR *M. tuberculosis* strains isolated in 4 regional TB laboratories in Serbia over the study period was 73. All strains were identified by biochemical tests, while the DST was performed by the proportional method on Löwenstein-Jensen medium. Since subcultures for 9 strains were not available, the number of isolates that were retested in the Supranational Reference Laboratory (SRL) at Forschungszentrum Borstel, Germany, was 64.

Out of these 64 isolates, 58 were confirmed as MDR *M. tuberculosis*. One strain was misidentified as *M. tuberculosis* and DNA sequencing revealed its true identity as *M. arupense*, while in the remaining 5 misidentified strains the results of DST were incorrect. All 64 strains were also examined by the MTBDR*plus* assay, which has only recently been introduced in Serbia. The results obtained by the MTBDR*plus* assay were in complete agreement with the results of SRL *i.e.* the molecular assay detected the same 58 MDR TB strains. Out of 6 remaining isolates, 1 was not identified as *M. tuberculosis*, while 5 were resistant to INH and susceptible to RMP. The predominant mutation in *rpoB* gene was MUT1 and it was detected in 44 MDR *M. tuberculosis* strains, while the most frequent mutation in *katG* gene, MUT1, was revealed in 55 MDR strains. The results of our survey, which included 87.7% of all MDR TB strains isolated in Serbia over the study period, clearly show that the GenoType MTBDR*plus* is a sensitive and specific tool for rapid detection of resistance to RIF and INH in *M. tuberculosis* strains. They also indicate that the assay is as effective in a laboratory in a resource-limited country as in technically superior laboratories in industrialized countries.

PP-32

USING GENOTYPE® MTBDR*plus* FOR DETECTION OF *M. TUBERCULOSIS* MULTI-DRUG RESISTANCE

Zalutskaya AM, Sahalchyk ER, Nikolenko AN, Skrahina AM

Republican Scientific and Practical Centre for Pulmonology and Phthisiology, Department of Laboratory Diagnostic and Treatment of Tuberculosis, Dolginovsky tr. 157, 220053, Minsk, Belarus
Contact: akasana@inbox.ru

The purpose of the study was the estimation of efficiency of *M. tuberculosis* MDR detection using GenoType® MTBDR*plus* in comparison with LJ medium.

We studied 78 Mtb strains isolated from sputum specimens of pulmonary TB patients. Coincidence of MDR detection results using LJ medium and GenoType® MTBDR*plus* was obtained in 75 cases (96.2±2.2%), including 40 MDR strains (51.3±5.7%), 34 (43.6±5.6%) susceptible strains and 1 strain (1.3±1.3%) resistant to INH. Using GenoType® MTBDR*plus*, we found MDR in 2 strains (2.6±1.8%) that were shown to be susceptible to INH and RIF and susceptibility to RIF in 1 strain (1.3±1.3%) that was shown to be resistant using the bacteriological method. In our study, we found the sensitivity of GenoType® MTBDR*plus* of 97.6%, specificity – 94.6%, PPV – 95.2%, NPV – 97.2%, efficiency – 96.2% compared with the ”gold standard” – bacteriological method.

Among 42 Mtb strains resistant to RIF, 14 (33.3±7.3%) had H526D mutation, 21 (50.0±7.7%) had S531L mutation, 2 (4.8±3.3%) had mutations in both 526 and 531 *rpoB* codons. Among 44 Mtb strains resistant to INH, 43 (97.7±2.3%) had S315T1 mutation in *katG* gene, 19 (43.2±7.5%) had C15T mutation in *inhA* gene, 8 (18.2±5.8%) had T8A mutation in *inhA* gene, 2 (4.5±3.1%) had both C15T and T8A mutations in *inhA* gene. 1 Mtb strain (2.3±2.3%) resistant to INH had a mutation in *inhA* gene only.

Thus, we found high efficiency of Mtb MDR detection using GenoType® MTBDR*plus* and high frequency of mutations localization in codons 526 and 531 of *rpoB* gene, codon 315 of *katG* gene, codons 15 and 8 of *inhA* gene.

PP-33

DEVELOPMENT OF THE NITRATE REDUCTASE ASSAY (NRA) FOR THE RAPID AND SIMULTANEOUS DETECTION OF MDR AND XDR-TB IN *M. TUBERCULOSIS*

Estel Ramos[1], Krista Fissette[1], Pim de Rijk[1], Ivan Hernández-Neuta[2], Patricia Del Portillo[2], Juan Carlos Palomino[1], Anandi Martin[1]

[1]Institute of Tropical Medicine, Nationalestraat, 155, 2000 Antwerp, Belgium
[2]Corpogen, Cra 5 N°66A-34 Bogota, Colombia
Contact: amartin@itg.be

Tuberculosis (TB) represents a major public health problem, especially in low-resource countries where the burden of the disease is more important. Multidrug-resistant (MDR-TB) and extensively drug resistant (XDR-TB) pose a serious problem to TB control programmes. The spread of MDR-TB and XDR-TB demands new tools for rapid and accurate drug susceptibility testing and stress the need for sensitive, quick and affordable diagnostic tools. The proportion method currently used for the detection of drug resistance in TB is time-consuming and slow requiring several weeks for definitive results.

The objective of this study was to evaluate the performance of the nitrate reductase assay (NRA) for the simultaneous detection of MDR- and XDR-TB in *M. tuberculosis*. Resistance to rifampicin (RIF) and isoniazid (INH) will identify MDR-TB patients and resistance to kanamycin (KAN) and ofloxacin (OFLO) are predictors of XDR-TB. We have evaluated 141 *M. tuberculosis* strains, 33 were fully susceptible, 88 were MDR, 12 XDR and 8 polyresistant, and we compared the results to the gold standard proportion method. The NRA assay was highly accurate in detecting resistance to RIF and INH, with sensitivity and specificity higher than 95%. For OFLO and KAN also good results were obtained with an overall sensitivity and specificity higher than 90%. The turnaround time of NRA was 11.6 days compared to 4 to 6 weeks for the LJ.

In conclusion, the NRA is simple to perform, results are easy to interpret by a visual change of color and provide accurate results for the simultaneous detection of MDR and XDR-TB. The assay could be easily implemented in countries with limited laboratory facilities.

PP-34

NONTUBERCULOUS MYCOBACTERIA IN PATIENTS WITH CYSTIC FIBROSIS AND THEIR SUSCEPTIBILITY TO ANTIBIOTICS

Deguillaume Y[1], Héry-Arnaud G[1,2], Rahajamanana L[3], Maugein J[3], Abalain ML[1,2]

[1]Laboratoire de Microbiologie, CHU de Brest, France
[2]Laboratoire Universitaire de Biodiversité et Ecologie Microbienne-EA3882, Faculté de Médecine, Brest
[3]Laboratoire de Bactériologie, CHU de Bordeaux, France
Contact: marie-louise.abalain@chu-brest.fr

Patients with cystic fibrosis (CF) are considered having a risk of pulmonary infections due to nontuberculous mycobacteria (NTM). In France, *Mycobacterium abscessus*, *M. avium*, *M. intracellulare* are the 3 most frequent NTM in these kind of infections and their pathogenicity in CF is now recognized. However, these species are difficult to eradicate because of their multiresistance to antibiotics. We have evaluated drug susceptibility testing of 36 NTM strains (17 *M. abscessus*, 9 *M. avium*, 10 *M. intracellulare*) isolated from 35 patients with CF. The minimum inhibitory concentration (MIC) of a panel of antibiotics was determined with the microdilution plate method (Trek diagnostic systems), using rapid growing mycobacterium plates for *M. abscessus* and slow growing mycobacterium plates for *M. avium* and *M. intracellulare*. Interpretation of MICs was done according to the guidelines of the Clinical and Laboratory Standard Institut (CLSI). The most active antibiotics against *M. abscessus* were clarithromycin, tigecyclin and amikacin with a susceptibility of 100%, 100% and 94% respectively. Susceptibility of *M. abscessus* to linezolid (41%) was limited. The most active antibiotics against *M. avium* and *M. intracellulare* were amikacin, clarithromycin and ethambutol with a susceptibility of 100%, 94% and 73% respectively. Susceptibility to rifabutin was better for *M. intracellulare* (90%) than for *M. avium* (44%).

Microdilution plate method appears as a convenient drug susceptibility testing technique with a simple and consistent endpoint determination. Easy set-up procedures and incubation provide results in 3-5 days for rapid growing mycobacteria to 14 days for slow growing isolates.

PP-35

TB DRUG RESISTANCE IN A LOW ENDEMIC AREA 2004-2009: QUANTITATIVE DRUG SUSCEPTIBILITY TESTING AND GENOTYPING

Lucke K[1,2], Ritter C[1,2], Friedel U[1,2], Böttger EC[1,2]

[1]Institut für Medizinische Mikrobiologie, University of Zurich, Switzerland
[2]Nationales Zentrum für Mykobakterien, University of Zurich, Switzerland
Contact: klucke@imm.uzh.ch

Tuberculosis continues to be a significant health care problem, not only in the developing countries. Worldwide, the present trend is characterized by an alarming emergence in drug resistance, raising concerns of future epidemics of virtually untreatable TB. Given the limited therapeutic options in MDR (and especially XDR) tuberculosis, it is essential to define the resistance levels and mechanisms present in clinical isolates categorized as drug resistant on the basis of critical concentration testing, in order to facilitate therapeutic decisions.

We determined quantitative resistance levels of drug resistant clinical isolates of *Mycobacterium tuberculosis* sampled in Switzerland over the past 6 years with the view to determine if therapeutic options with first-line drugs still exist in these isolates. Rifampicin resistant isolates unanimously showed a high-level drug resistant phenotype (>50 mg/L) associated with mutations in *rpoB*, indicating that rifampicin will have no efficacy in treatment. In contrast, a significant fraction of clinical TB isolates categorized as isoniazid resistant showed a low-level resistant phenotype (mostly mutations in *inhA*). Ethambutol resistance occurred mostly in MDR strains and was associated with alterations in *embB*, but resistance never exceeded 25 mg/L. These data lead us to suggest that some strains categorized by *in vitro* drug susceptibility testing as resistant to isoniazid or ethambutol may still respond to a treatment regimen including these agents.

PP-36

TLYA MUTATIONS IMPORTANT FOR CAPREOMYCIN RESISTANCE IN *IN VITRO* SELECTED MUTANTS BUT NOT IN CLINICAL ISOLATES OF *MYCOBACTERIUM TUBERCULOSIS*

Anna Engström[1,2], Nasrin Perskvist[1], Jim Werngren[1], Sven E Hoffner[1,2], Pontus Juréen[1]

[1]Swedish Institute for Infectious Disease Control (SMI), Nobels väg 18, SE-17182, Solna, Sweden
[2]Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Box 280, SE-171 77 Stockholm, Sweden
Contact: anna.engstrom@smi.se

The cyclic peptide capreomycin (CAP) and the aminoglycosides amikacin (AMK) and kanamycin (KAN) are key drugs for the treatment of multi-drug resistant tuberculosis (MDR-TB). Resistance to the aminoglycosides is associated with mutations in the 16S rRNA gene *rrs*. Both *tlyA* and *rrs* have been suggested to be involved in CAP resistance. *tlyA* encodes for methyltransferase that methylates the 16S and 23S rRNA. CAP inhibits mRNA–tRNA translocation on the ribosome. Previous reports on CAP resistance have mainly been based on *in vitro* selected CAP resistant mutants where *tlyA* mutations were commonly seen. However, in CAP resistant clinical isolates of *M. tuberculosis*, *tlyA* mutations are not as frequent. We wanted to investigate if CAP resistance caused by *tlyA* mutations mainly is an *in vitro* phenomenon.

We sequenced *rrs* and *tlyA* of 19 independent *in vitro* selected CAP resistant mutants and 42 CAP resistant *M. tuberculosis* clinical isolates. For all mutants and isolates, we also determined the minimal inhibitory concentration of CAP, AMK and KAN.

In general, both the *tlyA* and *rrs* genotypes, and the drug-resistance pattern were significantly different between the *in vitro* selected CAP resistant mutants and CAP resistant clinical isolates. Mutations in *tlyA* were mainly found in mutants, which were resistant to CAP only. The majority, 40 out of the 42 clinical isolates had a mutation at position 1401 in *rrs* and they were resistant to CAP, AMK and KAN. Only 2 of the 42 CAP resistant clinical isolates were found to have a *tlyA* mutation.

Our findings indicate that mutations in *rrs* are involved in resistance to CAP, AMK and KAN. This suggests that *tlyA* is not a suitable genetic marker for detection of CAP resistance in *M. tuberculosis* clinical isolates. A strain with a wildtype *tlyA* gene is not necessarily susceptible to CAP, as it may harbor an *rrs* mutation giving resistance to CAP, AMK and KAN.

PP-37

METHOD OF DETERMINING CRITICAL CONCENTRATIONS OF ANTI-TUBERCULOSIS DRUGS OF LAST RESORT TO ASSESS *MYCOBACTERIUM TUBERCULOSIS* DRUG SUSCEPTIBILITY BY ABSOLUTE CONCENTRATIONS METHOD USING LOWENSTEIN-JENSEN MEDIUM

Stepanshina VN[1], Mukhina TN[1], Nizova AV[1], Mironova RI[1], Shemyakin IG[1], Cegielski P[2]

[1]State Research Center for Applied Microbiology & Biotechnology, Obolensk, Moscow region, Russia
[2]Centers for Disease Control, CDC, Atlanta, USA
Contact: Tel. +7 (4967) 36 00 60, Fax. +7 (4967) 36 00 10, stepanshina@obolensk.org

A method for determining drug critical concentrations is urgently needed and is of practical significance because of the lack of scientifically justified criteria for assessing critical concentrations of drugs of last resort commonly used for anti-tuberculosis therapy. As a result, treating multi-drug resistant patients is mediated by empirical guidelines and is not always confirmed with validated data on the development of *M. tuberculosis* resistance to drugs of last resort.

To determine critical concentrations of kanamycin, capreomycin and ofloxacin a standard panel was used. The panel involved 100 genetically diverse *M. tuberculosis* cultures tested for mutations in target fragments of the bacterial genome. There were both "probably drug-susceptible" and "probably drug-resistant" *M. tuberculosis* cultures. "Probably drug-susceptible" strains were isolated from sputa of patients never treated with the drugs, carrying no mutations in target genes of *M. tuberculosis*. "Probably drug-resistant" cultures were from patients given with one of the drugs over a one month, carrying mutations in *M. tuberculosis* target genes. Critical concentrations were determined at inflection points in curves based on minimal inhibiting concentrations identified both for "probably drug-susceptible" and "probably drug-resistant" *M. tuberculosis* cultures through two-fold serial titration of the drugs in Lowenstein-Jensen medium. A critical concentration of the drug was the concentration that inhibited the growth of over 95% "probably drug-susceptible" clinical strains and provided the growth of more than 95% "probably drug-resistant" *M. tuberculosis* strains.

PP-38

USE OF HIGH-DOSE ISONIAZID THERAPY FOR ISONIAZID-RESISTANT TUBERCULOSIS IN LISBON, PORTUGAL

Rita Macedo, Irene Rodrigues, Arlinda Lopes, Edna Pereira

Public Health Laboratory: Micobacteriology/Tuberculosis, Public Health Department, Administração Regional de Saúde de Lisboa e Vale do Tejo, I.P.
Contact: rita.macedo@csalcantara.min-saude.pt

Portugal has an incidence of about 7% resistance to isoniazid (INH) amongst multidrug resistant (MDR) and non-MDR tuberculosis (TB). Unexpectedly the most prevalent mutation found conferring resistance to INH occurs in the promoter region of *mabA-inhA* gene, which is in contradiction with the majority of the published studies, where the most prevalent mutation found is in *katG* gene. We also verify a high level of cross-resistance between isoniazid and ethionamide. Since this cross-resistance is frequently associated with low-level INH resistance, usually due to a mutation in the regulatory region of the *mabA-inhA* operon, and this particular mutation exists at a high frequency in Portuguese INH-resistant isolates, it is very likely that these isolates are susceptible to high-doses of isoniazid (900 mg per day). If so, inclusion of high-dose INH as an adjuvant in the treatment regimen would probably improve the outcome.

The aim of our study was to evaluate the *in vitro* sensitivity to high-doses of isoniazid in the INH-resistant *Mycobacterium tuberculosis* strains isolated in our laboratory from patients living in Lisbon area.

We have analyzed 60 strains amongst MDR, poli-resistant and mono-resistant, and we have verified that 17 of these strains (28%) were susceptible to a high-dosage of INH.

Therefore, in Portugal, in presence of an INH-resistant case we encourage clinicians to request DST for isoniazid at 0.4 µg/ml.

PP-39

QUANTITATIV DRUG SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* USING MGIT AND EPICENTER SOFTWARE

Lucke K[1,2], Ritter C[1,2], Friedel U[1,2], Böttger EC[1,2]

[1]Institut für Medizinische Mikrobiologie, University of Zurich, Switzerland
[2]Nationales Zentrum für Mykobakterien, University of Zurich, Switzerland
Contact: klucke@imm.uzh.ch

In the diagnostic laboratory, drug susceptibility testing of mycobacteria is substantially different from general testing procedures used in bacteriology. Rather than to determine minimal inhibitory concentrations, mostly a single drug concentration, termed the "critical concentration", is used to categorize a clinical isolate as susceptible or resistant. However, genetic analyses have indicated that different resistance mutations are associated with different levels of phenotypic drug resistance, *i.e.* low-level, moderate-level, and high-level drug resistance. In addition, a molecular defined resistance mutation may be associated with various phenotypic resistance levels pointing to as yet unknown genetic modifiers of resistance expression. These findings testify to the limitations in current procedures for drug susceptibility testing (DST). We have established the conditions for quantitative DST for both first and second line agents using non-radiometric MGIT 960 instrumentation and EpiCenter software equipped with the TB eXiST module. Extended comparative analysis on a range of susceptible and resistant clinical isolates has allowed us to propose conditions for testing, and to develop criteria for interpretation, *e.g.* susceptible, intermediate, and resistant. Following evaluation we have successfully implemented MGIT 960/TB eXiST based quantitative DST for first and second line drugs in our diagnostic laboratory.

PP-40

FITNESS IN RIFAMPICIN AND STREPTOMYCIN RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM BRAZIL

Fernanda Spies[1], Andrea von Groll[2], Pedro Eduardo Almeida da Silva[2], Anandi Martin[3], Juan Carlos Palomino[3], Maria Lucia Rossetti[1], Arnaldo Zaha[1]

[1]Universidade Federal do Rio Grande do Sul – Avenida Bento Gonçalves, 9500 – Porto Alegre – Brazil
[2]Universidade Federal do Rio Grande - Rua Visconde de Paranaguá, 102 – Rio Grande – Brazil
[3]Intitute of Tropical Medicine – Nationalestraat 155 – Antwerpen - Belgium
Contact: nandaspies@gmail.com

Multidrug-resistant tuberculosis (TB) is one of the greatest threats to human health worldwide. Mutations leading to drug resistance development may influence the fitness of *Mycobacterium tuberculosis*. The definition of fitness includes the microorganism's ability to survive, reproduce and be transmitted. The aim of this study was to measure the fitness in 21 *M. tuberculosis* isolates with different mutations in the genes *rpoB*, *rpsL*, *rrs* and *gidB* (genes related to rifampicin and streptomycin resistance) and 5 susceptible isolates from TB patients in Brazil. First, sequencing of the genes was performed, and then, these 26 strains were selected for the study. The fitness was measured by the growth time of the isolates as indicated by resazurin reduction by mycobacterial metabolism quantified every 12 hours. After the readings at 620 nm, a growth curve could be established as a measure of optical density (OD) in function of time (h). The program SPSS version 16 was used in the statistical analyses (nonparametric Mann-Whitney test). The average time in hours to reach an OD of 0.4 starting at an OD of 0.2 for all the susceptible strains (n=5) was 17.3 hours, for the *rpsL*43 mutated strains (n=2) was 13 hours, for the *rpoB*531 mutated strains (n=9) was 16 hours, for the strains mutated in *rpoB* (other than *rpoB*531; n=3) was 36.6 hours and the strains with mutations in more than one gene (n=7) was 29.4 hours. Only the comparison between susceptible isolates and isolates mutated in *rpoB* outside of codon 531, showed a significant p value (p=0.053).

In conclusion, only isolates with mutations in *rpoB*, other than *rpoB*531, showed a decrease in growth compared to susceptible isolates. The most important fact is that the isolates with the most frequent mutations related to high level resistance (mutations in *rpoB*531 and *rpsL*43) had a growth rate not statistically different from the drug-susceptible isolates.

PP-41

IN VITRO FITNESS OF MULTIDRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS PREVAILING IN ARGENTINA

Lopez B[1], von Groll A[2], Martin A[2], Barrera L[1], Palomino JC[2], Ritacco V[1]

[1]INEI-ANLIS Dr. C. Malbrán, Velez Sarsfield 563, (1281) Buenos Aires, Argentina
[2]Institute of Tropical Medicine, Nationalestraat 155-2000, Antwerp, Belgium
Contact: bealopez@anlis.gov.ar

The burden of multidrug resistant (MDR) tuberculosis (TB) in Argentina is largely associated with transmission of four outbreak strains that in order of prevalence belong to lineages H2, LAM3, LAM5 and Tuscany. The aim of our study was to investigate whether these successful MDR strains differ in fitness from contemporaneous MDR strains of similar lineages that have been infrequent or disappeared in Argentina.

To this end, we conducted *in vitro* fitness studies on 29 isolates obtained between 1998 and 2007, each from an individual patient. Fifteen isolates had the genotypes of those four outbreak MDR strains; five isolates had highly infrequent MDR variants of the same lineages; as control, we used eight fully susceptible isolates obtained in the study period that had genotypes closely related to those of the outbreak strains. Growth kinetic parameters were assessed in triplicate in MGIT 960™. Statistical analysis was performed using the t-test for independent samples (Welch-test). No significant difference was observed between prosperous and sporadic MDR strains in terms of mean growth rates, measured as doubling times during the exponential growth phase (p: 0.98). As expected, MDR strains had significantly slower growth rates than fully susceptible strains (p: 0.05). Compared with H2, LAM3 and LAM5 strains, the strains of the Tuscany lineage had a significantly slower growth rate, irrespective of drug resistance profile (30.4 hours vs 18.0 $p<0.002$, 20.1 $p<0.008$, and 21.0 $p<0.02$, respectively) and also reached lower growth units in the stationary phase (16,534 vs 25,490 $p<0.0001$, 23,847 $p<0.0006$, and 23,517 $p<0.002$, respectively).

Our data revealed certain differences in growth between lineages but failed to provide evidence of a relationship between *in-vitro* fitness of MDR strains and ability to disseminate in the community.

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PP-42

APPLICATION OF DENATURING HPLC TO THE DETECTION OF *rpoB* MUTATIONS IN THE EVALUATION OF RIFAMPICIN RESISTANCE IN CLINICAL SAMPLES FROM THE CUMURA HOSPITAL IN GUINEA-BISSAU

Suzana David[1], Ana Filipa Sutre[1], Ana Cardoso[1], Alfredo Mané[2], Armando Sanca[2], Vítor Henriques[2], Abílio Antunes[3]

[1]Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA), Avenida Padre Cruz, 1649-016 Lisbon, Portugal
[2]Cumura Hospital, The Republic of Guinea-Bissau
[3]Instituto de Higiene e Medicina Tropical, Rua da Junqueira 96, 1349-008, Lisbon, Portugal
Contact: suzana.david@insa.min-saude.pt

There is limited data on the resistance to antituberculosis drugs in developing countries due to lack of laboratories with conditions to perform culture and drug susceptibility testing. Molecular methods for the detection of drug resistance have been considered useful in these settings and more so as these can be carried out in collaboration with another laboratory having the capacity to perform PCR. The detection of mutations in the 81 pb rifampicin resistance-determining region of the *rpoB* gene (enconding the beta subunit of RNA polymerase) of *Mycobacterium tuberculosis* is a predictor of multidrug-resistant.

Denaturing high-performance liquid chromatography (DHPLC) utilizes heteroduplex formation between wild-type and mutated DNA strands to identify point mutations. In this study it was used as a rapid screening technique in the evaluation of rifampicin resistance in tuberculosis patients of the Cumura Hospital in The Republic of Guinea-Bissau. Confirmation of tuberculosis was based on clinical examination and AFB microscopy. DNA extraction and molecular analysis was carried out at the INSA in Lisbon on bleach inactivated specimens from the Cumura Hospital. PCR products from strains with different chromatographic profiles were sequenced for confirmation of mutations. Preliminary results indicate high level rifampicin resistance. Our study shows that bleach inactivation can be used prior to molecular analysis and that DHPLC could be useful for genotypic screening for mutations associated with rifampicin resistance. The DHPLC screening technique that was developed can also be used for the rapid diagnosis or the confirmation of rifampicin-resistance in other contexts.

PP-43

CLUSTER DIFFERENTIATION BY *gyrA* MUTATION ANALYSIS IN EXTENSIVE DRUG-RESISTANT TUBERCULOSIS

Joao Perdigão[1], Rita Macedo[2], Laura Brum[2], Isabel Portugal[1]

[1]Centro de Patogénese Molecular, URIA, Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa Portugal
[2]Deparatamento de Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida Padre Cruz, 1649-016 Lisboa, Portugal
Contact: isabel.portugal@ff.ul.pt

Previous reports have shown that a high rate of extensively drug-resistant (XDR) tuberculosis (TB) exists in Lisbon Health Region. One of the factors contributing to this high rate is the high prevalence of specific clusters. Moreover, Portugal has the highest rate of fluoroquinolone prescription in the European Union. Such high usage may be selecting *Mycobacterium tuberculosis* strains resistant to fluoroquinolones in infected patients. In this study we intend to characterize which mutations are conferring fluoroquinolone resistance in Lisbon Health region and eventually correlate them with specific clusters.

We have analyzed 26 fluoroquinolone-resistant *M. tuberculosis* strains, all XDR-TB, isolated in Lisbon Health Region during the year of 2005. An internal fragment of *gyrA* gene of each isolate was amplified by PCR and characterized by sequencing analysis. All strains were also genotyped by 12 loci Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR).

We have found three different missense mutations in *gyrA* gene: S91P, D94A and D94G. The most common mutation was S91P, which was present in 11 isolates. One isolate did not have any mutation. The 26 isolates were included in two MIRU-VNTR clusters: Lisboa3 and Q1. Both have been previously associated with XDR-TB. Mutations S91P and D94G were associated with Lisboa3 cluster, while mutation D94A was associated with Q1.

We verified an association between the mutations conferring fluoroquinolone resistance and a specific genotype. Such mutations may be helpful in determining a clinical isolate's genotype and possible association with XDR-TB. We also verify that given the high clonality of the analyzed isolates, active XDR-TB transmission is taking place.

PP-44

LABORATORY DATA REPORT ON DRUG-RESISTANT TUBERCULOSIS IN LISBON HEALTH REGION BETWEEN 2001-2006

João Perdigão[1], Rita Macedo[2], Carla Silva[1], Claúdia Pinto[1], Cristina Furtado[2], Laura Brum[2], Isabel Portugal[1,2]

[1]Centro de Patogénese Molecular, URIA, Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa Portugal
[2]Departamento de Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida Padre Cruz, 1649-016 Lisboa, Portugal
Contact: isabel.portugal@ff.ul.pt

Multidrug-resistance (MDR) and extensive drug-resistance (XDR) pose a serious threat to tuberculosis (TB) management. In Portugal, the TB incidence rates (28.7 cases / 100 000 Pop in 2008) cause difficulties in the transmission control of this infectious disease. The country has high TB incidence rates in comparison with the other European Union countries. Furthermore, the high MDR- and XDR-TB rates in regions such as Lisbon Health Region hamper the TB management.

In this study we have retrospectively analyzed 3.025 *Mycobacterium tuberculosis* clinical isolates, recovered over a six-year period (2001-2006), in Lisbon Health Region. The analysis was focused on first-line and second-line drug-resistance. The clinical strains were isolated in the Portuguese National Institute of Health – Dr. Ricardo Jorge which receives the majority of clinical specimens from Lisbon Health Region. Moreover, the clonality of a subset of isolates was assessed by 12-loci Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR) genotyping.

We have found 22 different resistance profiles with MDR-TB rates ranging between 9.9-15.2% and, XDR-TB rates between 44.3-57.3% (excluding one year here considered as an outlier). Six MIRU-VNTR clusters were associated with MDR-TB and, of which three (Lisboa3, Lisboa4 and Q1) had XDR-TB isolates.

In the present study we conclude that transmission of MDR- and XDR-TB is occurring mainly due to a limited number of genetic clusters, the majority belonging to Lisboa family. Implementation of genotyping in the diagnostic routine would most probably be useful in a timely detection of more serious types of resistance, like XDR-TB. A stronger and more efficient contact-tracing program would also bring some advantages to the halt transmission of primary drug-resistant TB.

PP-45

PROGRESS TOWARDS A STANDARD FOR FULLY AUTOMATED QUANTITATIVE DRUG SUSCEPTIBILITY TESTING OF NONTUBERCULOUS MYCOBACTERIA

Lucke K[1,2], Ritter C[1,2], Friedel U[1,2], Böttger EC[1,2]

[1]Institut für Medizinische Mikrobiologie, University of Zurich, Switzerland
[2]Nationales Zentrum für Mykobakterien, University of Zurich, Switzerland
Contact: klucke@imm.uzh.ch

Nontuberculous mycobacteria (NTM) - in particular slow-growers, *e.g. M. avium*, *M. intracellulare*, and *M. kansasii* - are increasingly recovered as significant pathogens in the diagnostic laboratory. Due to their ubiquitous presence humans are continuously exposed to these opportunistic mycobacteria, which may result in diseases such as pneumonia, lymphadenitis, and soft tissue infections. The optimal antimicrobial therapy for corresponding infections has yet to be established and is hampered by the lack of procedures for standardized drug susceptibility testing. While future studies have to address the clinical predictive value of drug susceptibility testing (DST), *i.e.* by correlating phenotypic resistance levels and treatment response, a prerequisite for corresponding investigations is the possibility to exactly determine levels of quantitative drug susceptibility.

We assessed quantitative levels of drug susceptibility for *M. avium*, *M. intracellulare*, and *M. kansasii* by comparing radiometric BACTEC460 based DST with nonradiometric MGIT960/EpiCenter V5.53 equipped with the TB eXiST module. Extended comparative analysis on a representative set of susceptible and resistant clinical isolates allowed us to define the conditions for MGIT960 based quantitative DST and to develop criteria for interpretation. This resulted in a fully automated system for quantitative DST, which is compatible with expert systems for interpretation and electronic data management. Implementation of MGIT960 based quantitative DST using EpiCenter V5.53 TB eXiST module software will provide a wealth of standardized drug susceptibility data to correlate results from quantitative DST with clinical outcome.

PP-46

ANALYSIS OF *nat* GENE OF *M. TUBERCULOSIS* STRAINS ISOLATED FROM BRAZILIAN PULMONARY TUBERCULOSIS PATIENTS

Vasconcellos SEG[1], Pereira MA[1], Gomes HM[1], Rossetti MLR[2], Possuelo LG[2], Silva MS[2], Ramos MC[3], Panunto AC[3], Lima KV[4], Lopes L[4], Mello FCQ[5], Suffys PN[1], Santos AR[1]

[1]Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro
[2]Centro de Desenvolvimento Científico e Tecnológico - CDCT, Rio Grande do Sul
[3]Universidade Estadual de Campinas, UNICAM, SP
[4]Instituto Evandro Chagas, Belem
[5]Hospital Universitario Clementino Filho Fraga, Federal University of Rio de Janeiro, Brazil
Contact: adalbertorezende@hotmail.com

The isoniazid (INH) is one of the main drugs used in TB treatment and chemoprophylaxis. The molecular mechanisms of resistance to this drug are complex and frequently associated to the presence of mutations in the genes *katG*, *inhA*, *ahpC* and *kasA*. However, 25-50% of the INH-R clinical isolates do not present mutation in these genes, indicating the involvement of other genes or factors. Recently, a gene denominated *nat* was identified in *M. tuberculosis* and its product, N-acetyltransferase (NAT), seems to be involved in INH acetylation *in vitro*. This gene presents a major single nucleotide polymorphism at position 619 (619 G>A) and the main objective of this project was to verify the prevalence of this and other SNPs in this gene among clinical isolates from different regions of Brazil and to possible association with INH-R. We therefore performed sequence analysis of the *nat* gene of 381 clinical isolates from treated pulmonary TB patients from different regions of Brazil (198 resistant and 183 sensitive to INH). Twenty isolates (5,2%) of the total sample presented the mutant variant 619A, however the frequency of this mutation was significantly higher among the resistant than among the sensitive ones (8.6% and 16% respectively; p=0.002, OR=5.73, IC 1.55–25.02). The isolate analysis of the resistant samples stratified according to the type of resistance (multidrug resistant and resistant only to INH do not show significant difference regarding the frequency of the 619A variant (p=0.06). Additional analysis of the *M. tuberculosis* isolates by spoligotyping, demonstrated that the mutant variant 619A was not characteristic for a specific genotype family and was detected in 6 different families, including S, LAM, Haarlem, X, T and LAM3/S convergent, and in 2 more isolates with unidentified genotypes. Our sequencing approach allowed the identification of nine new SNPs in the *nat* gene, including five non-synonymous (C20T, A233G, G202A, C373A and T503G), all identified in INH-R isolates and four more, synonymous (C276T, T312C, C375G and G501C), C276T being the only one observed in a INH-S isolate. These data add to the possible role of *nat* and genetic variations in INH-R.

PP-47

MOLECULAR ANALYSIS OF *MYCOBACTERIUM TUBERCULOSIS* RESISTANCE TO AMINOGLYCOSIDES

Bauskenieks M[1], Skenders G[2], Jansone I[1], Nodieva A[3], Broka L[2], Pole I[2], Ozere I[3], Baumanis V[1]

[1]Latvian Biomedical Research and Study Centre, University of Latvia, University of Latvia
[2]Infectology Centre of Latvia, Clinic of Tuberculosis and Lung Diseases
[3]Riga Stradins University
Contact: mb1985@inbox.lv

Aminoglycosides - streptomycin, kanamycin and amikacin are among the first and second line drugs for treatment of tuberculosis. Resistance to them however is different – half of streptomycine resistant isolates are susceptible to kanamycin. Therefore studies of resistance mechanisms to them are of practical interest.

Using PCR, RFLP and DNA sequencing methods 124 kanamycin and streptomycin resistant *M. tuberculosis* isolates have been studied to mutations in 16S rRNA gene *rrs* region 1400. Mutations previously described here (A1400G), cause the high resistance (MIC>120mkg/ml) to 2-deoxystreptamine ring containing aminoglycosides - kanamycin and amikacin. 77 (62%) of them had this mutation. Sequence analysis of 11 of 47 *rrs* wild type isolates showed mutations in recently described aminoglycoside acetyltransferase gene *eis* promoter region, associated to overexpression of the gene. 6 (54%) isolates contained previously described mutations G-10A and G-37T, 2 (18%) contained previously unknown mutations C-8T and C-15G but remaining 3 (27%) were wild type. Almost all *eis* mutations containing isolates had low level kanamycin resistance (4240 mkg/ml). 5 kanamycin resistant isolates were also sequenced to examine elongation factor G gene *fusA1*. 1 of them was found to have polymorphism A1117C. We have also sequenced loop 530 of *rrs* gene for 28 streptomycin resistant isolates (26 of them were also kanamycin resistant). 7 (25%) was found to have A513C mutation – previously associated to both streptomycin and kanamycin resistance. In our case 2 (29%) of mutation containing isolates were sensitive to kanamycin. Although none of streptomycin sensitive samples (4 such controls were made) had A513C, the mutation seems more to be connected to evolution than drug resistance because all of mutation containing isolates had ST42 spoligotype pattern. None of remaining 21 (75%) isolate had such genotype. Remaining isolates with wild type sequences in the genes studied indicate on other unknown mutations and therefore need further investigations. The possible role of efflux pumps, RNA methyltransferase or another aminoglycoside modifying enzymes will be discussed.

DETECTION OF RESISTANCE TO RIFAMPICIN AND ISONIAZIDE WITH THIN LAYER AGAR (TLA) DIRECT METHOD IN CLINICAL SAMPLES

Ardizzoni E[1,2], Mulders W[1], Varaine F[2], Martin Anandi[1], Rigouts L[1,3]

[1]Institute of Tropical Medicine, 155 Nationalestraat, 2000 Antwerp, Belgium
[2]Médecins Sans Frontières, 8 rue St Sabin, 75011 Paris France
[3]University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium
Contact: eardizzoni@itg.be

In countries with high multidrug-resistant (MDR) tuberculosis reliable and fast drug susceptibility testing (DST) methods are necessary. Thin Layer Agar (TLA) is a simple non-commercial culture method performed in a plate with four quadrants for simultaneous isolation, detection of rifampicin (RMP) and isoniazid (INH) resistance and preliminary identification of *Mycobacterium tuberculosis*. Plates are read by a standard optical microscope at x100 without being opened. Our objective was to evaluate the performance and turnaround time of direct DST TLA for RMP and INH compared to indirect DST with MGIT 960 automated system as gold standard.

A total of 59 routine smear-positive samples were decontaminated with Nalc-NaOH, then 100 µl of the sediment was inoculated in MGIT 960; the remaining sediment was diluted 1:5 and 100 µl inoculated on the four quadrants of a TLA plate. TLA plates were incubated at 37°C and read starting from day 5 from inoculation, then every 5 days for a total of 10 readings, until day 40. DST on TLA was considered invalid if the growth control showed less then 10 colonies.
With MGIT 960, 52 (88.1%) of 59 samples were positive, 1 negative and 6 (10.1%) contaminated. Out of all positive cultures 51 (98%) gave valid DST results, 1 had to be repeated due to contamination of the growth control tube. DST results showed 30 fully susceptible strains, 18 were resistant to both RMP and INH and 4 mono-resistant to INH.
On TLA, 49 cultures (83%) were positive, 9 (15%) were negative and 1 was contaminated. Out of 49 positive cultures 47 (95.9%) gave valid DST results while 2 could not be interpreted because of insufficient growth in the control quadrant. Compared to MGIT 960 TLA correctly detected all resistant strains showing 100% sensitivity and 100 % susceptibility for both RMP and INH.
All *M. tuberculosis* cultures were correctly identified by PNB on TLA.
The mean turnaround time from decontamination to DST result was on average 10 days (95% CI 8.53-11.47) for TLA compared to 24 days for MGIT 960 gold standard.

These preliminary results show that TLA for direct DST is an accurate method with a sensitivity and specificity for detection of RMP and INH resistance comparable to MGIT 960. TLA is inexpensive and faster than MGIT 960, and if combined to culture on Löwenstein-Jensen to recover samples that failed to grow on TLA, it represents a valuable option to early detect MDR patients for laboratories in settings with limited resources.

PP-49

IN VITRO ACTIVITY OF CEM-101 COMPARED TO CLARITHROMYCIN AGAINST *NOCARDIA* SPECIES

Flores-Penalba S[1], Shoen C[2], DeStefano M[2], Cynamon M[2]

[1]Universitat de València. Facultat de Farmàcia, Dto. Química Orgànica, Vicent Andrés Estellés, Burjassot,46100, València, Spain
[2]Veterans Affairs Medical Center, 800 Irving Avenue, Syracuse, 13210, NY, USA
Contact: Sonia.Flores@uv.es

Sulfonamides are usually the treatment of choice for *Nocardia* infections. Central nervous system and disseminated nocardiosis, however, continues to be difficult to manage. Drug resistant species of *Nocardia* such as *N. farcinica* or *N. otitidiscaviarum*, and sulfonamide intolerance require the use of alternative drugs. Promising reports of the effectiveness of clarithromycin (CLA) have been published, although clinical experience is still limited. In this study, the activity of CEM-101, a new ketolide, was evaluated *in vitro* compared to CLA, a structurally related macrolide, against *Nocardia* spp.

CLA, obtained from Abbott Laboratories (Abbott Park, IL), was dissolved in DMSO at a concentration of 1 mg/mL. CEM-101, provided by Cempra Pharmaceuticals Inc. (Chapel Hill, NC), was dissolved in double distilled water with 3% glacial acetic acid at a concentration of 1 mg/mL. Thirty one isolates of *Nocardia*, belonging to seven species, were obtained from the American Type Culture Collection (Manassas, VA), Barbara Body, and Betty Ann Forbes. Isolates were grown in cation supplemented Mueller-Hinton (MH) broth and diluted in MH broth to yield a standard turbidity of 100 Klett units per mL (1.2x10^6-6.0x10^7 CFU/mL). An *in vitro* broth microdilution method similar to that recommended by the Clinical and Laboratory Standards Institute (CLSI) was utilized for susceptibility testing.

The MIC50 and MIC90 for CEM-101 were 0.062 mcg/mL and 32 mcg/mL, respectively, compared to 0.125 mcg/mL and 128 mcg/mL for CLA. Two groups of isolates could be clearly distinguished in terms of susceptibility. A resistant group, with MICs >=8 mcg/mL (14 isolates) and a susceptible group, with MICs <=0.25 mcg/mL (17 isolates). There was good concordance with the activity of CEM-101 and CLA in each of these groups.

CEM-101 was demonstrated to be active *in vitro* against *Nocardia* spp, showing equal or better activity than CLA against most of the isolates tested. CEM-101 should be further evaluated against Mycobacteria to define its spectrum of activity.

IN VITRO ACTIVITY OF TR-700 COMPARED TO LINEZOLID AGAINST *MYCOBACTERIUM KANSASII* AND *NOCARDIA* SPECIES

Flores-Penalba S[1], Shrestha B[2], Shoen C[2], DeStefano M[2], Cynamon M[2]

[1]Universitat de València. Facultat de Farmàcia, Dto. Química Orgànica, Vicent Andrés Estellés, Burjassot,46100, València, Spain
[2]Veterans Affairs Medical Center, 800 Irving Avenue, Syracuse, 13210, NY, USA
Contact: Sonia.Flores@uv.es

TR-700, a new oxazolidinone, has recently been shown to be active against several Gram-positive species, with a level of activity superior to that of the structurally related linezolid (LZD). Studies on *Mycobacterium tuberculosis* and *Nocardia brasiliensis* revealed good MICs for TR-700. In this study, the activity of TR-700 was evaluated *in vitro* compared to LZD, against *Mycobacterium kansasii* and *Nocardia* spp.

TR-700 was provided by Trius Therapeutics Inc. (San Diego, CA). LZD was obtained from Pharmacia & Upjohn Inc. (Kalamazoo, MI). TR-700 and linezolid were dissolved in DMSO at a concentration of 1 mg/mL. Thirty one isolates of *Nocardia* and nineteen isolates of *M. kansasii* obtained from the American Type Culture Collection (ATCC, Manassas, VA), Barbara Body, Betty Ann Forbes, and Richard Wallace. *Nocardia* isolates were grown in cation supplemented Mueller-Hinton (MH) broth and diluted to yield a standard turbidity of 100 Klett units per mL (1.2x10^6-6.0x10^7 CFU/mL). *M. kansasii* isolates were grown in 7H10 broth and diluted to yield a standard turbidity of 10 Klett units per mL (5x10^7 CFU/mL). An *in vitro* broth microdilution method similar to that recommended by the Clinical and Laboratory Standards Institute (CLSI) for each microorganism was utilized for susceptibility testing.

In the case of *Nocardia* spp., the MIC50 and MIC90 for TR-700 were 0.25 mcg/mL and 0.50 mcg/mL, respectively, compared to 1 mcg/mL and 4 mcg/mL for LZD. Regarding *M. kansasii*, the MIC50 and MIC90 for TR-700 were 0.03 mcg/mL and 0.25 mcg/mL, compared to 0.25 mcg/mL and 0.5 mcg/mL for LZD.

TR-700 is more active than LZD against *Nocardia* and *M. kansasii in vitro* showing better or equal activity than linezolid against all the isolates tested, and lower MIC50 and MIC90. Further evaluation in animal models and clinical experience is necessary to understand the clinical potential for this new agent.

PP-51

DETECTION OF RESISTANCE TO ISONIAZID IN *MYCOBACTERIUM TUBERCULOSIS* BY REAL-TIME PCR METHOD

Mahbobe Naderinasab, Abdolrahim Rezaee, Fatemeh Riyahi Zaniyani, Mohamad Reza Razavi

Faculty in Microbiology & Virology Research Center, Buali (Avicenna) Research Institute, Mashhad University of Medical Sciences, Iran
Contact: naderinasabm@mums.ac.ir

Background. Tuberculosis remains one of the leading infectious causes of death worldwild. The emergence of drug-resistant strains of *Mycobacterium tuberculosis* is a serious public health threat. Resistance to isoniazid (INH) is the most prevalent form of resistance in *M. tuberculosis* and is mainly caused by mutations in the catalase peroxidase gene (*katG*) and *mabA-inhA* regulatory region. Classical methods of detection of drug resistance in *Mycobacterium tuberculosis* are time consuming. In this study we describe the Real-time PCR Taqman probe ARMS and the Real-time PCR Taqman probe Allelic discrimination to detect mutations in the *katG* and the regulatory zone of *inhA*, respectively. The results of these methods were compared with the proportional method.

Method. In this study 30 isoniazid – resistant and 30 isoniazid – susceptible *Mycobacterium tuberculosis* isolates were used. Firstly, the related genes were downloaded and aligned. The primers and probes were designed by Beacon designer and then the Real-time PCR method was optimized. In this study, the Real-time PCR Taqman probe method for IS*6110* region was used to detect tuberculosis and as an amplification control in other methods, too. Real-time PCR Taqman probe ARMS was carried out to detect mutation in 315 codon of *katG* gene and Real-time PCR Taqman probe Allelic discrimination was carried out to detect mutation in -15 (C>T) in the regulatory zone of *mabA-inhA*, and then results were compared to the proportional method.

Results. Sensitivity of Real-time PCR Taqman probe method for detection of *Mycobacterium tuberculosis* complex is 100%. The sensitivity of the Real-time PCR Taqman probe ARMS method and the Real-time PCR Taqman probe Allelic discrimination method are 69% and 53% for detection of isoniazid-resistant *Mycobacterium tuberculosis*, respectively. Specificity of both was 100%. In assessment of both mentioned mutations in two genes sensitivity is 75%.

Conclusion. Classical methods of detection of drug resistance in *Mycobacterium tuberculosis* are time consuming, so in this study, we described the Real-time PCR as a fastest method. According to the results of this study, the Real-time PCR Taqman probe method can be used as a standard method for detection

and confirmation of Tuberculosis. The Real-time PCR Taqman probe ARMS and the Real-time PCR Taqman probe Allelic discrimination methods can detect mutations in 315 codon of *katG* gene and the regulatory zone of *mabA-inhA* , respectively. These two methods can be used as fast screening methods for detection of isoniazid-resistant *M. tuberculosis*.

Keywords: Isoniazid, *Mycobacterium tuberculosis*, Drug resistant, Real-time PCR Taqman probe.

PP-52

CHARACTERIZATION OF *rpoB* MUTATIONS IN RIFAMPIN-RESISTANT ISOLATES OF *MYCOBACTERIUM TUBERCULOSIS* FROM A NORTH-EASTERN AREA OF ITALY BY DNA SEQUENCING AND LINE PROBE ASSAY

Fallico L, Peracchi M, Manganelli R, Palù G

Department of Microbiology, Histology and Medical Biotechnlogies, University of Padua, Via Gabelli 63, 35129 Padua, Italy
Contact: loredana.fallico@tiscali.it

Emergence and spread of *Mycobacterium tuberculosis* (Mtb) resistant strains, such as multidrug-resistant (MDR-TB) or even extreme drug-resistant strains (XDR-TB) represents a serious threat for global tuberculosis control.
Resistance to Rifampin (RFM) is mainly caused by mutations in *rpoB*, the gene coding for the beta-subunit of the RNA polymerase.
The aim of this study is to evaluate the efficiency of Genotype MTBDR*plus* (Hain Lifescience) based on PCR and DNA•STRIP® technology, in detecting RFM resistance in our settings.
Eighteen RFM resistant Mtb strains isolated in a north-eastern area of Italy from January 2006 to December 2009 (of which 14 MDR, and one XDR) were subjected to both Genotype MTBDR*plus*, and sequence of the 318-bp DNA fragment of *rpoB* in which most or the mutation conferring RFM resistance are known to map.
Genotype MTBDR*plus* identified all the 18 strains as resistant. Ten strains were determined to have common mutations identifiable from the kit, while the other 8 strains had a banding pattern deviating from the wild type pattern resulting in any case in RFM resistance, even if it was not possible to identify the mutation responsible for the resistance.
DNA sequencing confirmed the results and led to the characterization of the mutation present in the 8 strains in which this could not be characterized using Genotype MTBDR*plus*.
Eleven resistant strains (61%) carried the mutation in position 531 of *rpoB*: 8 strains having the mutation TCG531TTG and 3 strains the mutation TCG531TGG. Others isolates had mutations in codons 516 (GAC516TAC and GAC516GTC), 522 (TCG522TTG and TCG522TGG), 526 (CAC526GAC and CAC526TAC), and one had the insertion 514TTC, all resulting in nine different mutations involving five codons.
Four of these (TCG531TGG, TCG522TTG, TCG522TGG, insertion 514TTC) at the best of our knowledge were not yet identified in Italy.
Genotype MTBDR*plus* showed a specificity of 100% in detecting RFM resistance, even if some of the circulating mutations were not directly recognizable from the kit.

MYCOBACTERIAL INFECTION: DEVELOPMENT OF A DILUTION TEST FOR MINIMAL INHIBITORY CONCENTRATIONS (MIC)

Havelková M, Koufaki O-N

National Institute of Public Health, National Reference Laboratory for Mycobacteria,
Srobarova 48, 100 42 Prague, Czech Republic
Contact: mhavelkova@szu.cz

The drug susceptibility test (DST) is of major importance during treatment of mycobacterial infection. At this point we would like to present a rapid detection method for second line DST.

The combination of at least 6 drugs using 96 well plate with standardised micro-dilution of different critical concentrations in liquid medium for 2nd line drugs (amikacin, ofloxacin, ciprofloxacin, rifabutin, capreomycin and clofazimin for *M. tuberculosis*, and amikacin, ofloxacin, ciprofloxacin, rifabutin, azithromycin and clarithromycin for the non tuberculosis mycobacteria) in combination with the minimal inoculum demand are the main advantages of this method.

This micro-dilution method was standardised and used for several years now in routine diagnosis.

The results are usually ready in 7-10 days this fact gives a prospective future in the use of this rapid and sensitive method for DST.

PP-54

CROSS-REACTION OF *M. KUMAMOTONENSE* WITH *M. TUBERCULOSIS* USING COMMERCIAL PROBES

Almudena Rodríguez-Aranda[1], Soledad Jiménez M[2], Jesús Yubero[3], Fernando Chaves[1], Rafael Rubio-García[1], Elia Palenque[1], María García J[3], Carmen Menéndez M[3]

[1]Hospital Doce de Octubre, Madrid, Spain
[2]Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
[3]Dpt. Medicina Preventiva, Salud Pública y Microbiología, Facultad de Medicina, Universidad Autónoma de Madrid, Spain
Contact: mariaj.garcia@uam.es

Commercial molecular probes are widely applied in the identification of mycobacteria in clinical samples. However the misidentification could be found due to cross-reaction between species and could carry main troubles in the diagnostic and treatment of the patients.

A clinical isolate, labelled as 1369, recovered from a patient diagnosed of lymphoid tuberculosis was identified as *Mycobacterium tuberculosis* complex (MTBC) by using AccuProbe (BioMérieux). Further characterization by using standard microbiological and phenotypical procedures showed unexpected results for MTBC, such as an unusual drug susceptibility panel. However, the isolate 1369 was again identified as MTBC by using Inno-LIPA Mycobacteria v2 (Innogenetics). On the contrary, it was identified as *Mycobacterium* sp. by using Geno-Type (Hain Lifescience).

Further molecular characterization (sequencing of the 16S-rDNA and PCR-RFLP of *hsp65* gene) identified the isolate as *M. kumamotonense* a novel slow grower mycobacteria.

M. kumamotonense is closely related to the *M. terrae* group. This group is unusual because several of their members carry two copies of the *rrn* operon per genome.

We have shown that the novel species also carries two copies of the *rrn* operon per genome. Moreover, the ribosomal internal transcribed spacer 1 (ITS-1) of *M. kumamotonense* was analyzed, and two different fragments of the same size were found showing 92% of similarity to each other.

According to our data of *M. kumamotonense*, and previous data of several members of the *M. terrae* group, it appears that the presence of a supplementary copy of the *rrn* operon, in slow grower mycobacteria, could be related to cross-reaction of their rDNA with the commercial probes targeted to MTBC.

PP-55

PERFORMANCE OF THE THIN LAYER AGAR (TLA) CULTURE METHOD USING THE COLORIMETRIC INDICATOR STC

Ardizzoni E[1,2], Mulders W[1], Varaine F[2], Martin Anandi[1], Rigouts L[1,3]

[1]Institute of Tropical Medicine, 155 Nationalestraat, 2000 Antwerp, Belgium
[2]Médecins Sans Frontières, 8 rue St Sabin, 75011 Paris France
[3]University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium
Contact: eardizzoni@itg.be

Gold standard techniques used for culture of *M. tuberculosis* are sensitive but slow or too expensive to be wildly adopted in most TB high-incidence settings. TLA is a rapid and inexpensive technique, providing simultaneously culture and preliminary identification of *M. tuberculosis*. Plates are examined by a microscope at x100, which allows faster detection. Our objective was to evaluate the use of TLA in combination with STC (2,3-diphenyl-5-(2-thienyl)tetrazolium chloride), a redox indicator which produces red spots when organisms grow in cultures, to read plates by naked eye facilitating colonies detection.

We decontaminated 56 smear-positive samples with Nalc-NaOH and inoculated the sediment on MGIT 960 automated method, TLA-STC plates and TLA plates without STC as control to investigate possible growth inhibition by STC. TLA plates included 2 compartments plain medium and 2 with PNB for identification. Plates were read blinded every 2 days starting by day 5 from inoculation until day 15, then every 5 days until day 40, for a total of 10 readings. Growth on PNB was read when the control compartment was positive. MGIT 960 tubes were read according to manufacturer's protocol.

Of 54 samples positive on MGIT 960, 47 (87%) were positive on TLA-STC plates and 45 on plates without STC.

TLA-STC plates showed growth at microscopy examination on average of 10 days, with 39 (83%) of them being positive within 15 days. By naked eye red spots produced by STC were detected on average of 18 days.

TLA plates without STC were positive on average of 10 days and MGIT 960 cultures at 16 days. Of the 8 TLA-STC cultures positive after 15 days, 6 showed few colonies missed at microscopy examination but detected by STC spots by naked eye; 2 of them were negative also on plates without STC.

All microscopy-positive TLA-STC plates were confirmed by red spots detectable by naked eye later. All *M. tuberculosis* were correctly identified by PNB on all TLA assays.

STC in our experience delayed sensibly the results when plates were read by naked eye compared to reading by microscope. However it helped in detecting growth when only few colonies were present. STC did not inhibit growth in any of the cultures. Its use could be helpful for laboratories with high workload to improve detection in scanty positive cultures and to limit the number of microscopy examination to 5 readings and continue final reading by naked eye for confirmation of negative result.

PP-56

REAL-TIME RIBONUCLEASE-P PCR FOR BROAD, DETECTION OF THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX AND MEDICALLY IMPORTANT NON-TUBERCULOUS MYCOBACTERIA

Erik Svensson[1], Guma Abdeldaim[2], Carl-Johan Rubin[2], Leif Kirsebom[3], Jonas Blomberg[2], Björn Herrmann[2]

[1]Institute for Biomedicine, University of Gothenburg
[2]Departments of Medical Sciences
[3]Cell and Molecular Biology, Uppsala University, Sweden
Contact: erik.svensson@vgregion.se

The *rnp* gene product, ribonuclease-P RNA (RNase P), is ubiquitously present. Variations in the *rnpB* sequence have been used as for identification of different bacterial species. We wanted to develop a real-time *rnpB* PCR for direct detection of the *M. tuberculosis* complex (MTBC) and non-tuberculous mycobacteria (NTM).

The *rnpB* sequences of 17 *Mycobacterium* spp. were determined and two quantitative real-time PCRs for detection of MTBC (Mytu PCR) and NTM (Myat PCR) were developed and combined into a single tube format. The specificity of the duplex PCR assay was tested with 21 mycobacterial species (55 strains), and 35 bacteria non-mycobacterial species. The assay was evaluated on 10 samples from a quality control panel from QCMD and on 442 clinical samples. The results were compared with the results of culture, direct microscopy and the Roche Cobas Amplicor PCR (RCA).

The *rnpB* sequences showed hypervariable regions enabling species identification and PCR design. The Mytu PCR specifically detected the MTBC and the Myat PCR detected all tested 17 NTM species, except 2 of 7 strains from the *M. avium* complex. The sensitivity for detection of the *M. tuberculosis* complex was <50 copies/reaction, while for NTM it was 500 copies/reaction. The PCR assay correctly detected all 10 samples from QCMD quality assurance panel. The assay was specific and did not detect any of the non-mycobacterial bacteria spp. Of 442 clinical specimens, MTBC was detected in 40 cases (9%) by Mytu PCR, in 38 cases (8%) by the RCA, in 46 cases (10%) by culture and in 23 (5%) cases by direct microscopic examination. The NTM were detected by Myat PCR in 18 (4%) cases, in 11 (2%) by culture cases, and in 8 cases (2%) by direct microscopic examination.

Sequence determination of the *rnpB* gene is useful for *Mycobacterium* species identification. Our duplex real-time PCR was shown to detect strains from both the MTBC and tested NTM species. The PCR assay has a broader detection range of mycobacteria than commercial standard PCR methods with a sensitivity similar to culture.

PP-57

SNP DETECTION BY MLPA IN TUBERCULOSIS: ONE METHOD, MULTIPLE APPLICATIONS

Bergval I, Sengstake S, Schuitema A, Klatser P, Anthony R

KIT Biomedical Research, Royal Tropical Institute, Meibergdreef 39, 1105 AZ Amsterdam, Netherlands
Contact: r.anthony@kit.nl

The cost of whole genome sequencing has dropped dramatically and as a consequence in the next years there will be an explosion of SNP (single nucleotide polymorphism) discovery.

The emerging epidemic of drug-resistant TB calls for rapid detection characterization and early detection of drug resistance of infecting strains. We have developed *Mycobacterium tuberculosis* (MTB)-specific multiplex assay, based on Multiplex Ligation-dependent Probe Amplification (MLPA). This method allows simultaneous specific detection of multiple dispersed genetic markers in the MTB genome, such as mutations associated with drug resistance or particular genetic clades. The detection of mycobacterial SNPs in real time, combined with knowledge of the clinical consequences of these mutations, in principle would allow the appropriate clinical response to specific genotypes to be undertaken.

The primary advantage of the MLPA is its ability to screen for dispersed mutations and targets associated with resistance to several important second-line drugs (*e.g.* fluoroquinolones) are included our latest generation MLPA assay, along with additional genotypic markers.

The newly designed MLPA-probes were specific for the targeted SNPs, allowing the detection of prevalent second-line drug resistance mutations and a further delineation of MTB complex members when compared to our original assay (Bergval *et al.* JCM 2008; 46: 689).

Classically, MLPA-products are analysed by capillary electrophoresis, a method that requires complex expensive equipment and is therefore difficult to implement outside reference laboratories. In an effort to address these issues, we have transferred the assay to a microbead-based liquid array (Luminex) by modifying current MLPA-probes and protocols.

Methods allowing informative SNPs to be rapidly detected will become increasingly valuable as whole genome data and their phenotypic implications become available. Furthermore, we feel that the new analysis method for MLPA will be of particular value in high burden countries with high endemicity of (M)DR-TB. The liquid array allows multi-parameter testing and critically could provide a standard platform for several diagnostic and screening tests, which are at present performed by multiple tuberculosis specific methods. Multiplex molecular testing has the potential to allow full characterization of MTB clinical isolates closer to the patient and more rapidly than is currently feasible.

PP-58

DIRECT CAPTURE OF MYCOBACTERIAL rRNA ON A LIQUID BEAD ARRAY FOR SPECIATION FROM CULTURES: THE BASIS FOR A SIMPLE AND RAPID ASSAY?

Hans de Ronde, Sarah Sengstake, Richard Anthony

Royal Tropical Institute, KIT-BR, Meibergdreef 39, Amsterdam, 1105 AZ, the Netherlands
Contact: r.anthony@kit.nl

Speciation of cultured mycobacteria is critical particularly when using liquid culture. Confirmation of the presence of a member of the *Mycobacterium tuberculosis* complex can be rapidly achieved by using a lateral flow test such as the Capilia TB test or BD TBc Identification Test, but identification of non-tuberculose mycobacteria (NTM) is currently most practically achieved using an amplification based assay such as a line probe assay (*e.g.* Hain Test).

We make use of the biological amplification of the bacterial culture itself thereby avoiding an additional amplification step. This approach allows the direct detection of mycobacterial RNA in culture lysate. We demonstrate rRNA from culture lysate can be hybridized to a DNA probe immobilized on a micro bead and simultaneously to a DNA probe labelled with a reporter molecule. The rRNA acts as a bridge between the micro bead and label thus accumulates at the bead surface. In this way each mycobacterial species could be targeted with a specific particle and labelled probe. Subsequently particles were analysed in a dedicated flow cytometer (Luminex). Each particle species is identified based on its unique signature and the reporter signal accumulated at the surface is measured.

We believe that further development/application of this approach is of considerable interest particularly as liquid array equipment will likely become more widely available and affordable in the near future.

PP-59

LATERAL FLOW PCR-DIPSTICK FOR FAST AND SENSITIVE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX FROM PULMONARY AND EXTRAPULMONARY SPECIMENS

Michael Weizenegger

Laboratory Group Limbach, Dept. of Microbiology and Molecular Genetic, Im Breitspiel 15, D-69126 Heidelberg, Germany
Contact: weizenegger@labor-limbache.de

Introduction. A beta-version of the GenoQuick MTB assay (Hain Lifescience, Nehren, Germany) was compared to results derived from cultures grown on 2 solid (Löwenstein Jensen media) and on liquid media (Bactec MIGIT 960, Becton Dickinson, Heidelberg, Germany). The PCR-dipstick is a lateral flow device and contains three lines. The first line is for the detection of *Mycobacterium tuberculosis* (MTB)-complex specific DNA, the second line for the detection of the amplification control (AC) and the third line is for monitoring the successful migration of the gold particles.

Material and methods. 102 pre selected specimens were collected and stored after NALC-NaOH digestion at –20°C. The mycobacterial DNA was isolated with the GenoLyse-Kit (Hain Lifescience). Shortly, 500 µl of the decontaminated specimen was centrifuged, the supernatant discarded and the pellet was resuspended in 100 µl lysis buffer. The tube was incubated for 5 minutes in a water bath at cca. 98°C. 100 µl of a second buffer was added, mixed and 5 µl was taken for PCR amplification. 0.1 thermolabile Uracil N-glycosilase (Epicentre, Madison, USA) was added prior amplification (10 min at 37°C). After amplification the PCR-dipstick was incubated in a mixture of 10 µl of the PCR amplicon and 100 µl running buffer. The result was evaluated by eye after ten minutes.

Results. 25 specimens (22 culture positive) were smear positive and 77 specimens (20 culture positive) were smear negative. All smear positives and smear negatives, culture positive specimens could be identified with the GenoQuick MTB assay (100% sensitivity), 13 (one smear positive, 12 smear-negative specimens) were PCR positive, culture negative (78.3% specificity, gold standard culture). Ten of them derived from patients with a recent Tb infection, three were unknown (94.5% gold standard confirmed TB infection).

Conclusion. The GenoQuick MTB assay showed a very high sensitivity compared to culture. The specificity values were moderate due to high percentages of specimens from Mtb infected patients under long term treatment. The PCR-dipstick is fast and easy to handle. It can be a cost effective alternative to "Real Time" systems.

PP-60

DETECTION OF *M. TUBERCULOSIS* AND RIFAMPICIN-RESISTANCE USING A COMMERCIAL PCR REAL TIME TECHNIQUE IN RESPIRATORY AND EXTRAPULMONARY SAMPLES

Tórtola MT, Nieto L, Codina MG, Martín-Casabona N

Microbiology laboratory. Hospital Vall d'Hebron. Pg. Vall d'Hebron 119. 08035 Barcelona, Spain
Contact: ttortola@vhebron.net

Cepheid Xpert MTB/RIF Assay is a molecular beacon-based real time PCR for study of mutations in the *rpoB* gene designed to detect *M. tuberculosis* and identify rifampicin-resistant cases in less than two hours. It is a sample processing cartridge system very simple to use but is validated only in sputum samples. Despite of the lung is the primary target organ for tuberculosis, any other organ and system can be affected. We evaluated this amplification technique in 119 respiratory and 164 extrapulmonary samples (10 abscesses, 22 lymph nodes, 49 tissue biopsies, 72 sterile liquids and 11 other samples). The results were compared by conventional techniques (Ziehl-Neelsen stain and culture in liquid medium) in the same sample.

Twenty three respiratory samples, 4 abscesses, 2 lymph nodes, 1 intestinal and 1 pleural biopsies were both culture and PCR positive (12 of which were negative for baciloscopy), and 233 samples were negative using those three techniques. In 10 PCR negative samples we cultured non tuberculous mycobacteria. Five samples were only PCR positive.

In any case we found no inhibition of the amplification. All positive samples were sensitive to rifampicin by PCR and phenotypic test.

For respiratory and extrapulmonary samples, sensitivity was 100 and 66.7%, specificity was 98.9 and 97.4%, positive predictive value was 95.6 and 66.7%, and negative predictive value was 100 and 97.4% respectively.

This amplification technique can be used in samples other than sputum, had no false positive results with non-tuberculous mycobacteria and results are valuable in all samples.

EVALUATION OF GENEXPERT MTB/RIF ASSAY FOR *MYCOBACTERIUM TUBERCULOSIS* DETECTION AND RIFAMPICIN RESISTANCE IDENTIFICATION IN PATIENTS WITH HIGH CLINICAL SUSPICION OF TB

Ioannidis P, Papaventsis D, Nikolaou S, Karabela S, Panagi M, Raftopoulou A, Konstantinidou E, Marinou I, Kanavaki S

National Reference Laboratory for Mycobacteria (NRLM), Sotiria Chest Diseases Hospital, Mesogion 152, 11527 Athens, Greece
Contact: reflabtbsotiria@yahoo.gr

The GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is a novel semi-quantitative PCR based method for the simultaneous detection of *Mycobacterium tuberculosis* and Rifampicin (RIF) resistance. The aim of the study was to evaluate the assay's performance in microscopically negative [AFB(-)] pulmonary and extra-pulmonary specimens of patients with high clinical suspision of TB.

The Xpert assay results were compared against: 1) the conventional culture methods on Löwenstein-Jensen and/or BACTEC™ MGIT™ 960 (Becton Dickinson, USA) for the diagnosis of tuberculosis and 2) the line-probe assay MTBDR*plus* (Hain Lifescience, Nehren, Germany) for RIF resistance. Additionally, a small number of randomly selected AFB(+) samples were also included in the study.

Culture results were available for 67 (40 pulmonary; 27 extra-pulmonary) AFB(-) and 10 (9 pulmonary and 1 extra-pulmonary) AFB(+) specimens. The overall sensitivity (Se), specificity (Sp), positive (PPV) and negative predictive values (NPV) of the GeneXpert MTB/RIF assay compared to culture were 92%, 94.5%, 89 % and 96.3%, respectively. For the pulmonary specimens, Se, Sp, PPV and NPV were 91.3%, 100%, 100% and 92.8%, whereas for the extra-pulmonary were 100%, 89.3%, 25% and 92.8% respectively. For the cohort of the AFB(-) specimens the Se, Sp, PPV and NPV values were 86.6%, 94.4%, 81.25% and 96.2% respectively. For the pulmonary AFB(-) specimens the respective values were 85.7%, 100%, 100% and 92.8%. MTB strains were isolated from 29/32 GeneXpert MTB/RIF positive specimens cultured on Lowenstein-Jensen and/or MGIT media. MTB strains were not grown from two inoculums (specimens "quantitated" as very low by the GeneXpert MTB/RIF assay), whereas one culture had been contaminated. 27/29 strains were characterized as wild type regarding the *rpoB* hot spot region, whereas 2 strains were characterized as resistant to RIF due to lack of probe B signal. With the exception of one strain, MTBDR*plus* analysis confirmed RIF resistant status. The one case that was missed by the GeneXpert MTB/RIF assay was most likely due to a complex heteroresistant pattern. Cultures grown from this patient's specimens harbored along with the wild type strain, strains with either the H526L or the S531L mutation.

These preliminary data indicate that the GeneXpert MTB/RIF assay is highly effective at detecting MTB at smear negative samples of patients with high clinical suspicion of TB as well as to identify RIF resistant status.

PP-62

IMPACT OF NUCLEIC ACID-BASED AMPLIFICATION TECHNIQUES ON CLINICAL DIAGNOSIS OF CHILDHOOD TUBERCULOSIS

Papaventsis D[1], Ioannidis P[1], Tsolia M[2], Karabela S[1], Nikolaou S[1], Konstantinidou E[1], Marinou I[1], Panagi M[1], Raftopoulou A[1], Kanavaki S[1]

[1]National Reference Laboratory for Mycobacteria (NRLM), Sotiria Chest Diseases Hospital, Mesogion 152, 11527 Athens, Greece
[2]2nd Department of Pedriatrics, University of Athens, Thivon & Leivadias, 11527 Athens, Greece
Contact: reflabtbsotiria@yahoo.gr

Diagnosis of childhood tuberculosis is often based solely on clinical history and examination, with no laboratory confirmation. Detection methods and in particular, commercially available, nucleic acid-based amplification techniques, have been shown invaluable for the precise and rapid confirmation of the clinical diagnosis. The aim of the study was to evaluate the diagnostic performance and the impact on clinical decisions of the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test (AMTD, Gen-Probe, San Diego, California) in comparison with conventional culture for the detection of *Mycobacterium tuberculosis* (MTB) in children.

We retrospectively studied 82 children at risk for TB infection (51 males, 31 females; mean age 7 years; range 1-16 years). Children enrolled were referred to the 2nd Department of Pedriatrics, University of Athens, Greece. Sample origin was mainly sputum (33%), gastric fluid (31%) and lymph nodes (24%). Samples were examined using acid-fast direct smear microscopy, the Gen-Probe technique and bacterial culture using the semi-automated BACTEC™ MGIT™ 960 liquid system (Becton Dickinson, USA) and Löwenstein-Jensen (LJ) solid media.

Only 3 samples were acid-fast positive. MTB was recovered from 14 children. It was not isolated from 8 and 21 additional children that were AMTD positive or presented high clinical suspicion of TB (and thus received standard anti-TB therapy), respectively. The remaining children (n=47) had other infectious or noninfectious diseases. Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of both Ziehl-Neelsen smears and the Gen-Probe technique compared to conventional culture were: 14.3%, 98.5%, 66.7%, 84.8%, vs 100%, 88.2%, 63.6% and 100%, respectively. Based on clinical diagnosis of TB, sensitivity, specificity, PPV and NPV of the AMTD test vs the bacterial culture was 58.3%, 97.8%, 95.4%, 75%, and 37.8%, 100%, 100% and 66.2%, respectively.

The results of this study suggest that nucleic acid amplification tests are very sensitive and specific methods for rapid detection of MTB. The Gen-Probe molecular method increases the number of laboratory confirmed TB cases in children by almost 150% compared to bacterial culture.

PP-63

CORRELATION IN QUICKLY DETECT MUTATIONS ASSOCIATED WITH RESISTANCE STRAINS OF *MYCOBACTERIUM TUBERCULOSIS* (RMP AND HIN), ISOLATED IN LIQUID MEDIA CULTURE AND SUSCEPTIBILITY, ASSOCIATED WITH DNA ANALYSIS (GENOTYPE)

Dumitru M[1], Banica D[2], Cojocaru V[3], Borgazi E[3]

[1]Romanian TB Patients Association,58, Popa Tatu, District 1, Bucharest, Romania, 010806
[2]Moleculary Mycobilology Laboratory, National TB Institute "Marius Nasta", 90, Viilor, District 4, Bucharest, Romania, 050159
[3]Pneumoftiziology Clinical Hospital, 40, Santinelei, Constanta, Romania, 90000
Contact: marius.dumitru@arb-tb.ro

The need to limit their transmission of resistant strains requires methods for rapid detection of mycobacteria for the recovery of viable units in liquid culture media, as quick, efficient, with high specificity and sensitivity, followed by mycobacteria DNA analysis, using molecular biology methods.

In our intention was to demonstrate high correlation between rapid culture results and DNA mutation responsible for (multi)drug-resistance and diagnostic algorithm sustainability.

Strains selected for study became from 156 patients (sputum, bronchial aspirate, pleural liquid), assisted in the National Institute of Pneumology "Marius Nasta" Bucharest and Constanta TB Hospital, with additional data on clinical and paraclinical evolution of pre-and post- therapy (medical record documents).

After isolating and susceptibility major drugs mycobacteria testing (streptomycin, Isoniazid, Rifampicin and Etambutol), using automat MGIT® 960 system, was proceed to examine fragments of the bacillary genome responsible for Rifampicin (*rpoB*) and Isoniazid (*katG*, *inhA*) resistance (Light Cycler- PCR, GenoType® DNA Strip®).

Was obtained 89,9% correlation index between MGIT (SIRE) and genotype resistance profile (HIN and RMP), confirmed data from literature.

Rapid methods for detection of MDR TB complement the spectrum of laboratory tests, with a particularly important contribution to certify the diagnosis of tuberculosis (poly) chemo-resistant, set up early administration of therapy associated with major benefits on hospitalization costs and improve patient management of TB.

PP-64

MOLECULAR DIAGNOSIS OF TUBERCULOUS MENINGITIS: A THREE DAYS EXPERIENCE!

Peracchi M[1], Scarin M[2], Viviani F[2], Manganelli R[1], Fallico L[1], Palù G[1]

[1]Department of Microbiology, Histology and Medical Biotechnlogies, University of Padua, Via Gabelli 63, 35129 Padua, Italy
[2]Hospital of Rovigo- V.le Tre Martiri 140-45100 Rovigo
Contact: marta.peracchi@sanita.padova.it

Tuberculous meningitis is a rare yet serious infectious disease. Very rapid diagnosis and prompt initiation of therapy are needed due to its high mortality rates. However, in most instances, *Mycobacterium tuberculosis* is not initially considered to be a likely cause.

The GeneXpert® MTB/RIF assay, for use with the Cepheid GeneXpert® System (Cepheid, Sunnyvale, CA), has been recently introduced for rapid identification of *M. tuberculosis* complex and determination of rifampicin resistance on respiratory samples by semi-quantitative nested real-time polymerase chain reaction.

The aim of this work is to describe the performance of this test in cerebrospinal fluid specimens (CSF), during three days (17th - 19th of march 2010) in which two different departments send us cerebrospinal fluid specimens (CSF) from two foreign-born patients, with requests for *M. tuberculosis* culture and microscopy examination.

CSF studies showed typical low glucose levels and elevated protein levels. Sediments were examined by fluorescence microscopy and cultured (35°C/8 weeks) on liquid and solid media.
PCR-amplification of a 400 bp fragment of *hsp65* (the gene encoding the *M. tuberculosis* 65-kDa heat shock protein) was positive only for one sample.
Both specimens were smear positive and *M. tuberculosis* was isolated from liquid cultures after eleven days.
When examined using the GeneXpert® MTB/RIF test, both CSF were positive for rifampicin-sensitive *M. tuberculosis*. Sensitivity of both isolates was later confirmed by drug susceptibility testing performed on MGIT 960 (Beckton Dickinson).
In these cases GeneXpert® MTB/RIF has provided rapid and specific detection of *M. tuberculosis* even in a non respiratory samples.

Further investigations are needed to prove the utility of this test in these kind of specimens.

PP-65

PRELIMINARY EVALUATION OF XPERT MTB/RIF KIT FOR TUBERCULOSIS DETECTION IN NON-RESPIRATORY SPECIMENS

Causse M, Gutierrez-Aroca JB, Mena C, Casal M

Mycobacteria Referente Center, Reina Sofia University Hospital, Córdoba, Spain
Contact: mancau@hotmail.com

Introduction. Rapid detection of *M. tuberculosis* (MTB) is one of the priority WHO´s goals and the use of nucleic acid amplification techniques is becoming more common in advanced laboratories, but most of these techniques are designed to study MTB in respiratory samples and its operation is controversial in non-respiratory ones where bacterial load is poor. Xpert kit MTB/RIF for Cepheid GeneXpert system allows MTB detection by real-time PCR and has 5 MTB probes for mutations associated with resistance to Rifampin. This system enables individual processing of each sample by using cartridges. The aim was to evaluate its performance in non-respiratory samples

Material and methods. We processed a total of 112 samples: 9 CSF, 25 synovial fluid, 3 ascitic fluid, 1 pleural fluid, 30 biopsies, 9 gastric aspirates and 35 miscellaneous (noting purulent exudates, abscesses and urine) between January 2009 and March 2010 by subjecting them to decontamination by Kubica method. Smears were made by thick drop staining with auramine and cultures on solid (Lowenstein) and liquid (MGIT Bactec 960) mediums. We realized molecular test following commercial kit protocol.

Results. 98 of the 112 samples were negative by PCR, only one culture of these samples was positive to *M. intracellulare*, it was considered as negative for statistic study as it is not detectable by this kit. Of the 14 positive PCRs, 9 were negative smears and 4 positive, in a sample was not performed. The positive samples corresponded to five biopsies, four purulent exudates, 2 abscesses, 2 gastric aspirates and one CSF. All the cultures of these samples were positive to MTB

Conclusions. This kit seems to be effective in the detection of MTB in non-respiratory samples and a good tool to test samples individually without mounting series, and that can be used to have rapid results.
The Agreement Kit-culture was 100%
Although we have few positive samples in our serie, we obtained values of 100% in sensitivity, specificity, PPV and NPV.
Larger series with more positive samples are necessary to determine real statistical parameters.

PP-66

EVALUATION OF GENEXPERT MTB/RIF ASSAY FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* AND RIFAMPICIN RESISTANCE IN A ROUTINE LABORATORY SETTING IN SLOVENIA

Nataša Fajfar, Urška Bidovec-Stojkovič, Manca Žolnir-Dovč

University Clinic of Respiratory and Allergic Diseases Golnik, Golnik 36, SI-4204 Golnik, Slovenia
Contact: Tel. +386 4 25 69 100, Fax. +386 4 25 69 117, natasa.fajfar@klinika-golnik.si

Nucleic acid amplification tests have improved tuberculosis diagnostics considerably. The latest development in the field of molecular diagnosis of tuberculosis is a quick fully automated real-time PCR diagnostic test, the Cepheid GeneXpert MTB/RIF assay (GX), for the detection of *Mycobycterium tuberculosis* and the detection of rifampicin resistance.

The aim of our prospective study was to evaluate the performance of GX in comparison to the Gen-Probe Amplified *M. tuberculosis* Direct test (MTD), using culture as a reference method.

A total of 63 specimens (56 pulmonary and 7 extra-pulmonary) from 55 untreated patients for whom there was high clinical suspicion of tuberculosis were included in our study. 42 of 63 specimens were culture positive and 21 culture negative.

35 specimens were positive and 22 negative by GX and MTD. 6 specimens were positive by GX only. From 42 culture positive specimens MTD did not detect *M. tuberculosis* in 7 specimens but GX did not detect *M. tuberculosis* in only one culture positive specimen with only 1 colony grown on Loewenstein-Jensen medium which indicates really high sensitivity of the GX test. In comparison to culture, the sensitivity, specificity, positive predictive value and negative predictive value were 97.7%, 100%, 100% and 95.5%, respectively, for the GX; the corresponding values were 85.7%, 100%, 100% and 75.0%, respectively, for the MTD.

From 41 GX *M. tuberculosis* positive specimens all of them were rifampicin sensitive by phenotypic drug susceptibility testing method, but GX test showed 39 rifampicin sensitive specimens and detected 1 rifampicin resistance and 1 result was indeterminate. After repeat testing GX test did not detect rifampicin resistance.

The GX test is rapid and has produced better results than MTD; it is more sensitive with significantly higher sensitivity and negative predictive value. It is suitable for routine detection of *M. tuberculosis* in specimens from untreated patients in small laboratories. Thus it can be easily included in our clinical laboratory in Slovenia. Further studies are needed to evaluate the performance of the GX test for the detection of rifampicin resistance since no rifampicin resistant specimens were included in this study.

PP-67

EVALUATION OF THIN LAYER 7H11 AGAR IN THE ISOLATION AND PRESUMPTIVE IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

Basoglu F, Uzun M

Istanbul University, Istanbul Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Istanbul, Turkey
Contact: meltemuzuntr@yahoo.com

Automated systems and molecular techniques are used in rapid diagnosis of tuberculosis (tb) and identification of Mycobacteria in laboratories around the world. However, these technologies are too expensive and not available in laboratories where resource-limited countries.

As a promising low-cost technique for culture of clinical samples in diagnosis of tb and presumptive identification of mycobacteria, the performance of Thin Layer 7H11 Agar (TLA) method was determined by comparing the results obtained from Lowenstein Jensen (LJ) medium and BACTEC MGIT 960 system (Becton Dickinson, Sparks, MD). With this aim 700 clinical samples were included in this study and 46 (6.57%) of them were positive for *Mycobacterium* species. Forty five (97.8%) of these bacteria were isolated by BACTEC MGIT 960 system, 38 (82.6%) by TLA method and 26 (56.5%) by LJ medium. The isolation time by BACTEC MGIT 960 system, TLA and LJ medium was found to be 8.94, 7.70, 22.25 days respectively for smear positive samples and 15.85, 14.28 and 27.85 days for smear negative samples. While no statistically significant difference was found between the isolation time of TLA medium and that of BACTEC MGIT 960 system ($p>0.05$), the isolation time of LJ medium was detected to be longer than that of those two media ($p<0.05$).

Irregular-edged microcolonies and cord formation were observed in 38 *Mycobacterium* species isolated from TLA media and all of these species were identified as *Mycobacterium tuberculosis* complex according to these datas. The results were confirmed by GenoType MTBC (Hain Lifescience, GmbH, Germany); 34 isolates were determined as *M. tuberculosis*, 2 as *M. bovis* ssp. *bovis* and 1 as BCG. Only one out of 38 was identified as nontuberculosis *Mycobacterium* (*M. abscessus* by GenoType *Mycobacterium* CM/AS, Hain Lifescience, GmbH, Germany).

At the end of the study it was concluded that TLA method is a rapid, inexpensive and reliable alternative for diagnosis of tb and presumptive identification of *Mycobacterium tuberculosis* complex.

PP-68

EVALUATION OF ALPHA TEC NAC-PAC™ MYCOBACTERIA DIGESTION AND DECONTAMINATION SYSTEM ON PULMONARY SAMPLES

Naidoo S, Barker A, Williams R

Lancet Laboratories, Lancet Corner, Corner Stanley and Menton, Richmond, Johannesburg 2090, South Africa - 011 358 0800
Contact: ROWilliams7@gmail.com

Clinical samples sent to the Mycobacteria Laboratory for culture confirmation of mycobacteria infection are contaminated by non-mycobacterial organisms and require digestion and decontamination to allow effective diagnosis. The optimal recovery of mycobacteria requires a tightly regulated pH. A basic pH quickly eliminates non-mycobacterial organisms from the patient sample. However, prolonged exposure to a high pH is toxic to mycobacterial organisms. A carefully controlled pH through-out samples preparation is essential to the recovery of viable mycobacteria.

We assessed the performance of the Alpha Tec NAC-PAC™ specimen preparation system in a high throughput laboratory in Johannesburg, South Africa, in 100 pulmonary samples. The NAC-PAC™ method was compared to the currently implemented BD MycoPrep™ Kit. The pH of the decontaminated specimen should be less than 8.1 immediately after buffering and maintained between 6.8-7.1 for culturing and diagnosis. At these pH levels optimum survival of the Mycobacterial organisms can be ensured. The Alpha Tec NAC-PAC™ system is the only commercially available system that can effectively achieve these pH levels and help reduce Mycobacteria die-off during the specimen preparation process. Samples were split and processed using BD's MycoPrep™ system and Alpha Tec's NAC-PAC™ system. Both methods were performed as per the manufacturers' procedure and assessed in the BACTEC™ System.

Of the 100 samples processed using the Alpha Tec NAC-PAC™ kit contamination levels decreased significantly: 90 samples were positive for TB, 8 were MOTTs and a 2% contamination rate was noted. The same 100 samples processed using the BD MycoPrep™ kit showed a 7% contamination rate, 90 samples were positive for Mycobacteria infection, of which 8% had to be re-cultured.

The advantage of the Alpha Tec NAC-PAC™ vs. BD MycoPrep™: contamination rate decreased from 7% to 2%; Recovery time improved by 3-5 days, decreasing TAT for results. NAC-PAC™ NALC-NaOH reagent is stable for up to 72 hours after preparation, whereas MycoPrep™ needs to be used within 24 hours of reconstitution.

PP-69

A COMPARISON OF TWO SPECIMEN PREPARATION METHODOLOGIES AND THEIR EFFECT ON THE OUTCOME OF DIAGNOSTIC PROTOCOLS FOR MYCOBACTERIA DETECTION

Šķenders Ģ, Prokopoviča I, Broka L, Williams R

Infectology Center of Latvia, Mycobacteriology department, Linezera iela 3 Riga, LV-1006
Contact: ROWilliams7@gmail.com

Sputum and other respiratory specimens are the primary means to diagnose pulmonary tuberculosis and other mycobacteria. In order to be useful for diagnostics, these specimens must be liquefied and decontaminated, but the failure to control pH using conventional specimen preparation protocols can reduce the number of viable mycobacteria in the patient sample and negatively affect analytical protocols. In this study we examined the effect of two separate specimen preparation methods on the outcome of common diagnostic procedures for AFB infection.

Pulmonary samples submitted for diagnosis were split evenly and subjected to two separate specimen processing methodologies; the conventional methodology using NALC, 3% NaOH and M/15 Phosphate Buffer, and a new methodology offered by the NAC-PAC™ EA3 (Alpha Tec Systems, Vancouver, WA). Once prepared for diagnosis each sample was screened via Auramine O & Rhodamine B staining, and, if positive, organism concentration was graded based on the WHO/IUATLD evaluation scale. Samples that were negative via microscopy were cultured using Lowenstein-Jensen medium at 36°C for 8 weeks. Specimens that were smear negative, culture positive were graded by the number of colony forming units present.

Of the 113 samples submitted for diagnostics 22 were positive for AFB by either microscopy or culture. Of these positive samples all 22 were detected using the Alpha Tec methodology and 20 were detected using conventional specimen preparation methodology. The two samples undetected using conventional methodologies were smear negative but culture positive using the Alpha Tec methodology. One sample that was smear negative but culture positive using the conventional methodology was smear positive using the Alpha Tec methodology. Four smear negative culture positive samples showed non-correlating growth, three samples using the Alpha Tec methodology showed a greater number of AFB colonies, one sample using the conventional methodology showed a greater number of colonies.

The Alpha Tec NAC-PAC™ EA3 AFB specimen processing reagent system identified positive smears and cultures otherwise missed by the conventional NALC/NaOH/M/15 Buffer methodology.

PP-70

EVALUATION OF THE BD MGIT™ TBc IDENTIFICATION TEST FOR THE RAPID DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX FROM CULTURES

Ramos J[1], Machado D[1,2], Couto I[1,3], Amaral L[1,2,4], Viveiros M[1,4]

[1]Unit of Mycobacteriology
[2]UPMM, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT/UNL), Rua da Junqueira, 100, 1349-008 Lisboa, Portugal
[3]Centro de Recursos Microbiológicos (CREM), Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[4]COST ACTION BM0701 (ATENS)
Contact: mviveiros@ihmt.unl.pt

Rapid identification of *M. tuberculosis* complex (MTbc) and its differentiation from nontuberculous mycobacteria (NTM) is crucial for the efficient control of tuberculosis worldwide, demanding for reliable, instrument-free, fast and easy to perform identification assays, especially for low-income countries.

The recently launched BD MGIT™ TBc Identification Test (Becton Dickinson) is designed to detect MTbc from liquid cultures in 15 minutes, using a single, easily handled device, requiring no other instrumentation than a micropipette. Results are read in the TBc device, through the self-development of colored lines in the case of a positive result. The test is based on an immunochromatographic assay that detects the MPT64 antigen, which is specifically secreted by the *M. tuberculosis* complex.

In this work, the performance of the BD MGIT™ TBc Identification Test was blindly tested in a panel of 25 acid-fast bacilli (AFB) positive cultures from patients of five hospitals of the Lisbon Health Region, which included cultures of MTbc (n=13) and of NTM (n=12). The primary isolation cultures were done with the BD MGIT™ 960 system. The cultures were further identified by AccuProbe (Gen-Probe) assays and, when necessary, by the GenoType *Mycobacterium* CM/AS (Hain) systems.

Thirteen cultures were identified by the BD MGIT™ TBc Identification Test as positive for MTbc, whereas 12 cultures gave negative results and were identified as NTMs. These correspond, in this study, to a sensitivity and specificity of 100% and identical positive and negative predictive values.

Albeit additional testing with more cultures of both MTbc and NTM is required, the assay showed an excellent performance, being also very easy to perform and interpret. In the case of the negative results, the existence of a control line assured that the assay was correctly performed and that there was enough material to obtain a result.

The BD MGIT™ TBc Identification Test showed potential to be a possible alternative to the more demanding, expensive and time-consuming molecular biology based identification assays, principally in resource-limited countries, where the implementation and usage of molecular methods is often not possible.

PP-71

EVALUATION OF THE BD MGIT™ TBc IDENTIFICATION TEST (TBc ID), A RAPID IMMUNOASSAY FOR THE DETECTION OF *M. TUBERCULOSIS* COMPLEX FROM LIQUID CULTURE

Anandi Martin[1], Deirdre Bombeeck[1], Krista Fissette[1], Pim de Rijk[1], André De Bock[2], Ivan Hernández-Neuta[3], Patricia Del Portillo[3], Juan Carlos Palomino[1]

[1]Institute for Tropical Medecine, Nationalestraat,155, 2000 Antwerp, Belgium
[2]BD Diagnostics, Diagnostic Systems Erembodegem Dorp, 86, Belgium
[3]Corpogen, Cra 5 N°66A-34 Bogota, Colombia
Contact: amartin@itg.be

Rapid identification of mycobacteria in cultured samples is extremely important with the aim of starting the appropriate treatment in infected patients. This has become more urgent since the introduction of liquid culture-based automated systems for the rapid detection of *M. tuberculosis* such as the Bactec MGIT 960. For any positive liquid culture obtained, laboratories need to use a rapid identification test to differentiate *M. tuberculosis* complex from non tuberculous mycobacteria (NTM). NTM have been associated with human pulmonary disease and due to their clinical significance, rapid identification of mycobacteria has become more important.

This study was designed to evaluate the performance characteristics of the new BD MGIT™ TBc Identification Test (TBc ID) for the rapid identification of *M. tuberculosis* complex in clinical samples when performed directly from an MGIT 960 positive tube. We compared the results with those obtained with the reference PCR test specific for *M. tuberculosis* complex. We tested 66 *M. tuberculosis* clinical samples and 26 NTM. The sensitivity and specificity of the TBc ID test was 98.5% and 100% respectively compared to the PCR.

The TBc ID test is an easy and sensitive method for the identification of *M. tuberculosis* complex in liquid culture medium. All positive liquid cultures can be used directly in the TBc ID test without any further preparation or dilution. The test is very simple and does not require a high level of skills, neither the use of any specific equipment.

PP-72

EVALUATION OF THE MGIT™ TBc ID FOR *M. TUBERCULOSIS* COMPLEX DETECTION FROM LIQUID AND SOLID CULTURES IN A ROUTINE SETTING

Rita Macedo, Arlinda Lopes, Irene Rodrigues, Edna Pereira

Public Health Laboratory: Micobacteriology/Tuberculosis, Public Health Department, Administração Regional de Saúde de Lisboa e Vale do Tejo, I.P.
Contact: rita.macedo@csalcantara.min-saude.pt

There is a growing need for rapid and cost-effective methods to detect *Mycobacterium tuberculosis* complex organisms from positive cultures.

The purpose of this study was to compare the sensitivity and specificity of three identification devices in a routine setting: BD MGIT TBc Identification Test (BD Diagnostics, Sparks, USA), Accuprobe *Mycobacterium tuberculosis* Culture Identification Test (Gen Probe, San Diego, USA) and the Genotype *Mycobacterium* CM/AS (HAIN lifescience, Nehren, Germany).

Positive liquid (BACTEC™ MGIT™ 960 7 mL tubes) and solid (BBL Lowenstein-Jensen slants) cultures were screened for *M. tuberculosis* detection according to manufacturer's instructions.

All three devices detected each of the predominant non-tuberculous mycobacteria (NTM) isolated in our laboratory (100% specificity).

Sensitivities for the *M. tuberculosis* complex detection were as it follows: 100% for the Genotype *Mycobacterium* CM/AS device, 93.8% for the TBc ID device, and 78,1% for the Accuprobe device.

Sensitivity and specificity results for each device were identical both for solid and liquid media.

Although the Genotype *Mycobacterium* CM/AS device showed the best results (100% sensitivity and specificity), the use of the TBc ID device may be a more cost effective approach, allowing high sensitivities, and a faster (15 minutes from a positive AFB culture to *M. tuberculosis* complex detection) and cheaper (no need for PCR-based methods) diagnosis.

PP-73

EVALUATION OF GENOTYPE MYCOBACTERIA DIRECT ASSAY IN COMPARISON WITH GEN-PROBE *MYCOBACTERIUM TUBERCULOSIS* AMPLIFIED DIRECT TEST AND GENOTYPE MTBDR*PLUS* FOR DIRECT DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX IN CLINICAL SAMPLES

Kontos Fanourios[1], Kosmadakis Georgios[2], Bazigos Stavros[2], Gitti Zoe[2]

[1]Clinical Microbiology Laboratory, Medical School of Athens, Attikon University Hospital, Athens, Greece
[2]Microbiology Laboratory, University Hospital of Heraklion, Greece
Contact: fankon68@hotmail.com

Early diagnoses together with adequate therapy are essential for TB control. Commercial molecular assays for direct detection of *Mycobacterium tuberculosis* in clinical samples lead to considerable improvement in the diagnostic rate.

Three commercial molecular assays: Gen-Probe Amplification Direct test (AMTD), (bioMérieux, Gen-Probe Inc., San Diego, CA), GenoType Mycobacteria Direct (GTDIR), and GenoType MTBDR*Plus* (GTPlus) (Hain Lifescience, Nehren, Germany), were evaluated for direct detection of *Mycobacterium tuberculosis* complex in 125 respiratory and 22 non-respiratory samples.

A total of 147 samples from 132 patients who were highly suspicious for TB were included. Samples were collected over a three-year period (2006-2008). The samples were processed by Nalc-NaOH procedure, Z-N technique for smear staining, inoculated into BacT/Alert 3D bottles, onto L-J slants and three aliquots of each sediment were further used to perform the three genetic assays. The three tests were simultaneously applied to 147 samples (n=125 respiratory) and (n=22 extrapulmonary). Isolates were identified to species level by GenoType MTBC, CM and AS assays (Hain-Lifescience). The results, together with the results of acid-fast staining were compared to those of culture, in liquid and solid medium. The overall sensitivities obtained were as follows: GTPlus, 97.9%; GTDIR, 93.7%; AMTD, 89.6%. The specificity of the assays used was 100%.

It was concluded that although the three nucleic acids assays are rapid, sensitive and specific for the detection of *Mycobacterium tuberculosis* in all kind of clinical samples in a routine laboratory, easier in performance was the assay AMTD, more sensitive were the GenoType Assays. GTPLUS offers the simultaneous detection of the most common resistance mutations in the *rpoB* (rifampin resistance), *katG*, and *inhA* (isoniazid resistance) genes. GTDIR should be used in areas with high incidence of infections with NTM.

PP-74

PCR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX AND LABORATORY CONFIRMATION OF TUBERCULOSIS

Paula Cristovão, Inês João, Luísa Jordão, Suzana David

National Health Institute Dr. Ricardo Jorge, Avenida Padre Cruz, 1640-016, Lisbon, Portugal
Contact: suzana.david@insa.min-saude.pt

The use of nucleic acid amplification (NAA) for the detection of *Mycobacterium tuberculosis* complex (MTC) in biological specimens is frequently requested for the rapid diagnosis of tuberculosis (TB). Results obtained in the laboratory using NAA were analyzed and classified according to the definition of a TB case at the laboratory level: upon the detection of nucleic acids of the MTC a TB case is confirmed when either positive acid-fast bacilli (AFB) microscopy or culture is obtained. TB is considered probable, with either positive AFB microscopy or detection of nucleic acids of the MTC in the biological specimen.

Laboratory confirmation of TB was evaluated in a preliminary study based on 186 specimens (95 cerebrospinal fluid, 45 respiratory, 46 of other origins). NAA was carried out using a commercial real time PCR kit. AFB microscopy was carried out using the Ziehl-Neelsen stain. Culture was performed on Lowenstein-Jensen medium. Of the 186 specimens analyzed, 156 (83.8%) were negative for NAA, AFB microscopy and culture and were thus classified as improbable cases of TB, 12 (6.5%) were confirmed cases and 18 (9.7%) probable TB cases.

Results obtained are analyzed and discussed as regards quality assurance for this type of NAA technique.

PP-75

TWO REAL-TIME PCR METHOD FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

Khaje Karamadini M, Amel Jamehdar S, Derakhshan M, Darban Hosseini M, Noemani H

Microbiology and Virology Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Iran
Contact: karamadinim@mums.ac.ir

Introduction. Tuberculosis remains one of the major public health problems worldwide particularly due to the appearance of drug-resistant *Mycobacterium tuberculosis* strains that render TB control programs more cumbersome. A number of nucleic acid test methods have been developed for rapid detection of *Mycobacterium tuberculosis* complex in clinical specimens. In the present study, specific detection of *Mycobacterium tuberculosis* complex was shown by Real Time PCR using SYBR Green-I and Taq Man probe.

Materials and Methods. *Mycobacterium tuberculosis* DNA was extract by Qiagen kit and PCR reaction was done. For preparation of calibrator PCR product was cloned into the pCRII plasmid. Calibrator plasmid was purified with the Qiagen plasmid maxi kit and used to evaluation of tests sensitivity. Repeatability and reproducibility were estimated by computing the coefficient of variation.

Results. Intra-assay and Inter-assay variability for two tests were always below 10% and 20%, respectively. The results demonstrated that Taq Man and SYBR Green technique respectively detected one and five copy of DNA in reaction.

Conclusion. Since respiratory tract specimens are naturally contaminated by many different species of commensal and pathogenic microorganisms, a high degree of specificity for *M. tuberculosis* recognition is mandatory. Sensitive and specific techniques to detect and identify *Mycobacterium tuberculosis* are important for the diagnosis and management of patients with TB. This two Real Time PCR assays represent a good system for the sensitive detection of TB and can be used in setting of a routine university hospital laboratory.

Key Words. *Mycobacterium tuberculosis* Complex, Real-Time PCR, Taq Man Probe, SYBR Green I

PP-76

STUDY OF THE POTENTIAL OF THE LIPASE LipY TO THE DIAGNOSIS OF ACTIVE PULMONARY TUBERCULOSIS

Brust B[1,2], Kremer L[1], Valverde V[2]

[1]Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Universités de Montpellier II et I, CNRS; UMR 5235, case 107, Place Eugène Bataillon3, 34095 Montpellier Cedex 05, France
[2]Bio-Rad, route de Cassel, 59114 Steenvoorde, France
Contact: belinda.brust@univ-montp2.fr

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major public health problem worldwide, with 9.4 million new cases and 1.8 million deaths annually, despite the use of the bacilli Calmette-Guerin (BCG) vaccine and effective antibiotics. Indeed, the problem has been raised by the increase emergence of multidrug-resistant strains and co-infection with human immunodeficiency virus (HIV). Currently, microscopy, culture and molecular tools are used to diagnosis disease but unsuited to emergent countries. A rapid, cheap and performing test needs to be developed. Immunologic test can be a solution. However, the difficulty is to find appropriate antigens. To date, many antigens have been studied but their performances are not sufficient for an efficient diagnostic test. In this regard, the lipase LipY that is only present in pathogenic species, induced during infection and strongly associated to the mycobacterial cell wall may represent an attractive candidate. We developed two ELISA tests to detect respectively the LipY antigen and the specific antibodies in the serum.

We have been tested 134 sera whose 55 BCG vaccinated healthy donors, 40 non-vaccinated healthy donors and 39 patients suffering from active pulmonary tuberculosis were tested. The detection of LipY specific antibodies showed a sensitivity of 43.6% (34.08%-53.29%) with a specificity of 90% (82.38%-95.10%). There was a significant impact of the vaccination in the response ($p<0.0003$).

Our sandwich ELISA did not allow to detect circulating LipY in the serum of infected patients. The results obtained for the detection of LipY specific antibodies are in accordance with those of other described antigens. It could be interesting to test LipY in combination with other antigens to improve the detection of specific antibodies.

PP-77

MYCOBACTERIA ISOLATED FROM CATTLE TISSUE SAMPLES IN POLAND 2004-2009

Marek Lipiec, Marcin Weiner, Monika Krajewska

National Veterinary Research Institute, 57 Partyzantow Str, 24-100 Pulawy, Poland
Contact: mlipiec@piwet.pulawy.pl

Introduction. Bovine tuberculosis is a chronic disease of cattle that sometimes affects other species of mammals. This disease is a significant zoonosis which can spread to humans. This disease is an effect of *Mycobacterium bovis* infection. Bovine tuberculosis is characterized by the formation of granulomas (tubercles) where bacteria have localized The laboratory diagnosis can be made by post mortem, microscopic, culture and biological examinations on laboratory animals. The identity of the microorganisms can be confirmed with biochemical or genetic techniques which can distinguish different mycobacteria strains from *Mycobacterium bovis*.

Materials and methods. The period 2004-2009 was the decisive for classification of Poland as a country free from bovine tuberculosis. In these six years totally 2073 cattle tissue samples were tested. According to Polish and all laboratory tests can made in National Reference Laboratory for bovine tuberculosis and following samples have to be send: lungs, liver, spleen and retropharyngeal, bronchial, mediastinal, submandibular and mesenteric lymph nodes. The tubercles were most common in the lymph nodes of the thorax, particularly the mediastinal lymph nodes. All tissue samples (or other tissue when veterinarian has suspicion) were prepared using 5% oxalic acids and microstomacher. After 30 minutes of incubation the leavings of the acid were rinsed with physiologic salt solution and inoculated on the set of Stonebrink and Lowenstein-Jensen media. Direct smears from all samples were stained with the Ziehl-Neelsen stain. The cultures were incubated for eight weeks; growth usually becomes visible in 3 to 6 weeks. The identity of the organism can be confirmed with Hain GenoType MTBC, CM or AS tests. In each case biological tests on guinea pigs were made.

Results. Totally 891 strains classified as *Mycobacterium bovis* were isolated. 12 strains were described as *Mycobacterium terrae*, *Mycobacterium tuberculosis* and *Mycobacterium fortuitum*. It is evident that form 20,0% to 54,7% of all samples originated from tuberculin positive animals were negative in laboratory tests. The number of samples with tuberculous lesions did not exceed 65,0% in any of the documented years.

Conclusions. It was concluded that other mycobacteria besides *Mycobacterium bovis* can be isolated form tuberculin positive cattle and they can caused false positive results of tuberculin tests.

PP-78

BOVINE TUBERCULOSIS IN POLAND 2004-2009. ERADICATION AND FREE STATUS OBTAINMENT

Marek Lipiec, Marcin Weiner, Monika Krajewska

National Veterinary Research Institute, Partyzantow 57, 24-100 Pulawy, Poland
Contact: mpweiner@piwet.pulawy.pl

Introduction. Bovine tuberculosis (bovine TB) is a contagious chronic disease of cattle caused by *Mycobacterium bovis* and associated with progressive emaciation and tubercle (granuloma) formation involving most usually the respiratory system but also other organs. As well as being of great economic importance to the livestock industry. Bovine TB is widespread throughout the world. It is subject to control programs in a number of countries.

Materials and methods. In Poland as in other European countries exist the special control program of bovine TB. It provides to test using bovine and avian PPD tuberculins 1/3 of the cattle population in each voivodeship also with comparative tuberculin testing to differentiate false positive reactions. All positive animals are eliminated under special conditions and suitable tissue samples are send to the reference lab in NVRI (National Veterinary Research Institute) in Pulawy, in which all samples are tested using microscopic, culture, biological (on laboratory animals) and typing procedures.

Results. In years 2004-2009 (the analyzed period for establish the country status) the number of bovine TB outbreaks oscillated from 44 in 2004 to 12 in 2009. The outbreaks were localized specially in the central part of Poland. The number of cattle eliminated because of positive result of tuberculin tests decreased in the same time from 554 to 111. The positive results of laboratory tests (isolation of *Mycobacterium bovis* strain) were obtained in 45.3–80.0%, depending from the year.

Conclusions. Relatively low percentage of positive results means that in many cases tuberculin test gave false positive results. It is the cost of an employment the tuberculin test in the veterinary practice. It was concluded that microscopic examinations had limited value in case of bovine TB because in 95.0% of microscopic specimens were negative. The biological test (on guinea pigs) was very sensible and reliable procedure. The isolation procedure was completed with the mycobacteria strains typing using Hain GenoType kits. The obtained status of Poland to be free from bovine TB testifies that the used procedures and TB eradication system fulfill its task and allow to control this zoonosis. All methods are compatible with Manual OIE and Directive 64/432.

PP-79

DETECTION OF *MYCOBACTERIUM BOVIS* INFECTION IN FARM AND COMPANION ANIMALS IN GREAT BRITAIN

Brewer JI, Worth DR, Wood EJ, Brown J, Okker M

Veterinary Laboratories Agency, Woodham Lane, New Haw, Surrey KT15 3NB, United Kingdom
Contact: d.r.worth@vla.defra.gsi.gov.uk

The number of incidents of bovine tuberculosis (*Mycobacterium bovis*) in cattle in Great Britain remains high. A change in the Defra submissions criteria in 2009 has meant a drop in reactor submissions whilst the number of culture positives remains high.

Diagnosis in cattle depends largely on statutory tuberculin testing of herds and routine inspection of carcases at slaughter. Although suspicion of TB in the carcases of other domestic species became notifiable in GB in 2006, identification of the disease is dependent on veterinarians recognising the signs in the live animal or at post mortem.

Methods for confirmation of infection in cattle and other domestic species are similar and largely rely on the identification of *M. bovis* culture on a combination of liquid and solid media. Work on DNA extraction and PCR from tissue and fixed histological material is ongoing.

Other mycobacterium species occur in the environment that can cause granulomas and infection in companion animals especially cats that may be considered zoonotic to their owners especially if immuno-compromised, and these need to be identified. This is currently through use of a multiplexed PCR and the Hain test but use of other rapid technologies are being investigated.

All VLA diagnostic work is accredited to IS17025 and 9001 standards.

PP-80

BOVINE TUBERCULOSIS IN WILD ANIMALS IN POLAND

Marek Lipiec, Marcin Weiner, Monika Krajewska

National Veterinary Research Institute, Partyzantow 57, 24-100 Pulawy, Poland
Contact: monika.krajewska@piwet.pulawy.pl

Introduction. Tuberculosis caused by *Mycobacterium bovis* has been described in many species of domestic and free living animals all over the world. The prevalence of tuberculosis in Poland in six past years, allow to receive according to current regulations a status of country free from bovine TB. It means that percentage of tuberculin positive herds is under 0.1%. At the same time in few cases the suspicion of bovine TB in wild animals was undertaken.

Materials and methods. The aim of the research was an estimation the range of mycobacterial infections in wild, hunted animals. In 5 years period of the program realization, totally 837 samples from wild animals were examined. The source of all samples was tissue of hunted free living animals. These samples were tested according OIE official methods, it means: 5% oxalic acid was used for first step of preparation then microscopy examinations, culture test on Lowenstein-Jensen and Stonebrink media and biological test on laboratory animals (guinea pigs) were ran.

Results. Total 10 *M. bovis* and 35 *M. avium* strains were isolated. *M. bovis* strains were isolated form 5 tested samples from roe-deer (*Capreolus capreolus*) (1,7% of all samples) and in 4 cases from deer (*Cervus elaphus*) (3,0%). All strains (35, 8,7%) isolated from wild boars (*Sus scrofa*) were identified as *Mycobacterium avium* strains.

Conclusions. It evidences that the wild animals could be the reservoir of basic type of mycobacteria *Mycobacterium bovis* and *Mycobacterium avium* and the species most of all infected is wild boar. Similarly to pigs tuberculosis the dominant type is *Mycobacterium avium*, which is not contagious for the cattle and human with the normal status of immunological system. Geographic localization of these outbreaks show that they did not have the direct connections with the bovine tuberculosis cases. At the animal-to-animal level *Mycobacterium bovis* is transmitted probably as an aerosol in the same herd. Poland has no problem (as UK) with badgers tuberculosis and transmission of the infection to cattle herds. The evaluation of degree of mycobacterial infection will conduct in next years, also in other species of wild animals.

PP-81

MYCOBACTERIUM CAPRAE AND *MYCOBACTERIUM BOVIS* INFECTION IN CATTLE IN CROATIA

Špičić S, Zdelar-Tuk M, Duvnjak S, Račić I, Kompes G, Habrun B, Cvetnić Ž

Croatian Veterinary Institute Zagreb, Laboratory for bacterial zoonosis and molecular diagnostics of bacterial diseases, Savska cesta 143, Zagreb, Croatia
Contact: spicic@veinst.hr

In the period from 2001 to 2006 we monitored the circulation of tuberculosis in cows within the regular annual tuberculosis checking in the Republic of Croatia. All cattle older then 6 weeks are subject to tuberculin skin test with bovine tuberculin and after 6 weeks suspecious and positive cattle were retested by avian and bovine tuberculin (comparative method). Positive reactors were slaughtered and matherial for bacterial testing were collected. Isolates were identified and typed by molecular methods: 65kD antigen (PCR), GenoType CM and MTBC- Hain (specific hybridization) and MIRU-VNTR.

During that period the positive reactions on bovine tuberculine were found in 95 (9.4%) and suspicious ones in 34 (3.4%) tested cows. A pathoanatomic examination upon slaughter was carried out on 123 cows, which were found positive or suspicious of tuberculine. Mycobacteria were extracted from 83 (67.5%) cows in 6 counties. *Mycobacterium* (*M.*) *caprae* was isolated in 71 cows from 7 flocks and *M. bovis* was isolated from 11 cows in 4 flocks and 1 isolat belong to *M. avium* complex.

By mutual comparison of the isolate genotypes and other epizootiological facts we determined the sources and routes of spreading the infection by types *M. bovis* and *M. caprae* in cows. We also found *M. caprae* as a dominant couse of bovine tuberculosis in Croatia. The defined genotypes of our mycobacteria isolates are comparable to other results of this type in the world thus providing us the basis for further research from the point of view of epizootiology and epidemiology.

PP-82

AN OUTBREAK OF BOVINE TUBERCULOSIS CAUSED BY *MYCOBACTERIUM CAPRAE* IN BOSNIA AND HERZEGOVINA

Besirovic H[1], Alic A[1], Spicic S[2], Cvetnic Z[2], Prasovic S[1]

[1]University of Sarajevo, Veterinary Faculty, Department of Pathology, Zmaja od Bosne 90, 71000 Sarajevo, Bosnia and Herzegovina
[2]Croatian Veterinary Institute, Zagreb, Department of Bacteriology and Parasitology, Laboratory for Bacterial Zoonosis and Molecular Diagnosis od Bacterial Diseases, Savska Street 143, Zagreb, Republic of Croatia
Contact: hajrudin.besirovic@vfs.unsa.ba

Bovine tuberculosis is a chronic disease of a zoonotic character that occurs in cattle and a wide range of domestic and wild animals. It is caused by *Mycobacterium bovis* and other members of *M. tuberculosis* complex which includes *M. tuberculosis*, *M. caprae*, *M. microtii*, *M. canettii*, *M. africanum*, *M. pinnipedii*. This work describes an outbreak of bovine tuberculosis caused by *M. caprae* in a five small dairy herds in south western Bosnia and Herzegovina.

For the identification of the disease and causative agent comparative tuberculin skin test (TST), pathomorphology, microbiology and molecular methods were applied.

Total of twenty five cattle were euthanized after comparative TST revealed fifteen positive and seven suspicious animals. In seven out of nine animals positive to comparative TST that were subjected to field necropsy lesions consistent with tuberculosis were observed on lymph nodes of thoracic cavity and lung. Histopathologic examination by hematoxilin and eosin staining confirmed the presence of specific granulomatous lesions while Ziehl-Neelsen staining of both cytologic and histopathologic slides demonstrated the presence of a very few acid fast bacteria. Acid fast bacteria isolated from four out of six submitted samples of cattle lymph nodes were identified as *M. caprae* by biochemical and molecular methods.

Our findings represent the first documented outbreak of cattle tuberculosis in Bosnia and Herzegovina since 1999. Also, this is the first evidence of bovine tuberculosis caused by *M. caprae* in Bosnia and Herzegovina.

PP-83

ISOLATION AND IDENTIFICATION OF THE *MYCOBACTERIUM AVIUM* SUBSP. *HOMINISSUIS* STRAINS FROM PIGS IN ESTONIAN SLAUGHTERHOUSES

Mihhail Sudakov, Silva Sütt, Sirje Kokassaar

Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Laboratory of Mycobacterioses, Kreutzwaldi 62, 51014 Tartu, Estonia
Contact: mihhail.sudakov@emu.ee

M. avium subsp. *avium* is the causative agent of avian tuberculosis and the most prevalent atypical mycobacteria. *M. avium* may cause the infection in cattle, pigs and HIV-infected people. In farm animals pigs are most susceptible for the infection of *M. avium* causing the tuberculosis–like lesions in mesentheric lymph nodes. We used the conventional pathomorphological methods to investigate the pathological materials from the mesenteric lymph nodes of 4698 pigs. The pig's lesions were examined bacteriologically on Löwenstein-Jensen medium. During 2004-2009 373 (7,9%) swine carcasses tuberculosis-like lymphadenitis of mesenteric lymph nodes were observed. We isolated 245 cultures of *Mycobacterium* spp. and 88 from them were diagnosed as *M. avium*.

We have detected infections of *M. avium* by PCR based on the detection of insertion sequence IS*1245*. PCR test was used to differentiate *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*. We have analyzed probes from swine mesentheric lymph nodes and all these cultures were IS*1245* positive and IS*901* negative. According to this classification the cultures which earlier were detected as *M. avium* were in fact *M. avium* subsp. *hominissuis*. Therefore we can assume that *M. avium* subsp. *hominissuis* is essential cause of Mycobacteriosis of swine herd in Estonia.

PP-84

NON-TUBERCULOUS MYCOBACTERIA IN ANIMALS IN HUNGARY

Rónai Zs[1], Csivincsik Á[2], Dán Á[1], Jánosi Sz[1]

[1]Central Agricultural Office, Veterinary Diagnostic Directorate, Tábornok u. 2., 1149 Budapest, Hungary
[2]Somogy County Agricultural Office, Cseri Major, 7400 Kaposvár, Hungary
Contact: janosis@oai.hu

Non-tuberculous Mycobacteria (NTM) are worldwide distributed environmental opportunist pathogens which can infect both humans and animals. NTM infections can have various clinical manifestations (especially in human) and may cause allergic sensitisation of the infected animals. Besides the potential zoonotic risk these infections in animals can interfere with intradermal tuberculin test and hamper the *in vivo* diagnosis of bovine tuberculosis resulting in significant economic losses due to unnecessary restrictions and culling of reactor animals.

In the last five years we analysed nearly 4500 samples from various wild and domestic animal species by bacterial cultivation. *M. caprae* or *M. bovis* was isolated from less than 5% of the samples and more than 80% of all tested samples were negative.

From 14% of the samples non-tuberculosis Mycobacteria were isolated. Growth rate, pigment production and temperature range were determined and the strains were further tested by molecular methods (AccuProbe, GenoType).

60% of the NTM strains proved to belong to the *M. avium* complex (MAC). The remaining strains could be only partially identified.

Almost 65% of the strains isolated from cattle samples belonged to the MAC and more than 80% of these isolates were *M. avium* ssp. *paratuberculosis*. The vast majority of these strains were isolated from the lymphonodes of intradermal tuberculin test positive cattles confirming their role in the in vivo diagnosis of bovine tuberculosis. The strains isolated from swine were predominantly *M. avium*. In wild animals (wild boar, red deer, roe deer, fellow deer, fox) both *M. avium* and *M. avium* ssp. *paratuberculosis* were identified but 70% of the isolates did not belong to MAC. Among these isolates we recognised some *M. kansasii*, *M. fortuitum* or *M. gordonae* strains but 75-80% of them could not be properly identified by the applied methods.

PP-85

NEW SPOLIGOTYPES OF *MYCOBACTERIUM BOVIS* ISOLATES OF PIGS FROM ARGENTINA

Soledad Barandiaran[1], Marcela Martínez Vivot[1], Eduardo Vicente Moras[1], Angel Cataldi[2], Martín José Zumárraga[2]

[1]Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina
[2]Instituto de Biotecnología, CICVyA-INTA-Castelar, Buenos Aires, Argentina
Contact: sbaran@fvet.uba.ar; mzumarraga@cnia.inta.gov.ar

Tuberculosis is a chronic disease affecting domestic and wild animals as well as human. Pigs are susceptible to *M. tuberculosis*, *M. bovis* and *M. avium* subsp. *avium*. In Argentina, where bovine tuberculosis (BTB) has not been eradicated, the prevalence of *M. bovis* in pigs shows the same values as the one registered in the local cattle population.

The aim of this study was to identify spoligotypes of *M. bovis* isolates from pigs.

One hundred thirty nine tuberculosis lesions were collected during the veterinary inspection in slaughterhouses during 2007-2009. The molecular typing of the *M. bovis* isolates was carried out using the reverse hybridization technique of Spoligotyping.

Twenty eight different spoligotypes were identified and 121 (87%) isolates were grouped into 11 clusters. There were two main clusters designated SB0140 and SB0120 containing 76 (54.6%) and 17 (12.2%) isolates respectively. This spoligotype was also the most frequently detected in cattle from Argentina. One hundred thirty isolates (93.5%) revealed identical spoligotypes to cattle, showing the source of infection. There were 9 spoligotypes (8 unique and one cluster with 2 isolates) exclusively from pigs because they were not found neither in cattle nor other hosts from Argentina. Moreover, seven of them were not detected previously in the International data-base of Sussex University. Five of seven (71.4%) of new spoligotypes were related with spoligotype SB0140 and 2 (28.6%) with SB0120 because they were differentiated by deletion of 2 to 6 spacers and 1 to 3 respectively.
The finding of new spilogotypes of isolates from pigs could be due to the partial screening of bovines with BTB in Argentina or the existence of *M. bovis* clones circulating among pigs. On the other hand, the transmission between different hosts could provoke rearrangements of then DR region showing different spoligotypes related to the previously described.

PP-86

GENOTYPING OF PORTUGUESE MTC (*MYCOBACTERIUM TUBERCULOSIS* COMPLEX) ANIMAL STRAINS

Duarte EL[1], Domingos M[2], Amado A[2], Botelho A[2]

[1]Universidade de Évora, Laboratório de Sanidade Animal/ICAAM, Apartado 94, 7002-554 Évora codex Portugal
Contact: emld@uevora.pt
[2]Laboratório Nacional de Investigação Veterinária, INRB- Estrada de Benfica, 701, 1549-011 Lisbon, Portugal
Contact: a.amado.45@gmail.com

During five years (2002-2007), 295 MTC animal isolates, representing 25% of the total isolations from the bovine TB national eradication programme, were genotyped by spoligotyping and MIRU-VNTR typing. Main purposes were to assess the discriminatory power of different typing techniques and their usefulness for the Portuguese epidemiological scenario, to confirm transmission hypothesis between different cattle herds, and to evaluate strain sharing between different animal species, especially between ruminants and wildlife.

Isolates, previously identified by PCR-REA *gyrB* as *M. bovis* (n=283), *M. caprae* (n=10) and *M. tuberculosis* (n=2), from different animal species (cattle, n=258; goat= 8; deer, *Cervus elaphus*, n=21; wild boar, *Sus scrofa*, n=6 and mandrills, *Mandrillus sphinx*, n=2) were spoligotyped using 43 spacers home made membranes.

A sub panel of 177 isolates, representing the most frequent spoligotypes and those shared between domestic and wildlife species, of the same geographical region, were MIRU-VNTR typed, using an 8 VNTR loci set (3232, 2164, 2461, 2996, 2163b, 2163a, 0577, 0580).

SB0121 was the most frequent spoligotype (26.3%). Discriminatory power of both spoligotyping (Hunter-Gaston index, h=0.89) and MIRU-VNTR typing (h=0.97) was high, concordant with a low prevalence setting. Ten new spoligotypes were found and most had evolved through additional spacer deletions from SB0121. Four VNTR loci discriminated superiorly (3232, 2165, 2461, 2163a), and retained, together, 99% discriminating ability of the entire set. Genotyping and animal movements allowed tracing transmission routes between different herds. Still, for eight isolates, with identical genotypes, from four different herds of the same area, no cattle movements were recorded appointing for an unknown common infection source. Strain sharing between domestic and feral species was recorded in two regions, proving evidence of strains transmission between cattle and wild boar, and cattle and deer.

Future goals are to use genotyping as a supportive tool for epidemiological monitoring and effective eradication measures, as well as to further evaluate if feral species could hamper bTB eradication, clarifying whether if they act as spillover or maintenance hosts.

PP-87

MOLECULAR TECHNIQUES USED TO CHARACTERIZE *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*, AN UP-DATE

Castellanos E[1,2], de Juan L[1,2], Rodríguez S[1,2], Álvarez J[1], Alende T[1], Gutiérrez A[1], Mateos A[1,2], Aranaz A[1,2], Domínguez L[1,2]

[1]Animal Health Surveillance Center (VISAVET), Complutense University of Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain
[2]Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid
Contact: dejuan@visavet.ucm.es

Mycobacterium avium subspecies *paratuberculosis* is a world-wide microorganism that causes paratuberculosis or Johne's disease in many animal species, mainly ruminants. The role of this agent in the development of Crohn's disease in humans has been discussed although this hypothesis still remains controversial.

M. a. paratuberculosis is difficult to isolate due to long time incubation periods and culture requirements and it has been divided into three groups or clusters: Type I ('sheep' or 'S' type), Type II ('cattle' or 'C' type) and Type III ('intermediate' or 'I'). This classification has been achieved by means of different molecular techniques such as IS*900*-RFLP, PFGE, PCR-REA of *gyrB*, PCR-REA of *inhA*, PCR and denaturing gradient gel electrophoresis (DGGE) of MAP1506, PCR-sequencing of *recF*, PCR-sequencing of IS*900*, comparative genomic hybridization comparison (CGH), and high resolution melt analysis (HRM).

However, in occasions the slow-growing phenotypes (types I and III) are not typable by some of the above techniques (PFGE and IS*900*-RFLP) due to DNA quality and quantity requirements. In addition, these two tools are complex and difficult to apply as routine tests to characterize *M. a. paratuberculosis*.

For this reason, the techniques that are recommended in order to divide *M. a. paratuberculosis* strains are dependent on the capabilities of each laboratory. Thus, to avoid further steps such as gel electrophoresis or sequencing, HRM would be the first choice due to its speed and reproducibility. However, for those groups with no HRM technology available, the use of the PCRs specific for each type, derived from the CGH data would be another option. Therefore, by targeting MAV4125 or MAV4126, specific for types I and III and MAP3584 or MAP1435, type III-specific deletions, strains could be easily differentiated. As a result, type I strains would show amplification for all genes, type II for MAP3584 and MAP1435 genes and type III strains just for genes MAV4125 and MAV4126.

PP-88

MYCOBACTERIUM AVIUM SUBSP. *PARATUBERCULOSIS* DETECTION BY QUANTITATIVE REAL TIME PCR AND CULTIVATION IN MILK AND CHEESE

Slana I, Kralik P, Pavlik I

Veterinary Research Institute, Brno, Czech Republic
Contact: slana@vri.cz

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiologic agent of paratuberculosis, a disease with considerable economic impact, principally on dairy cattle herds. Animals with paratuberculosis shed viable MAP especially in their milk, faeces and semen. MAP may have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products.

The aim of the study was to develop two duplex real time quantitative PCR (qPCR) systems specific for MAP detection (MAP-specific targets IS*900* and F57). Both assays incorporate an internal amplification control amplified with the same primers as the targets and the same probes are used in both assays. The developed qPCR method was used to detect MAP in cows' bulk tank milk (BTM; 220 samples) and cheeses (28 samples) prepared from sheep, goat and mixed sheep and goat milks. By qPCR, MAP DNA was detected in 63 (28.6%) of 220 cows' BTM samples and in seven (25%) cheese samples. However no viable MAP was detected either in BTM or cheese samples by conventional culture method.

This study demonstrates that qPCR method can be used effectively to investigate levels of MAP contamination that may pose a risk to human health.

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PP-89

REAL-TIME QUANTITATIVE PCR DETECTION OF *MYCOBACTERIUM AVIUM* SPECIES IN RAW AND PROCESSED BEEF PRODUCTS

Klanicova B, Slana I, Kaevska M, Pavlik I

Veterinary Research Institute, Brno, Czech Republic
Contact: klanicova@vri.cz

The member of the group *Mycobacterium avium* complex (MAC), *M. avium* species, is considered to be the main human and animal pathogen within the MAC group. *M. avium* is divided into four subspecies; *M. a. avium* (MAA), *M. a. paratuberculosis* (MAP), *M. a. hominissuis* (MAH), and *M. a. silvaticum*. MAA and MAH can cause diseases such as tuberculosis-like illness, disseminated infections, osteomyelitis, lymphadenitis in immuno-compromised patients and mammals. Subspecies MAP evocates paratuberculosis in ruminants (Johne's disease) and furthermore it has been proposed, that MAP could be involved in Crohn's disease, chronic inflammation of the gastrointestinal tract in humans.

The aim of this work was to examine various raw and processed beef meat products purchased in three supermarkets and in one butcher and to find out if any traces of MAP, MAA and MAH subspecies could be detected in these commodities.

The analysis of the beef products was performed using real time quantitative PCR (qPCR) method for the detection of specific insertion sequences; duplex qPCR for the detection of IS*900* specific for MAP, and triplex qPCR for the detection of IS*901* specific for MAA, and IS*1245* specific for MAH.

Out of the 33 tested beef samples, nine (27%) were found to contain MAP, two (6%) samples contained MAA and in eight (24%) beef samples MAH was detected. The concentration of MAP and MAH in several beef products exceeded 103 genome/g; in some products the concentration of MAA was overlapping 104 genome/g.

By the assay of beef products, we have provided the evidence that *M. avium* is present in elevated amounts in commodities which are sold to ordinary consumers. Therefore further investigation should be considered to establish the risk of *M. avium* contamination.

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PP-90

COMPARISON OF MILK AND SERUM ELISA, FAECAL CULTURE AND PCR IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTED HERDS

Darja Kušar, Katarina Logar, Urška Zajc, Brane Krt, Jana Avberšek, Matjaž Ocepek

University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Gerbičeva 60, SI-1115 Ljubljana, Slovenia
Contact: matjaz.ocepek@vf.uni-lj.si

Mycobacterium avium subsp. *paratuberculosis* (Map) is the causative agent of paratuberculosis, a chronic gastroenteritis in many species, including ruminants, that causes great economic losses in animal production systems. In Slovenia, a systematic screening of paratuberculosis began in 1995. Detection of Map-antibodies by serum or milk ELISA is a commonly used screening tool for identifying the potentially Map-positive herds. The aim of our study was to select the best method for the confirmation of Map-positive animals in the infected herds by comparing different Map-detection methods on large pool of animals originating from serologically Map-positive bovine herds.

Four different methods were compared: commercial serum and milk ELISA kits (Pourquier® ELISA Paratuberculosis; Institut Pourquier, France), faecal IS*900*-PCR assay, and faecal culture on solid selective media. We collected 483 serum, milk and faecal samples from cows originating from 15 herds with at least one serologically Map-positive animal.

Out of 15 herds, four (26.7%) herds with 214/483 (44.3%) animals were considered to be Map-positive since at least one animal from the herd tested positive by faecal culture and/or PCR in the present study or the herd had a clinical history of the disease. From these herds, 72/214 (33.6%) animals were Map-positive by faecal culture and/or PCR, and out of these animals, 16/72 (22.2%) tested positive to Map serologically as well (10/16 by serum and milk, 3/16 by serum only and 3/16 by milk ELISA only). In Map-positive herds, 67/214 (31.3%) animals tested positive by faecal culture, 20/214 (9.3%) by milk ELISA, 17/214 (7.9%) by serum ELISA, and 11/214 (5.1%) by faecal PCR. In Map-negative herds, 32/269 (11.9%) animals tested positive by serum and/or milk ELISA.

Our results indicate that serological Map-testing might lack sensitivity and specificity. On one hand, approx. only 1/5 animals assigned with Map-positive result of faecal culture/PCR and on the other hand, approx. 1/10 animals originating from herds without the present or previous reports on the presence of Map, tested serologically positive. In our case, faecal culture showed to be the most reliable method for the detection of Map-positive animals in herds with paratuberculosis. PCR detection exerted lower sensitivity than cultivation, however it enabled Map-detection in some samples not positive by faecal culture.

PP-91

PARATUBERCULOSIS IN FREE RANGING DEER HERD

Marek Lipiec, Marcin Weiner, Monika Krajewska

National Veterinary Research Institute, Partyzantow 57, 24-100 Pulawy, Poland
Contact: monika.krajewska@piwet.pulawy.pl

Introduction. Paratuberculosis (Johne's disease) is a chronic disease of domestic and wild ruminants. It is caused by *Mycobacterium avium* subsp. *paratuberculosis* which proliferates in the mucosa of the intestines and belonging lymph nodes. It is characterized by loss of weight and body condition. Paratuberculosis is well documented in cattle, sheep, and goats. The direct costs of Johne's disease in dairy herds consist of reduced milk productivity, increased susceptibility to other diseases and infertility. Some cases of paratuberculosis is encountered in other ranging ruminants. In Polish conditions there are red deer (*Cervus elaphus*) or fallow deer (*Dama dama*).

Materials and methods. The tested red deer herd had about 1000 animals breeding for meat and in basic herd. The herd, in which paratuberculosis was diagnosed, based on 250 hinds and 80 stags.
First serologic screening test was performed on 5 blood samples collected from animals with clinical signs as follows: diarrhea, emaciation and infertility. For all tests the ELISA paratuberculosis screening and verification kits were used (ELISA Paratuberculosis Antibody Screening/Verification – Institut Pourquier, France). All animals positive in screening test were retested using verification test system. The results were obtained on Multiscan Ascent ELISA reader. In the next stage of test all deer in infected farm (1015 animals) were examined. The feaces from these animals were collected and reserved for PCR and culture tests. From the intestinal mucosa microscopic specimens were prepared (Ziehl-Neelsen staining) and culture tests were performed with Herrold egg yolk medium (HEYM).

Results. Post mortem examination demonstrate in all 13 cases the anatomopathological changes in the cecum and colon, but without any macroscopic lesions in adjacent lymph nodes. Positive results of microscopic examinations were obtained in 13 cases. On HEYM medium the colony of *Mycobacterium avium* subsp. *paratuberculosis* were visible after 18 weeks of incubation in 37°C. The DNA of *M. avium* subsp. *paratuberculosis* was find in feaces of infected animals.

Conclusions. The Johne's disease infection in described deer herd was confined and controlled. The next ELISA screening test (57 animals) were performed 6 month later with negative results. Taking into account the nature of *Mycobacterium avium* subsp. *paratuberculosis* and its resistance, this herd need persistent control.

PP-92

DETECTION OF MYCOBACTERIA IN FISH BY CULTURE AND MOLECULAR METHODS

Darja Kušar, Urška Zajc, Mateja Pate, Matjaž Ocepek

University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Gerbičeva 60, SI-1115 Ljubljana, Slovenia
Contact: darja.ferme@vf.uni-lj.si (Darja Kušar)

Chronic to subacute mycobacterial infections are commonly found in fish and are referred to as piscine tuberculosis. They affect the skin and fish internal organs, and severely reduce the production and market value of aquarium and consumable fish. Certain fish mycobacterial species like *Mycobacterium marinum*, *Mycobacterium fortuitum* and *Mycobacterium chelonae* can be transmitted to humans, where they cause contagious skin infections and are difficult to treat.

Thirty-five aquarium fish each from a different fish tank from eight randomly selected pet shops were examined by culture and molecular methods for the presence of mycobacteria. Samples from angelfish (*Pterophyllum scalare*), green swordtail (*Xiphophorus helleri*), southern platyfish (*Xiphophorus maculatus*), goldfish (*Carassius auratus auratus*), three spot gourami (*Trichogaster trichopterus*), and guppy (*Poecilia reticulata*) were collected. Fish internal organs were examined microscopically after Ziehl-Neelsen (ZN) staining, and prepared for the bacteriological examination on selective media. Cultures were examined for their morphology. ZN-positive cultures were subjected to molecular determination using the GenoType (GT) *Mycobacterium* CM and AS assays (Hain Lifescience, Germany).

Five samples out of 35 tested negative on mycobacteria (three tested ZN/culture-negative and two ZN/culture-positive but GT-negative). However, 30 samples (85.7%) tested positive on fish tuberculosis. *M. fortuitum* (of the group 1 according to GT) was found in one half (53.3%) of the positive samples, *M. marinum* in one fourth (23.3%), *M. fortuitum* of the group 2 (or with lower probability *Mycobacterium mageritense*) in 10%, and *M. chelonae* (or with lower probability *Mycobacterium immunogenum*) in 6.7%. Two fish exerted a mixed infection: one tested positive on *M. fortuitum* 1 and on *M. fortuitum* 2 or *M. mageritense*, and one on *M. fortuitum* 1 and on *Mycobacterium peregrinum*.

Since a high portion of the randomly selected ornamental fish tested positive on mycobacteria that are known to be pathogenic for humans as well, fish tuberculosis spread in pet shops should not be overlooked and safety precautions should be discussed.

PP-93

IS *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* THE TRIGGER IN TYPE 1 DIABETES MELLITUS AND MULTIPLE SCLEROSIS?

Daniela Paccagnini, Speranza Masala, Valentina Rosu, Andrea Cossu, Davide Cossu, Stefania Zanetti, Leonardo Sechi A

Dipartimento di Scienze Biomediche, Viale San Pietro 43, University of Sassari, Italy
Contact: sechila@uniss.it (Leonardo Sechi A)

Mycobacterium avium subspecies *paratuberculosis* (MAP), an obligate pathogen is the causative agent of a chronic, inflammatory bowel disease referred to as Johne's disease of ruminants. Infected animals excrete MAP in their milk and excrete MAP in their feces. MAP is also implicated to cause a similar type of enteritis in humans called Crohn's disease.

Sardinia, a genetic isolate with alleles and haplotypes that are rare or absent elsewhere poses as a high endemic zone of autoimmune disorders such as Type 1 Diabetes Mellitus (T1DM) and Multiple Sclerosis (MS). An interplay of genetic and environmental factors in Sardinia possibly could make it a hotspot to study autoimmune disorders. Our group have already shown an association between MAP and T1DM by showing the presence of MAP DNA and antibodies against specie-specific proteins of MAP in the blood of T1DM patients. There are several evidences indicating an association between early exposure to cow's milk and increased risk of T1DM. Interestingly, it has also been observed that children at risk for T1DM who were breast fed exclusively for more than six months were less likely to have T1DM later in life than children at similar risk who were weaned on to cow's milk. Such observations led to the foundation of the TRIGR study: Trial to Reduce Insulin Dependent Diabetes Mellitus in the Genetically at Risk. Here we report the results of the search for MAP specific antibodies in the blood of Sardinian children within the TRIGR study. Moreover, we show a final evidence in terms of culture of MAP bacilli from the blood of four of T1DM patients from Sardinia.

Regarding MS, the other autoimmune disease that in Sardinia reach one of the highest incidence in the world (along with Scandinavian countries), we report the results of a pilot study searching for the presence of IS*900* DNA and MAP specific antibodies in the blood of MS patients in comparison to controls.

PP-94

DETERMINING THE DISINFECTION EFFECT OF A LAUNDERING PROCEDURE FOR HOSPITAL TEXTILES USING THE INDICATOR BACTERIA *MYCOBACTERIUM TERRAE*

Fijan S[1,2], Šostar Turk S[2]

[1]University of Maribor, Faculty of Mechanical Engineering, Institute of Engineering Materials and Design, Maribor, Slovenia
[2]University of Maribor, Faculty of Health sciences, Žitna ulica 15, 2000 Maribor
Contact: sabina.fijan@uni-mb.si

Laundered hospital textiles should not contain microorganisms that cause diseases because their users are patients with a low immune system and should therefore be protected from infections from inappropriately washed textiles. The laundering procedure should have an anti-microbial effect, especially when washing hospital textiles that contain many kinds of pathogenic bacteria, fungi and viruses. Recent studies confirm the increase of nosocomial infections and microbial resistance and that one of the possible causes are infected textiles due to inappropriate laundering procedures. Since great quantities of hospital laundry are washed every day, it is important that laundering is ecological as well as appropriately disinfected. Ecological laundering means lowering the temperature used to disinfect hospital textiles and increase the washing and disinfecting agents, since high temperature laundering results in high energy and water consumption. However, decreasing the temperature of laundering procedures enhances the possibility of pathogenic microorganisms to survive the laundering procedure; therefore special caution is needed to find the optimum laundering conditions. In our research we determined the antimicrobial laundering effect by simulating a common laundering procedure for hospital textiles in the laboratory washing machine at different temperatures by the use of the indicator bacteria: *Mycobacterium terrae* as a representative of mycobacterium family. Sheep blood was used as a substrate for simulating human excrements and was inoculated together with the chosen microorganism onto cotton pieces to simulate real laundering conditions. It was found that *M. terrae* survived laundering at 35° and 45°C but was completely inactivated at 60°C. The chosen laundering procedure has an effective disinfection effect and at the same is more ecological and economical than thermal laundering procedures.

PP-95

TREND OF POSITIVITY RATES OF MICROSCOPY, CULTURE AND CULTURES CONTAMINATION FOR TUBERCULOSIS BETWEEN YEARS 2006-2009 IN CLUJ-NAPOCA LABORATORY

Daniela Homorodean[1], Andreea Jodal[1], Alin Dascalu[2]

[1]Clinical Hospital of Pneumology, B.P.Hasdeu street No 6, 400371-Cluj Napoca, Romania
[2]Laboratory medicine resident, University of Medicine, Emil Isac Street 5, Cluj Napoca, Romania
Contact: dhomorodean@yahoo.com

The positivity rate microscopy (M), culture (C) and culture contamination (CC) rate trends are indicators in internal quality control in tuberculosis laboratory.

We assessed monthly the workload in the last four years, 2006-2009, and the positivity rate for M and C. CC rate had been calculated monthly in the last three years.

The yearly average number of smear M has been 12164 (13115, 10495, 12643, 12403 respectively). The average of positivity rate M has been 8.2% (7.6%, 9%, 9.3%, 7% respectively). For cultures, the average number has been 12536 (13124, 12277, 12485, 12257 respectively). In the last three years the average rate of CC has been 4.9% (4%, 5.1%, 5.8%-respectively).

The monthly assessment of these indicators help us to maintain the process under control.

PP-96

MOLECULAR CHARACTERIZATION OF *M. TUBERCULOSIS* ISOLATES FROM NEWLY DETECTED TB PATIENTS IN THE STOCKHOLM AREA

Alexandra Pennhag, Nasrin Perskvist, Ramona Groenheit, Sven Hoffner

Department of Bacteriology, Swedish Institute for Infectious Disease Control, Nobels väg 18, 171 82 Solna, Sweden
Contact: alexandra.pennhag@smi.se

Altogether we saw 643 new tuberculosis (TB) cases in Sweden in 2009, which represents a 16% increase since 2008. Most of the new cases were seen in the Stockholm area.

The aim of this study was to characterize the clustering and global strain lineages among the isolates from new TB patients in the Stockholm area in 2009.

A total of 163 isolates were collected for molecular characterization using spoligotyping and IS*6110*-RFLP. Of the studies isolates, 83 were from male and 80 female patients, 21 were born in Sweden and 142 were born abroad.

Among the 163 isolates, we found a broad spectrum of different lineages; 36 were of the T-type, 31 CAS, 23 Beijing, 17 EAI, 17 H, 6 LAM, 6 U, 1 AFRI, 1 S, 1 X and 24 of different undefined types. Twenty-nine (17.8%) of the isolates were drug-resistant (DR) and of these 6 were multidrug-resistant (MDR). The majority of the DR strains belonged to the T- and Beijing-lineages. Among the 6 MDR isolates, 3 were of the Beijing genotype, 2 of T1 and one was of an undefined type. Thirty-four (21%) of the isolates had identical IS*6110*-RFLP pattern and belonged to a cluster. In total we found 15 different clusters and the cluster, each including 2-3 patients in 2009. The majority of the foreign-born patients came from Africa, especially Somalia. The majority of the patients with DR strains originated from the Middle East Central Asia region.

The increase in number of new TB cases is most likely related to increased immigration. Molecular characterization is important for understanding of the transmission of strains and migration between regions. The lineages of isolates from foreign-born patients reflected to a great extent lineages common in their country of origin. For example, 18 of the 23 patients with strains of the Beijing lineage came from Asia and the dominance of CAS (58%) and EAI (70.5%) lineages among patients from the Horn of Africa may reflect the vicinity of Asia.

PP-97

AUTOMATED MIRU-VNTR-TYPING OF *MYCOBACTERIUM TUBERCULOSIS* AND COMPARISON WITH SPOLIGO TYPING BY MEANS OF PARTITION MAPPING ANALYSIS

Lysiane Hauben, Dolf Michielsen, Luc Vauterin

Applied Maths NV, Keistraat 120, 9830 Sint-Martens-Latem, Belgium
Contact: lysiane_hauben@applied-maths.com

Genotyping of *Mycobacterium tuberculosis* (MTBC) has long been obstructed by the conserved character of the genome. Identification of genetic markers like large sequence polymorphisms (LSPs), regions of difference (RDs) and single nucleotide polymorphisms (SNPs) initiated a long progress in understanding the overall phylogenetic structures of the species. Deeper phylogenetic classification became possible using more variable independent markers. Nowadays, a typing system based on VNTR-typing (Variable Number of Tandem Repeats) applying genetic elements called MIRUs (Mycobacterial Interspersed Repetitive Units) as genetic markers has been found a suitable tool to analyze the genetic diversity of clinical isolates (Supply *et al.* J Clin Microbiol 44: 4498-4510).

In this study, we describe the use of the MIRU plugin in the BioNumerics software to automatically process sequencer trace files, interpret and analyze MIRU-VNTR data and interact with the MIRU-VNTR*plus* website (http://www.miru-vntrplus.org) for typing and global epidemiology of Mycobacteria (Allix-Béguec *et al.* J Clin Microbiol 46(8): 2692-2699). The synchronization with the MIRU-VNTR*plus* server allows types to be assigned automatically and new types to be submitted.

MIRU-VNTR data and spoligo data from 186 strains available on the MIRU-VNTR*plus* website were downloaded into the BioNumerics software and subjected to a partition mapping analysis, which allowed a statistically supported comparison to be conducted between the types (partitions) resulting from both techniques. The congruence between these two typing techniques was further illustrated by creating contingency tables, mapping rules and devising statistical parameters.

As a result of this comparison, it appeared to be possible to define a set of mapping rules to predict one partition from the other with no single violation. With MIRU-VNTR being the finer typing technique, it was possible to predict all spoligo types from MIRU types.

PP-98

A FIRST ASSESSMENT OF THE GENETIC DIVERSITY OF *MYCOBACTERIUM TUBERCULOSIS* IN CAMBODIA

Jian Zhang[1], Seiha Heng[2], Stéphanie Le Moullec[1], Guislaine Refregier[1], Brigitte Gicquel[3], Bertrand Guillard[2], Christophe Sola[1,3]

[1]Institut de Génétique et Microbiologie, UMR8621 CNRS-UniverSud, Equipe Infection, Génétique et Evolution des Pathogènes Emergents, Bat. 400, F-91405 Orsay-Cedex, France
[2]Institut Pasteur du Cambodge, Pnom-Penh, 5 boulevard Monivong, BP 983, Phnom-Penh, Royaume du Cambodge
[3]Unité de Recherche en Génétique Mycobactérienne, Institut Pasteur, Paris, France
Contact: jian.zhang@igmors.u-psud.fr, christophe.sola@igmors.u-psud.fr

We characterized 105 *Mycobacterium tuberculosis* clinical isolates collected between 2007 and 2008 in the region of Phnom-Penh, Cambodia, and including 28 multidrug-resistant isolates. Classical and extended spoligotyping (43 spacers and 68 spacers, respectively) and multi locus of VNTR analysis (MLVA) were performed. Classical spoligotyping indicated that EAI lineage is highly prevalent in Cambodia and discriminated 42 patterns with 85 clinical isolates being distributed in 14 clusters. Extended spoligotyping discriminated 51 patterns (+21%) which confirms the higher discriminatory power of extended spoligotyping, although discrimination still is insufficient to allow epidemiological inferences: the 24 loci MIRU-VNTR typing scheme distinguished 90 patterns with only 13 multi-isolates clusters covering 28 isolates. We also detected a higher prevalence of Beijing lineage among unrelated MDR isolates, suggesting higher ability of Beijing isolates to acquire multi-drug resistance.

Noteworthy, Manu isolates included in the study exhibited an extended spoligotyping profile suggesting that they are related to modern strains. We thus validate the usefulness of extended spoligotyping and propose a simplified spoligo-MLVA combined method for TB genotyping in East Asia.

PP-99

OCCURRENCE OF TUBERCULOSIS IN LARGEST SLOVENIAN CORRECTIONAL FACILITY ZPKZ DOB IN THE PERIOD FROM 2000 TO 2008

Koren Pucelj N[1], Hudoklin I[2]

[1]University Medical Centre Ljubljana, Zaloška cesta 2, 1000 Ljubljana, Slovenia
[2]Out-patient Practice for Respiratory Diseases, Goljev trg 3,8210 Trebnje, Slovenia
Contact: nadja.koren@gmail.com

Inmates are known to be more vulnerable to infection with tuberculosis (TB) compared to general population (crowded environment, presence of persons of low socioeconomic status, foreign-born individuals, users of illicit substances, persons with human immunodeficiency virus infection). According to the Slovenian national central register TB did not occur often in correctional facilities until 2000 (1-2 cases annually).

In 2000, after first symptomatic case was found, altogether four cases of pulmonary TB were detected in epidemiological investigation in largest Slovenian correctional facility ZPKZ Dob (ZPKZ Dob) with 345 male long term inmates. ZPKZ Dob had been overcrowded for many years (official capacity is 233 persons). As incidence of TB in general population was 18.9 per 100,000 inhabitants in the same year, the need for enhanced surveillance for TB in this correctional facility was recognized.

From beginning of 2001 until the end of 2008 all new inmates were screened for symptoms of TB and had tuberculin skin test and chest x-ray carried out. On average 162 (min 118, max 210, SD 29.1) new inmates were examined annually (altogether 1251 in 8 years). All were male, on average 36.2 years old (min 19, max 77, SD 10.4). In the period of eight years two cases of pulmonary TB (in 2002 and in 2004) and one person with inactive TB and documented history of adequate treatment for TB were found. Furthermore 169 cases of latent TB infection, with tuberculin skin test more or equal 10 mm, were detected. Both cases of active and 95.4% cases of latent TB infection (161 of 169) were treated with standard regimens for active and latent infection respectively.

We believe that the danger of outbreaks of TB in ZPKZ Dob was greatly reduced with treatment of active and latent TB among new inmates. We also recognize the need to implement the surveillance protocol for infection with TB among new inmates, already incarcerated inmates and correctional officers in all correctional facilities in Slovenia because the risk of TB is known to be higher in correctional facilities compared to general population. This is even more relevant for our country as for many years correctional facilities have been overcrowded and in last ten years 11-16% of inmates have been foreign-born (including those born in countries with high prevalence of TB and multidrug-resistant TB).

PP-100

IS*6110* INSERTION IN THE *dnaA-dnaN* INTERGENIC REGION OF *MYCOBACTERIUM TUBERCULOSIS* ZARAGOZA (MTZ)

Millán MI[1,2], Lezcano MA[2], Vitoria MA[2], Iglesias MJ[2], Martín C[2], Sandoval A[3], Reyes Muñoz A[3,4], del Portillo P[3], Samper S[1,2]

[1]Instituto Aragonés de Ciencias de la Salud, Hospital Miguel Servet, Zaragoza, Spain
[2]Grupo de Genética de Micobacterias, Departamento de Microbiología, Medicina Preventiva y Salud Pública, Universidad de Zaragoza, Zaragoza, Spain
[3]Corpogen. Cra 5 No. 66A-34, Bogotá, Colombia
4Washington University, St.Louis, 4444 Forest Pk Av St Louis, USA
Contact: samper@unizar.es

An outbreak of *Mycobacterium tuberculosis* strain named MTZ was detected in Zaragoza from 2001 to 2004 (Int J Tuberc Lung Dis 2007; 11(10): 1080-1086). MTZ strain was highly transmitted among the population. In one isolate of the MTZ strain one copy of IS*6110* was detected in the origin of replication, and we aimed to evaluate the presence of IS*6110* in the *dnaA-dnaN* intergenic region in MTZ strain in other isolates.

A total of 149 MTZ isolates were selected from 2001 to 2008, and performed a PCR to amplify the IS*6110* inserted in the *dnaA-dnaN* region. Even though all isolates had identical IS*6110*-RFLP, we found the IS*6110* in *dnaA-dnaN* in 7 (4.70%) isolates: 4 isolates in 2006, 2 in 2007 and 1 in 2008. We reviewed the medical records of these patients and 3 of them had family links and employment relationship with another person.

The amplified fragment was sequenced. The exact point of IS*6110* insertion in MTZ subgroup (1,625-27 pb of H37Rv) was different that the found for the Beijing Lineage (1,592 pb of H37Rv).

Our findings support that this insertion of IS*6110* in the *dnaA-dnaN* region detected a subgroup in MTZ strain. However, this subgroup cannot be identified by RFLP. The first isolate was found in 2006, while the spread of the strain began at least five years before, suggesting that IS*6110* insertion in *dnaA-dnaN* is not associated with high transmission of MTZ strain.

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PP-101

MOLECULAR CHARACTERIZATION OF *M. TUBERCULOSIS* STRAINS ISOLATED IN REPUBLIC OF BELARUS

Zalutskaya AM[1], Wijkander M[2], Skrahina AM[1],
Sahalchyk ER[1], Nikolenko AN[1], Hoffner S[2]

[1]Republican Scientific and Practical Centre for Pulmonology and Phthisiology, Department of Laboratory Diagnostic and Treatment of Tuberculosis, Dolginovsky tr. 157, 220053, Minsk, Belarus
[2]Swedish Institute for Infectious Disease Control, Department of Bacteriology, SE-171 82 Solna, Sweden
Contact: akasana@inbox.ru

The aim of this study was to define the most frequent geno-families of susceptible and resistant *M. tuberculosis* (Mtb) strains isolated from patients with pulmonary tuberculosis in Belarus. A total of 122 Mtb strains were isolated from sputum specimens of patients treated in the clinic of the Center in 2009. Mtb strains were isolated on LJ medium and in the BACTEC MGIT 960 system. Growth of mycobacteria was confirmed by ZN microscopy. Mtb strains were identified using biochemical and growth characteristics. DST was performed using the BACTEC MGIT 960 system. Among the 122 strains, 78 were MDR and 44 were susceptible to anti-tuberculous drugs. Genotyping of Mtb isolates was done by spoligotyping and the obtained spoligotypes were compared to the international database (SpolDB4.0).

In total, 73 (93.6%) MDR strains and 32 (72.7%) susceptible strains were clustered within 8 spoligotypes (Beijing, LAM9, T1, T2, H1, H4, Manu 2 and U). The most frequent spoligotype among both MDR and susceptible strains was Beijing family: 32/78 MDR strains (41.0±8.6%) and 14/44 susceptible strains (31.8±12.9%). We found no significant difference in the frequency of Beijing genotype in MDR and susceptible strains (p=0.3376). 30/78 (38.5±9.0%) of MDR strains and 10/44 (22.7±13.9%) susceptible strains belonged to T1 family (p=0.1076), 5/78 of MDR strains and 4/44 of susceptible strains belonged to LAM9 family.

Thus, we found that the large majority of the Mtb strains isolated in Belarus belonged to three geno-families: Beijing, T1 and LAM9. We found no significant differences in the frequency of these genofamilies in MDR and fully susceptible strains.

PP-102

TUBERCULOSIS FROM CONTACTS

Tomaž Hafner, Petra Svetina Šorli

University Clinic of Pulmonary and Allergic Diseases Golnik, Slovenia
Contact: tomaz.hafner@klinika-golnik.si

Introduction. Tuberculosis is an infectios disease passed from patient to people with infected droplets in the air usually from coughs and sneezes. Transmission usually occur between people who are in close and longstanding contacts. Not all infected people present with clinical disease, most of them have latent infection. Because of this active investigation of contacts of patients with tuberculosis is needed.

Materials and methods. In our study we retrospectively investigated how many cases of active tuberculosis and latent infections were found from investigations of contacts of patients with tuberculosis. Data were retrieved from Slovene national tuberculosis registry. We included contacts screened from years 2001 to 2009. Data were collected and manipulated using Microsoft Excel spreadsheet. We wondered how many contacts have been identified, what was the response rate to medical examination, how many latent and active tuberculosis infection were diagnosed, did contacts had any risk factors, and how much time passed between treatment of primary patient and discovered active tuberculosis of their contacts.

Results. From years 2001 to 2009 16815 contacts were identified and invited to medical examination. 12847 (76.4%) responded. From them 1937 (15%) were treated for latent tuberculosis infection and 94 (0.7%) were treated for active tuberculosis infection. 20 (21.3%) of patients with active disease had one of the risk factors for tuberculosis: alcoholics, drug users, HIV infection, living or working in correctional or nursing institution, homeless people, people with diabetes, cancer or patients on systemic corticosteroid treatment. 3 (3.2%) had two risk factors. Leading risk factor for active tuberculosis were alcohol with 10 (50%) alcoholics. 65 (69.1%) of contacts with active tuberculosis started treatment within one year after the primary patient. In 9 (9.6%) contacts elapsed time was between one and two years. And in 20 (21.3%) contacts more than two years have passed.

Conclusions. Screening of contacts of patients with active tuberculosis is an important part of diagnosing latent and active tuberculosis infections and is also part of prevention of spread of tuberculosis. More work needs to be done to achieve higher response rate of identified contacts to actually come to medical examination.

PP-103

TUBERCULOSIS IN SLOVENIA IN YEARS FROM 2000 TO 2009

Katja Triller Vadnal[1], Petra Svetina Šorli[2]

[1]General Hospital Izola, Polje 35, Izola, Slovenia
[2]The University Clinic of pulmonary and Allergic Diseases Golnik, Golnik 36, Golnik, Slovenia
Contact: katja_triller@yahoo.com

All newly diagnosed patients with tuberculosis (TB) have been reported to Central Slovenian Registry for tuberculosis. TB patients are currently treated in almost all Slovenian hospitals: University Clinic of pulmonary and Allergic Diseases Golnik, University Clinical Center Maribor, Clinical Center Ljubljana, General hospital Topolšica, Novo mesto, Murska Sobota and some others.

The aim of our observational study was to summarize the data of TB patients in Slovenia diagnosed in last ten years period. We also investigated trends of TB incidence since 2000.

We analyzed data of 3042 TB patients registered by Slovenian Central Registry for tuberculosis in ten years period (from 2000 to 2009). Methods of descriptive statistics were used.

In observed period TB incidence rate decreased for more than 50 percent. TB incidence in 2000 was 20.0, while in 2009 it was only 9.25. Sex ratio was 2 to 1 with male predominance and was not changed over this period. The incidence rate peak was between 25 and 54 years of age, and people over 75 years of age are increasingly threatened, which is significant for all countries with very low incidence. Among all patients diagnosed with TB in Slovenia 17 to 32 percent are foreign residents. Most of them are citizens of Bosnia and Herzegovina, Croatia, Serbia and Kosovo. Almost half of the patients diagnosed with TB were treated at University Clinic of pulmonary and Allergic Diseases Golnik (45.2%) all others at University Clinical Center Maribor (15.1%), General Hospital Topolšica (9.4%), General hospital Murska Sobota (7.6%), General Hospital Novo mesto (6.8%), Clinical Center Ljubljana (6.5%) and in other hospitals (6.1%).

Slovenia is the country with very low TB incidence comparing to other countries in the world. We believe that such low incidence was achieved because of a good TB-control program. Because of the low incidence and relatively small number of Slovenian residents the next goal is that all Slovenian TB patients should be quick diagnosed and treated in specialized pulmonary departments and should also promptly investigate all suspected exposures to each and every case of TB.

PP-104

MYCOBACTERIUM TUBERCULOSIS STRAIN ADAPTATION: FAMILY LISBOA AND MAPPING OF IS*6110*

João Perdigão, Cláudia Pinto, José Moniz-Pereira, Isabel Portugal

Centro de Patogénese Molecular, URIA, Faculdade de Farmácia da Universidade de Lisboa
Contact: isabel.portugal@ff.ul.pt

Tuberculosis (TB) is responsible for about 2 million deaths worldwide. National TB control programs have to cope with the enhanced virulence of some *Mycobacterium tuberculosis* (*M. tuberculosis*) strains. It is the case in Lisbon Health region, where a specific strain family, designated Lisboa family, is responsible for the high number of multidrug and extensively drug resistant TB (MDR-TB and XDR-TB, respectively) cases. Given the high prevalence of such strains one may ask what genomic factors may be triggering such high virulence and/or transmission. Genetic expression plasticity may be conferred by the differential location of several genomic mobile elements, of which the most notorious is insertion sequence IS*6110*. This study intends to map these insertion sequences in a Lisboa family strain to possibly infer on its phenotypic consequences.

The genomic position of IS*6110* in a representative Lisboa family strain, previously characterized by 24 loci Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR), was analyzed through Ligation-Mediated PCR and Heminested Inverse PCR with restriction endonucleases and specific primers directed at IS*6110*. Amplified fragments were characterized by DNA sequencing analysis.

In this study, using the above methodology, we were able to map the location of nine IS*6110*. The insertion sites were mapped to positions 483580, 1932202, 1998809, 2456838, 3480373, 4183430, 1481529, 3120524 relative to *M. tuberculosis* H37Rv genome and, 3722295 relative to *M. tuberculosis* F11. Five of these insertion sites were located at intergenic sites, while the others were inserted in Rv0403c (mmpS1), Rv3113, Rv3732 and Rv1319c open reading frames. The latter encode a hypothetical protein with unknown function, a possible phosphatase, a membrane protein and an adenylate cyclase. On the other hand, two of the intergenic IS*6110* may influence the expression of the upstream genes.

In conclusion, we have identified the location of nine IS*6110* in the genome of a Lisboa family strain, of which 6 may influence the strain's metabolism. The identification of more insertion sites will allow a better characterization of the influence of these mobile elements in the metabolism and adaptation of these strains. Further search for other insertion sites is ongoing.

PP-105

APPLICATION OF PCR-BASED FINGERPRINTING FOR DETECTION OF NONTUBERCULOUS MYCOBACTERIA AMONG PATIENTS REFERRED TO TUBERCULOSIS REFERENCE CENTER OF KHUZESTAN PROVINCE, IRAN

Khosravi AD[1, 2], Seghatoleslami S[2], Hashemzadeh M[2]

[1]Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Dept. of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
Contact: khosraviaz@yahoo.com

There is an increased morbidity and mortality associated with non-tuberculous mycobacterial (NTM) infections in recent years. Since there are a few clinical and radiological differential findings between NTM infections and tuberculosis, the precise and rapid diagnostic tests are needed for differentiation between them. The present study was conducted to determine the frequency of NTM by application of PCR-based restriction fragment length polymorphism (PCR-RFLP) among suspected tuberculosis patients.

In present study, 150 clinical isolates from patients referred to TB reference laboratory, were screened. The majority of samples were comprised of 84 sputum (no. 84) and 52 urine (no. 52). Culture and biochemical tests were performed as per standard procedure. Template DNA was extracted and PCR-RFLP method based on amplification of a 439 bp fragment of *hsp* gene involving genus specific primers was performed. PCR products were digested with *Hae*III and *Bst*EII restriction enzymes.

Of total isolates tested, 90 isolates were culture positive (66.6%). Eighty out of 88 isolates that were subjected to RFLP, showed the identical restriction patterns similar to *Mycobacterium tuberculosis* (90.9%). The remaining eight isolates were identified as NTM and comprised of six isolates with restriction pattern compatible to *M. intracellularae* and two isolates, showed restriction pattern equal to that reported for *M. gordonae* I. Therefore, combining LJ medium culture and RFLP, 9.1% of the isolates were determined to be NTM. While the rate of isolated NTM in sputum in total number of *M. tuberculosis* isolates was about 6%, in the urine samples the isolated NTM was consisted of half of the isolates recovered from urine. No NTM was isolated from other minority samples included in this study.

In conclusion, RFLP as a fast, cheap and accurate technique is a valid alternative for phenotypic identification of pathogenic and potentially pathogenic mycobacteria in the routine laboratory.

PP-106

PHYLOGENETIC CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* LISBOA STRAINS BY SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS

João Perdigão, Carla Silva, Isabel Portugal

Centro de Patogénese Molecular, URIA, Faculdade de Farmácia da Universidade de Lisboa,
Av. Prof. Gama Pinto, 1649-003 Lisboa Portugal
Contact: isabel.portugal@ff.ul.pt

Multidrug and extensive drug resistant tuberculosis poses a very serious threat for public health. Lisbon Health Region has one of the world's most serious situations regarding this problem. Such, is the result of a continued circulation of an endemic and predominant strains of a particular genetic family – Lisboa family. Little is known regarding the phylogeny, relative virulence and genetic background of these strains. The loss or deletion of specific genomic regions constitutes the most important way by which *Mycobacterium tuberculosis* (*M. tuberculosis*) diverges and adapts. Several deletions, named Regions of Difference (RD), have already been described and associated with phylogeographic lineages. Alternatively, single nucleotide polymorphism (SNP) analysis allows a more precise positioning in the global *M. tuberculosis* phylogeny. The characterization of Lisboa family in this manner may elucidate its origin and perhaps be helpful in explaining its high prevalence.

Three representative clinical isolates of different genetic clusters of *M. tuberculosis* strains circulating in Lisbon Health Region were characterized by SNP analysis was performed through the analysis of 9 SNP regions, amplified by PCR and characterized by sequencing analysis. Furthermore, the isolates were also screened for the presence of 16 distinct RDs. Deletion detection was performed by PCR carried out using primers flanking each RD. Confirmation was performed by sequencing analysis.

All three isolates were found to possess four of the tested deletions: TbD1, pks15/1, RD174 and RDRIO. It was not possible to discriminate between the strains using this deletion typing approach. However, it was possible to infer on the phylogeography of these strains. The presence of TbD1 and pks15/1 deletion positions the strains in the modern and Euro-American lineage, respectively. Furthermore, RD174 suggests that the analyzed strains are related to the West-African sub-lineage.

SNP analysis revealed that all strains under analysis belong to SNP Cluster Group (SCG) 5.

The present study points toward the fact that Lisboa strains and others circulating in Lisbon belong to modern lineages of *M. tuberculosis*, specifically a widespread lineage extremely prevalent in West African countries. Also, the position of Lisbon-circulating strains in the global SNP phylogenetic tree became established. This study therefore reveals more on Lisboa family's origin and genomic distinctiveness.

PP-107

MOLECULAR TYPING OF CLINICAL ISOLATES OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX OBTAINED IN BOGOTÁ (COLOMBIA) BASED ON SPOLIGOTYPING AND MIRU-VNTRs

Cerezo I[1], Jiménez YJ[1], Hernández J[1], Zozio T[2], Rastogi N[2], Murcia MI[1]

[1]Departamento de Microbiología, Facultad de Medicina. Universidad Nacional de Colombia, Bogotá Carrera 30 No 45 – 03
[2]Unité de la Tuberculose et des Mycobactéries, Institut Pasteur de Guadeloupe, Abymes 97183 Guadeloupe
Contact: mimurciaa@unal.edu.co (Martha Isabel Murcia Aranguren)

The present study focused on molecular typing of *M. tuberculosis* strains isolated from TB patients diagnosed in Bogotá-Colombia between 1995 to 2006. Our primary aim was to have a first insight on the population structure of tubercle bacilli in Bogotá. We studied 154 clinical isolates collected from different hospitals in Bogotá. The strains were genotyped using spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats MIRU-VNTRs (classical 12 loci format) in order to determine the percentage of unique and clustered isolates. Spoligotypes in binary format and 12-digit MIRU patterns were entered in the SITVIT2 proprietary database of the Pasteur Institute of Guadeloupe to assign major phylogenetic clades and sub-lineages for *M. tuberculosis* isolates. In this database, SIT (Spoligotype International Type) designates spoligotyping shared by 2 or more patient isolates, whereas MIT (MIRU International Type) designates 12-locus MIRU patterns shared by 2 or more patient isolates, as opposed to "orphan" patterns designating patterns reported for a single isolate. The discriminatory power of the methods used was calculated using the Hunter Gaston Discriminatory Index (HGDI).

Using Spoligotyping as first molecular marker and MIRU-VNTRs as second marker we obtained 100 single patterns and 54 grouped strains into 15 clusters. The lineages found in our sample were in the following order: Latin American & Mediterranean (LAM) 49.25%; Harlem, 25.97%; ill-defined T, 12.33%; S family 1.3%; X lineage, 1.3%; Beijing, 0.65%, and unknown, 7.14%. The MIRU-VNTRs patterns corresponded to 51 MITs for 111 strains and 43 orphan patterns. The most frequent patterns were MIT190 (n=12), MIT45 (n=10), and MIT25 (n=9). The HGDI of both methodologies gave a value of 0.983. In our setting, the HGDI of a five loci subset (MIRU-26, 40, 10, 31, 23) contributed most to the discriminatory power of the 12-loci format used. *M. tuberculosis* lineage distribution showed that more than ¾ of strains in Bogotá are commonly found in Latin-America, Caribbean, and Europe. This observation reflects the common post-Columbus history of Colombia and its Latin-American neighbors as well as the strains brought by 20th century immigrants from Europe. We also show the usefulness of MIRU-VNTRs to detect polyclonal infections, and high stability across time allowing us the detection of chronic infections and endogenous reactivations.

PP-108

MOLECULAR EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS* IN AN ITALIAN NORTH-EASTERN AREA

Fallico L[1], Peracchi M[1], Rassu M[2], Pascarella M[2], Manganelli R[1], Palù G[1]

[1]Department of Mycrobiology, Hystology and Medical Biotechnlogy, University of Padua, Via Gabelli 63, 35129 Padua, Italy
[2] Department of Microbiology, S. Bortolo Hospital, Via Rodolfi 37-36100 Vicenza, Italy
Contact: loredana.fallico@tiscali.it

720 strains of *Mycobacterium tuberculosis* (Mtb) were isolated in the Laboratories of Microbiology and Virology of the Hospital of Padua and Vicenza (Jan. 2006 - Dec. 2009) from patients with pulmonary and extra pulmonary tuberculosis (TB) and then genotyped by spoligotyping.

132 strains were collected during 2006, 177 during 2007, 197 during 2008 and 214 during 2009.

The spoligotype ST designations were attributed by comparing the patterns obtained with those included in the International Spoligotype Database (SpolDB4), available at the following link: http//www.pasteur-guadeloupe.fr/tb /bd_myco.html.

We found six major spoligotypes (ST) (≥5 isolates each): ST1378 (CAS family) with 89 isolates; ST1 (Beijing family) with 28 isolates; ST26 (CAS1_Delhi family) with 22 isolates; ST482 (Bovis1_BCG family) with 9 isolates; ST1498 and ST46 (U families) with 6 and 5 isolates respectively.

33 isolates were grouped in 13 minor ST (< 5 isolates each) distributed among the CAS, U, AFRI_1, EAI families.

Furthermore, we detected 24 strains with already known ST represented only one in our group.

We detected 504 orphans (not yet present in the international database) that we named ST0. Among these, 158 isolates clustered in 11 major ST (≥5 isolates each); among these the largest groups included 38, 35, 31 and 10 strains. 146 isolates clustered in 56 minor ST and 200 isolates were true orphans (represented only once in our group).

Our results demonstrate a characteristic epidemiological pattern of Mtb in our area, represented by two well known major ST (ST1378 and ST1) and 11 major ST not yet reported in the SpolDB4 database.

The identification of large clusters of isolates belonging to still unreported ST suggests the possibility of local outbreaks with ongoing transmission.

At the best of our knowledge, this is the first report showing the presence of isolates belonging to the Beijing family in northeastern Italy.

PP-109

INVESTIGATION OF TUBERCULOSIS PREVALENCE IN PATIENTS REFERRED TO AHVAZ REFERENCE CENTER IN RELATED TO SOCIAL FACTORS AND LIVING CONDITIONS

Khosravi AD[1,2], Barazandeh B[2], Afzali M[3]

[1]Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Dept. of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]School of Medicine, Iran University of Medical Sciences, Tehran, Iran
Contact: marjanafzali85@yahoo.com

Tuberculosis (TB) causes approximately 2 million deaths per year globally; 98% occur in low-income countries. The understanding of TB requires effective links to be made between advances in biomedical knowledge and the wider social and economic dynamics of disease epidemiology. The TB epidemic situation is both a public health problem and a socio-economic issue in many countries

The purpose of this study was to investigate different criteria such as age, sex, ethnic origin and living conditions in tuberculosis patients, that may have some epidemiological value.

A total of 180 clinical isolates belonging to patients having pulmonary and extra-pulmonary TB were collected from TB reference unit, PHLS in Ahwaz, Iran. All ages and both sexes were included in the study. Acid fast staining was performed for the isolates and they were identified as *Mycobacterium tuberculosis* by culture on Lowenstein Johnsen medium and biochemical tests comprised niacin, catalase activity and nitrate reduction.

The results showed that prevalence of TB was more significant in youth age, and in women compared to men. The significance was even more among patients with extrapulmonary disease. The prevalence was also more significant in region native ethnics compared to other ethnic groups such as persian, or migrant turkish and kordish population. The disease was more significant among prisoners compared to common people. The majority of TB patients were youngesters and this may be related to the fact that they comprised a significant country population. Besides they are more high risk due to their social activities and more close contacts in the community. Some of the factors that are contributed to rapid increase of the disease in the region are: family overcrowding, traditional close cultural contacts, living in limited spaces, unemployment, drug addiction, social crimes with jail sentence, refuse to refer to public health centers due to long distances from their living area and not affording the transportation fares.

PP-110

THE DYNAMICS OF URO-GENITAL TUBERCULOSIS IN A CENTRAL COUNTY OF ROMANIA VERSUS THE ENTIRE COUNTRY

Ionela Muntean[1], Oana Ticau[1], Tania Macavei[1], Elmira Ibraim[2], Cocei H[2]

[1]Pneumophtisiology Hospital Brasov, Sitei street 17, 500 260
[2]Pneumophtisiology Institute "Marius Nasta" Bucharest, Sos. Viilor 90, sector 5, Bucharest, Romania
Contact: ionelasorina40@yahoo.com

Background. In Romania the incidence of TB cases is above the mid European incidence. Compared with whole country, the central county Brasov has an half-incidence (50.9‰ in 2009). The Brasov Pneumophtisiology Hospital is performing medical services for 600,000 inhabitants. We noticed a high percentage of urogenital TB and we decided to study this topic.

Objective. Study the evolution of the urogenital TB cases in Brasov county compared with the situation of the whole country, in the period of 2002 and 2009.

Material and methods. It is a retrospective study of 37 cases of urogenital TB treated in our county hospital in 8 years. We used data from patient's files and compared them with electronic national TB register which contains for the same period 1108 uro-genital TB patients.

Results. We noticed that the incidence of extra pulmonary TB was higher in our county in the first 6 years and then, in the next 2 years, was lower than the national data. In the same time, was seen a constant increase of the urogenital TB in our hospital despite the constant level in the country. From 2003, the percentage of the urogenital TB patients among all extra pulmonary TB cases in Brasov county was roughly double compared with the national data.
Regarding the laboratory findings, we didn't notice any special resistance profile of the strains from Brasov county.

Conclusions. In Brasov county, despite the low incidence of TB compared with the entire country, we noticed a high incidence of uro-genital TB. This finding could not be related to microorganism susceptibilities and further epidemiological studies are needed to explain the causes.

PP-111

MOLECULAR BIODIVERSITY OF MDR-TB GENOTYPES IN BULGARIA. A THREE-YEAR SURVEY

Panaiotov S, Bachiyska E, Ivanova A, Atanasova Y, Brankova N, Levterova V

National Center of Infectious and Parasitic Diseases, 1504 Sofia, Bulgaria
Contact: spanaiotov@yahoo.com

Introduction. Population-based genotyping has demonstrated that a small percentage of strains cause a large number of cases, implying that some strains are spread more effectively than others. Biodiversity of multi drug-resistant *Mycobacterium tuberculosis* (MTB) isolates in Bulgaria has not been studied. Our previous studies on more than 200 drug sensitive MTB strains analyzed by spoligo- and VNTR typing demonstrated that in the country circulate at least 61 spoligotypes, 26 of them are clustered. Six spoligotypes represent more than 50% of the isolates and four of them were found to be geographically specific for Bulgaria. Goal of our study was to isolate and characterize MDR strains isolated in Bulgaria by molecular typing methods.

Materials and Methods. 109 MDR-TB strains were collected between 2007-2009, 31, 29 and 49 MDR strains respectively. Drug resistance to isoniazid and rifampicin of all strains was confirmed by phenotypic methods (BACTEC 960). Strains were grouped by year of isolation, patients age, sex, HIV status, new or old case and geographic origin. Spligo and VNTR analysis were applied for typing.

Results. 24 spoligotypes were identified, 9 of them formed clusters. Spoligotypes ST41 and ST53 represent 61% in the studied MDR population. Two MDR Beijing strains have been identified. The patients´ median age was 46 years with a range of 19-88 years. Stratification according to age showed that 5 (5.0%) patients were aged ≤25, 69 (69.7%) were between 26-54, while 24 (24.2%) were above 55 years old. Three patterns corresponded to orphan strains that were unique among the strains recorded in the SpolDB4 database. The 4 most predominant lineages in our study comprised PGG2/3 lineages: LAM (n=43, 43.4%), Ghana, an evolutionary recent T clade (n=30, 30.3%), Haarlem (n=12, 12.1%), and the Uganda (n=6, 6.1%).

Conclusions. The analysis allowed to identify a strain-clustering rate (or recent transmission index) of 75.8%. Concerning the two most represented SITs ST41 belongs the LAM lineage, whereas the ST53 belongs to the evolutionary recent T clade, together with the ST284 and ST144 and ST125. Both ST41 and ST53 are considered ubiquitous worldwide. No significant differences among age nor new/old cases distribution was observed for these two SITs in the present study. Concerning DST, 52.5% of ST41 isolates were MDR resistant also to both STR and EMB. ST53 isolates were MDR resistant to both STR and EMB in 15% of cases. Together these two SITs account for 67.6% of fully resistant strains (MDR + STR-R and EMB-R). Spoligotype ST41 in drug sensitive strains is present in about 3%, while among the MDR strains is present in 40%. ST41 is and example for a successfully transmitted spoligotype in MDR strains from Bulgaria. ST41 is a low copy number of the IS*6110* element as identified by RFLP-IS*6110* typing. In this study for the first time we identify Beijing spoligotype in Bulgaria.

PP-112

MLVAplus.net: A NEW DATABASE AND ANALYSIS TOOL FOR MULTIPLE TYPING DATA OF MICROBIAL STRAINS

Justina Krawczyk[1], Thomas Weniger[2], Philip Supply[3], Dag Harmsen[2], Stefan Niemann[1]

[1]Forschungszentrum Borstel, National Reference Center Mycobacteria, Borstel, Germany
[2]Department of Periodontology, University Hospital Münster, Münster, Germany
[3]Institute Pasteur de Lille, Lille, France
Contact: justina.krawczyk@uni-muenster.de

Molecular typing of *Mycobacterium tuberculosis* complex (MTBC) isolates is crucial for identifying specific phylogenetic lineages or outbreak strains (clusters), in order to better disclose and prevent their spread in a community. No online-accessible database was available so far for analysis and storage of user genotyping data. Therefore, we have developed a new generalized and expandable database for polyphasic analysis of genotypic data of MTBC as well as other bacterial species.

MLVAplus.net is a web-based application implemented in Java that can be accessed via any standard web browser. The web pages are generated by JavaServer Faces. A PostgreSQL database system is used for data storage and retrieval.

MIRU-VNTR*plus* is the MTBC species part of the database. For this, different typing methods like LSPs, SNPs, MIRU-VNTR or spoligotyping are used alone or in combination to achieve analysis at different evolutionary levels, like identification at the phylogenetic lineage or down to strain level. The database will be linked with a geographical information system, allowing for combined data investigations and geographical case mapping. The MLVAplus.net application allows users to create online-accessible databases for combination of MIRU-VNTR, spoligotyping, or other categorical data. Access rights for each database can be modified to grant read and write access to the public or to specific users only. Users can upload their strain data to the server and, if authorized, store them in the database. The available analysis features include polyphasic distance calculation based on a weighted combination of different typing data as well as drawing and exporting of graphs based on UPGMA-, neighbor-joining, and minimum spanning tree algorithms.

MLVAplus.net is a powerful tool for analysis of microbial strains based on different categorical and binary typing data. It can be used to provide easy access to extendable strain collections for the scientific community. The application is freely accessible at http://www.mlvaplus.net.

PP-113

MYCOBACTERIUM TUBERCULOSIS INFECTION IN CATTLE

Romero B[1,2], Rodríguez S[1,2], de Juan L[1,2], Castellanos E[1,2], Bezos J[1,2], Moya N[1], Lozano FJ[1], Mínguez O[3], Díaz R[4], Fernández E[5], Mateos A[1,2], Domínguez L[1,2]

[1]VISAVET Health Surveillance Centre
[2]Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Avenida Puerta de Hierro, s/n, 28040 Madrid, Spain
[3]Dirección General de Producción Agropecuaria, Junta de Castilla y León, 47014 Valladolid, Spain
[4]Dirección General de Medio Ambiente, Consejería de Medio Ambiente, Vivienda y Ordenación del Territorio, 28012 Madrid, Spain
[5]Dirección General de Ganadería y Agroalimentación, Consejería de Medio Rural y Pesca, 33005 Oviedo, Spain
Contact: sabrina.rodriguez@visavet.ucm.es

According to the EU Bovine Tuberculosis Task Force, bovine tuberculosis is the infection in cattle caused by any mycobacterial species within the *Mycobacterium tuberculosis* complex. However, *M. bovis* is considered the main etiological agent of the disease in animals. Tuberculosis in cattle due to the human pathogen, *M. tuberculosis*, is a rare event but the detection of this infection is increasing in countries where the prevalence of tuberculosis in humans is high.

In Spain, *M. tuberculosis* infection was detected in three cattle farms during compulsory skin test and/or bacteriological culture. Samples from lung and associated lymph nodes were taken at the abattoir and sent to the regional veterinary laboratories to confirm *M. tuberculosis* complex infection. Samples were cultured by the automated BACTEC™ MGIT™ 960 System. Identification and molecular characterization were done by PCR and spoligotyping (spolDB4 database).

Positive cultures were obtained by the liquid culture system, PCR classified all isolates as members of the *M. tuberculosis* complex and spoligotyping identified three isolates as *M. tuberculosis*. They were only recovered from three animals (one from each farm) with three different spoligotypes (SIT58, SIT1564, SIT undetermined). In addition, *M. bovis* coexisted in a farm. A detailed epidemiological investigation was conducted and personnel closely related with the farms seemed to be the source of infection.

Although few *M. tuberculosis* has been isolated this infection is receiving attention in cattle due the risk of transmission to other animals or to humans. Moreover, the automated BACTEC™ MGIT™ 960 System is recommended for the isolation of *M. tuberculosis*.

PP-114

MIRU-VNTR TYPING OF *MYCOBACTERIUM AVIUM* IN ANIMALS AND HUMANS: HETEROGENEITY OF *M. AVIUM* SUBSP. *HOMINISSUIS* VERSUS HOMOGENEITY OF *M. AVIUM* SUBSP. *AVIUM* STRAINS

Mateja Pate[1], Darja Kušar[1], Manca Žolnir – Dovč[2], Matjaž Ocepek[1]

[1]University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Gerbičeva 60, SI-1115 Ljubljana, Slovenia
[2]National Reference Laboratory for Mycobacteria, University Clinic for Respiratory and Allergic Diseases, Golnik 36, SI-4204 Golnik, Slovenia
darja.ferme@vf.uni-lj.si (Darja Kušar)

Epidemiological studies on *Mycobacterium avium* are requisite for revealing the sources of infection and disease transmission. They are based upon genotyping methods like RFLP (restriction fragment length polymorphism) and MIRU-VNTR (mycobacterial interspersed repetitive units – variable number of tandem repeats).

MIRU-VNTR is one of the novel rapid genotyping techniques. In the present study, it was applied to 121 previously RFLP typed *Mycobacterium avium* field isolates to compare the discriminatory power of both methods. The applicability of MIRU-VNTR typing was studied for isolates from a limited geographic area, namely 41 *M. avium* subsp. *avium* isolates from pigs, poultry and cattle, 43 *M. avium* subsp. *hominissuis* isolates from pigs and 37 *M. avium* subsp. *hominissuis* isolates from humans.

Among the 41 *M. avium* subsp. *avium* isolates exhibiting 12 IS*901* RFLP types, five MIRU-VNTR types were found, with discriminatory index of 0.716. Among the 80 *M. avium* subsp. *hominissuis* isolates exhibiting 56 IS*1245* RFLP types, MIRU-VNTR typing revealed 18 distinct patterns, resulting in discriminatory index of 0.866. Concomitant use of both typing methods increased the discriminatory indices to 0.981 (*M. avium* subsp. *avium* isolates) and 0.995 (*M. avium* subsp. *hominissuis* isolates), respectively.

MIRU-VNTR typing based on the markers employed in the present study provided discernible discrimination among *M. avium* subsp. *hominissuis* isolates, however further investigations are necessary to develop a suitable MIRU-VNTR typing scheme for *M. avium* subsp. *avium*.

PP-115

USE OF MULTILOCUS SEQUENCE ANALYSIS FOR TYPING OF *M. AVIUM* SUBSP. *HOMINISSUIS* STRAINS

Kolb J[1], Hillemann D[1], Rüsch-Gerdes S[1], Möbius P[2], Richter E[1]

[1]Research Center Borstel, Nationales Referenzzentrum für Mykobakterien, Parkallee 18, 23845 Borstel, Germany
[2]Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Naumburger Str. 96a, 07743 Jena, Germany
Contact: jkolb@fz-borstel.de

One of the most frequent isolated species within the NTM is *M. avium*, which is ubiquitously found in the environment and has been shown to be responsible for a variety of human diseases, mainly in immunocomprised patients, but also in patients with no obvious immune deficiencies. *M. avium* comprises the four subspecies *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *hominissuis*. *M. avium* subsp. *hominissuis* is usually responsible for diseases in humans which can present as pulmonary and extrapulmonary localization. Genetic characterization of the infectious strains is a prerequisite for deeper insights into the pathogenicity and also for understanding of transmission mechanisms. The aim of our study was to assess the feasibility of Multilocus Sequence Typing (MLST) for *M. avium* strains, a technique, which is well established for many other species. For this purpose we analyzed fragments of eight housekeeping genes by sequence analysis. Comparing the DNA sequences of each gene locus we estimated the degree of variability and its applicability to distinguish different *M. avium* strains. By analysis of more than 70 clinical *M. avium* subsp. *hominissuis* isolates 140 variable positions could be detected showing a high variability between the strains. Of the eight genes investigated five genes were found with a high range of variation, and these can be recommended for further investigations.

PP-116

FIRST RESULTS OF 24 LOCUS MIRU-VNTR GENOTYPING IN SLOVENIA

Urška Bidovec-Stojkovič, Nataša Fajfar, Manca Žolnir-Dovč

University Clinic of Respiratory and Allergic Diseases, Laboratory for Mycobacteria, SI-4204 Golnik, Slovenia
Contact: Tel. +386 4 2569411, Fax. +386 4 2569117, ursa.bidovec@klinika-golnik.si

The incidence of TB in Slovenia for year 2008 was 10.4/100.000 inhabitants. Molecular genotyping of *Mycobacterium (M.) tuberculosis* started in year 1999 with IS*6110*-RFLP and continued with the introduction of spoligotyping as additional method in year 2003. With introducing 24 loci MIRU-VNTR typing in year 2009 we expect that our knowledge in understanding of TB transmission will improve even more.
The aim of our one year nation-wide study was to characterize all culture positive isolates in year 2008 by 24 locus MIRU-VNTR method. All MDR strains from Slovenian MDR collection isolated in the period 1996-2009 were also included in the same evaluation.

A total of 219 Slovenian *M. tuberculosis* isolates were characterized by 24 loci MIRU-VNTR typing method, using MIRU-VNTR Genotyping Kit (Genoscreen, France). The samples were subjected to electrophoresis using ABI 3130 genetic analyzer (Applied Biosystems, USA). Sizing of PCR fragments and assignment of the alleles of the 24 loci was done using the MIRU-VNTR Calibration Kit (Genoscreen, France), as well as the GeneMapper software ver. 4.0 (Applied Biosystems, USA).
Isolates were obtained from 219 culture positive TB patients, registered at Slovenian Central Registry for Tuberculosis Golnik. Among 219 isolates, 22 isolates were MDR strains from patients, registered between 1996 and 2009; other 197 isolates were isolated from 197 TB patients registered in year 2008.

Among 219 *M. tuberculosis* isolates, 160 different MIRU-VNTR genotypes were obtained. Unique MIRU-VNTR type was found in 127 isolates (58.0%) while 92 (42.0%) isolates were in clusters (2-7 isolates/cluster). Two largest clusters were present among MDR strains, first cluster with 7 strains/cluster and second cluster with 6 strains/cluster. There was only one more cluster with size of 6 strains/cluster, all other clusters were smaller, most of them with 2-3 isolates/cluster.

Our first results with MIRU-VNTR typing showed high discriminatory power of 24 loci MIRU-VNTR typing on Slovenian strains. Especially results of MDR strains showed some information of transmission of TB of great importance. Further analyzes and comparison with other molecular genotyping methods (IS*6110*-RFLP, spoligotyping) will give us more information about its powerful meaning.

PP-117

NATION-WIDE EVALUATION OF THREE MOLECULAR GENOTYPING METHODS IN SLOVENIA

Urška Bidovec-Stojkovič, Nataša Fajfar, Manca Žolnir-Dovč

University Clinic of Respiratory and Allergic Diseases, Laboratory for Mycobacteria, SI-4204 Golnik, Slovenia
Contact: urska.bidovec-stojkovic@klinika-golnik.si

High discriminatory power of genotyping method is of great importance however some other requirements such as low cost and timeliness are also relevant. The aim of our one year nation-wide study was to evaluate three different methods for genotyping *Mycobacterium (M.) tuberculosis* on all culture positive isolates in Slovenia in year 2008. All MDR strains in the period 1996 and 2009 were also evaluated in this study. We tried to determine discriminatory power of different genotyping methods.

A total of 212 Slovenian *M. tuberculosis* isolates were typed by three different genotyping methods: IS*6110*-RFLP (RFLP), 24 loci MIRU-VNTR (MIRU-VNTR) and spoligotyping. Analysis of typing results was performed with BioNumerics program ver. 5.2 (Applied Maths, Belgium) and Hunter-Gaston discriminatory index (HGDI) was calculated as well.
190 isolates were obtained from 190 TB patients registered in 2008 at Slovenian Central Registry for TB Golnik. Additional 22 MDR strains were included in the study and they represent all (100%) MDR cases in the period 1996-2009.

Among 212 *M. tuberculosis* isolates 160, 157, 63 distinct patterns; 135 (63.7%), 126 (59.4%), 36 (17.0%) unique isolates; 77 (36.3%), 86 (40.6%), 176 (83%) clustered isolates; 25, 31, 27 clusters were found with RFLP, MIRU-VNTR and spoligotyping method, respectively.
Number of isolates per cluster was 2-8, 2-7, 2-46 for RFLP, MIRU-VNTR and spoligotyping, respectively. HGDI showed even more surprising results since we obtained discriminatory index 0.9947, 0.9953, 0.9183 for RFLP, MIRU-VNTR and spoligotyping, respectively.
Evaluation of MDR strains gave us some interesting information. Among 22 *M. tuberculosis* MDR isolates 9, 11 and 8 distinct patterns were found in RFLP, MIRU-VNTR and spoligotyping, respectively.

Our one year nation-wide study showed that the highest discriminatory power has IS*6110*-RFLP method. Nevertheless 24 loci MIRU-VNTR has shown very high discriminatory power as well, in some cases even higher than IS*6110*-RFLP (MDR isolates). Spoligotyping method showed very low discriminatory power but has its advantage in recognizing some important strains lineage (Beijing, Haarlem, LAM type…).

PP-118

TUBERCULOSIS OUTBREAK IN A NORTHERN SARDINIA HIGH SCHOOL

Zanetti S[1], Bua A[1], Molicotti P[1], Zanetti A[2], Hussain TS[1], Sechi LA[1]

[1]Department of Biomedical Science, University of Sassari
[2]Public Health Service ASL of Sassari
Contact: sechila@uniss.it

The aim of this study was to investigate the transmission of *Mycobacterium tuberculosis* among high school student and teachers populations in a high school of the Northern Sardinia.

The epidemiologic investigation included: tuberculin skin-test screening, chest-x-rays, Quantiferon-TB Gold in the individuals with negative skin test, microbiological examinations on sputum specimens in subjects with suspected active tuberculosis and VNTR analysis of *M. tuberculosis* isolates.

The tuberculin skin test was positive in 153 subjects and negative in 421. The QuantiFERON-TB Gold was positive in two persons of the 83 students with negative tuberculin skin test. Ten subjects developed active tuberculosis and they were infected by the same *M. tuberculosis* strain.

Our study indicated the effectiveness of the epidemiological investigation, including a rapid mobilization of the Public Health Service and a prompt diagnosis that would have reduced the severity of the illness in the patient and potentially prevented widespread school-based transmission.

PP-119

CLINICAL UTILITY OF THE QUANTIFERON-TB GOLD TEST IN THE DIAGNOSIS OF ACTIVE TUBERCULOSIS

Losi M[1], Peracchi M[2], Dal Monte P[3], Fabio A[4], Meacci M[4], Bocchino ML[5], Crespi I[6], Del Giovane C[7], Fallico L[2], Lombardi G[3], Sanduzzi A[5], Fortina G[6], D'amico R[7], Apice M[4], Manganelli R[2], Pellegrino MT[3], Molinari GL[6], Camaggi A[6], Meccugni B[4], Palù G[2], Richeldi L[1], Fabbri LM[1], Rumpianesi F[4]

[1]Section of Respiratory Diseases,Department of Oncology, Hematology and Respiratory Disease, University of Modena and Reggio Emilia, Via del Pozzo 71, 41100, Modena, Italy
[2]Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Via Gabelli 63, 35121, Padua, Italy
[3]Department of Hematology and Oncologic Sciences "L and A Seragnoli", University of Bologna, Via Massarenti 9, 40138, Bologna, Italy
[4]Microbiology and Virology Laboratory, Azienda Ospedaliero-Universitaria, Policlinico Hospital, Via del Pozzo 71, 41100, Modena, Italy
[5]Department of Clinical and Experimental Medicine "Federico II", University of Naples, Via Bianchi 5, 80131, Naples, Italy
[6]Department of Microbiology and Virology, "Maggiore della Carità",University Hospital, Corso Mazzini 18, 28100,Novara, Italy
[7]Unit of Biostatistics,Department of Oncology, Hematology and Respiratory Disease, University of Modena and Reggio Emilia, Via del Pozzo 71, 41100, Modena, Italy
Contact: monica.losi@unimore.it

Rapid diagnosis of pulmonary (pTB) and extra-pulmonary (eTB) active tuberculosis might be difficult when conventional microbiological methods are negative, in particular in extra-pulmonary cases. Interferon-gamma (IFN-gamma) assays (IGRA) may play a role for the diagnosis of cases of active TB, although efficacy data are not completely consistent across different studies.
In a multicenter study, we retrospectively evaluated 342 patients with culture-confirmed active TB tested with QuantiFERON-TB Gold In-Tube (QFT-IT) (Cellestis Ltd, Carnegie, VIC, Australia) before to start anti-TB treatment in 5 different centers in Italy.

256 out of 342 (74.9%) patients were diagnosed with pulmonary TB (pTB) and 86 (25.1%) with extra-pulmonary TB (eTB), respectively. Mean age was 42.2±19.5 years; most patients were males (n=197; 57.9%) and 139 (40.6%) individuals were foreign-born. Tuberculin skin test results were available in 170 (49.7%) patients. Indeterminate QFT-IT were 5.5% in pTB and 4.6% in eTB; QFT-IT tested positive in 77.7% of pTB and 82.6% of eTB (p=ns). IFN-gammạlevels were similar in the two groups (median UI/ml: 2UI/ml in pTB *vs* 2.3UI/ml in eTB, p=ns). Interestingly, 54 out of 342 (15.8%) patients tested QFT-IT negative: 43 (79.6%) had pulmonary disease and was more frequent among people born in Italy.

These preliminary data suggest that QFT-IT might be a reliable aid in diagnosing active TB, both in pulmonary and extra-pulmonary cases, although a substantial fraction of patients with active TB showed a negative QFT-IT results. Further studies are needed to focus on this IFN-gammạnegative group of active TB cases.

PP-120

EVALUATION OF QUANTIFERON-TB GOLD IN CLINICAL PRACTICE

Erik Svensson[1], Björn Samuelsson[2], Patrik Peterson[2]

[1]Department for Bacteriology, Institute of Biomedicine
[2]Medical School; University of Gothenburg, Sweden
Contact: erik.svensson@vgregion.se

Tuberculin test (TST) has for decades been used for the detection of infection of tuberculosis (TB). Unfortunately, the TST has low specificity, and in immunocompromised persons, also a low sensitivity. The new Interferon Gamma Release Assays (IGRA) have been developed to overcome some of those shortcomings. In 2007 the IGRA test QuantiFERON-TB-Gold (QFN) was introduced at Sahlgrenska University Hospital, as a complement in TB-investigations. We wanted to know how QFN was used in the clinic, if disease or drugs affected the test results and QFN added useful information in the assessment of suspected TB infection.

The study was based on the 557 samples that were collected on 501 patients in 2007 and the first half of 2008. We used data in contact tracing records, journal entries and lists of laboratory results. Compilation and processing of data was done in EpiData and Excel. The QFN results were related to TST and clinical data in the records.

Our results show that QFN seems to exclude infection better than TST. There were no major differences between QFN and TST in detecting active TB. Indeterminate results were associated with auto-immune diseases and use of steroids.

PP-121

GAMMA INTERFERON TEST IN COWS WITH POSITIVE RESULTS OF TUBERCULIN TEST

Marek Lipiec, Marcin Weiner, Monika Krajewska

National Veterinary Research Institute, Partyzantow 57, 24-100 Pulawy, Poland
Contact: mpweiner@piwet.pulawy.pl

Introduction. Bovine tuberculosis (bovine TB) diagnose in the live animal is based on skin testing (intradermal tuberculin testing) and clinical examination. These tests with the final isolation in culture and identification of the agent are the basis of bovine TB control and eradication programe in Poland. According to our earlier results of laboratory tests, about 60% of all laboratory tests were negative (without *Mycobacterium bovis* isolation). Even than in area where *Mycobacterium avium* is frequent and comparative tuberculin testing is used to differentiate false positive results of single intradermal test, the diagnosis is difficult and sometimes false. In veterinary practice in some countries, the gamma interferon test is used for the bovine TB control, especially in sheep herds. The aim of the investigations was to estimate the usefulness of gamma interferon test in cattle herd for the bovine TB diagnosis.

Materials and methods. The investigations were performed in two cattle herds: first with 50 healthy cows, without any bovine TB history and second one – 25 cows diagnosed in the single intradermal tuberculin test as TB infected. The single intradermal and comparative tuberculin test were performed with bovine and avian PPD tuberculin (single dose of PPDs was 3250 IU and 2500 IU respectively). The samples of blood from all animals were collected and gamma interferon test were performed (Bovigam, Prionics kit). Tissue samples from animals found as infected were collected and tested using standard OIE accepted laboratory methods.

Results. In healthy herd all results in single and comparative tuberculin test were negative. In infected herd the comparative tuberculin test, performed after 42 days, gave positive results in all animals. In gamma interferon trial, among 25 blood samples 17 were classified as positive and 8 as negative. From 25 tissue samples 24 had anathomopathological TB lesions. 24 strains of *Mycobacterium bovis* were isolated. In one case culture test gave negative result.

Conclusions. The obtained results testify that gamma interferon test can give false negative results, even Tb lesions were find and *M. bovis* strains were isolated. It was concluded that gamma interferon test can't use as an only test for the diagnosis of bovine TB for individual animals and can use as and additional herd test.

PP-122

SALICYLANILIDE ESTERS WITH PROMISING *IN VITRO* ACTIVITY AGAINST ATYPICAL AND MDR MYCOBACTERIAL STRAINS

Martin Krátký[1], Jarmila Vinšová[1], Szilvia Bösze[2], Jiřina Stolaříková[3]

[1]Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, the Czech Republic
[2]Research Group of Peptide Chemistry, Eötvös Lórand University, Hungarian Academy of Science, Pázmány Péter Sétány 1/A, Budapest, H-1117, Hungary
[3]Laboratory for TBC, Regional Institute of Public Health in Ostrava, Partyzánské náměstí 7, 702 00 Ostrava, the Czech Republic
Contact: jarmila.vinsova@faf.cuni.cz

Improper use of antibiotics in chemotherapy of drug-susceptible TB patients leads to the progression of resistance. Multidrug-resistant tuberculosis (MDR-TB) and non treatable extremely drug resistance (XDR) has become a very serious problem in several last years. Thus the research of new anti-tuberculosis drugs is very urgent and is directed mainly to find some new structures of compounds with a new mechanism of activity (1).

Salicylanilides (2-hydroxy-N-phenylbenzamides) have shown a wide range of biological activities, *e.g.* antiprotozoal, antibacterial, antifungal (2), antiviral and potential anticancer activity (3). We have found their promising antimycobacterial activity as well against several atypical strains of *Mycobacterium* (4) and MDR strains (5).

We will present three series of halogenated salicylanilide esters with different organic acids, as potential antituberculotics, showing high *in vitro* activity against *M.* tbc. H37Rv, five MDR-TB and one of XDR-TB strains with different resistance patterns and three isoniazid-resistant atypical mycobacteria strains (*M. avium*, *M. kansasii*). Generally, MIC values against drug-sensitive *M.* tbc range from 0.125 to 8 μmol/L, MDR- and XDR-TB 0.125 – 2 μmol/L, *M. avium* 2 – 16 μmol/L and *M. kansasii* 0.5 – 8 μmol/L. With the respect of their excellent activity mainly against resistant TB strains, these derivatives are perspective candidates for new antituberculotics with a relatively low cytotoxicity.

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PP-123

DEVELOPMENT OF ANTIMYCOBACTERIAL ACTIVE SUBSTITUTED N-PHENYLPYRAZINE-2-CARBOXAMIDES

Doležal M[1], Svobodová M[2], Zitko J[1], Janďourek O[1], Eibinová D[1]

[1]Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Department of Medicinal Chemistry, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[2]Department of Microbiology, Regional Hospital, Kyjevská 44, Pardubice, 532 03, Czech Republic
Contact: martin.dolezal@faf.cuni.cz

Current treatment of tuberculosis consists in the combination of two to four drugs with different modes of action for three to nine months of continuous therapy. Pyrazinamide (PZA), the first-line TB drug, was discovered through an effort to find antitubercular nicotinamide derivatives. The activity of PZA appears to be pH dependent, since it is bactericidal at pH 5.5, but inactive at neutral pH. It is especially effective against semi-dormant mycobacteria. Its mechanism of action appears to involve its hydrolysis to pyrazinoic acid via the bacterial enzyme pmcA. Today is an urgent need for new drugs with novel modes of action and higher selectivity and antimycobacterial activity. A different analog of PZA, 5-chloropyrazine-2-carboxamide, has previously been shown to inhibit mycobacterial fatty acid synthase I (FAS I).

Our research is focused on the development of PZA analogues with a -CONH- bridge connecting the pyrazine and benzene rings. This moiety can form centrosymmetric dimer pairs with the peptidic carboxamido group of some peptides, needed for binding to the receptor site, possibly by formation of hydrogen bonds. All substituted amides of pyrazinecarboxylic acid studied can be interpreted as more lipophilic azaanalogues of nicotinamide. In antituberculosis assay of prepared compounds were used strains *Mycobacterium tuberculosis* H37Rv, *M. kansasii* Hauduroy CNCTC My 235/80, *M. avium* My CNCTC 80/72 and *M. avium* 152/74. The culture was ten days old and the culture medium used was Šula's medium at pH 5.5 and 37°C; microdilution panel method was performed.

Several synthesized compounds exerted minimal four times higher antimycobacterial activity (MIC≤2 mg/L) against *M. tuberculosis* in comparison with the standard PZA (MIC=8 mg/L) and moderate activity against minor *Mycobacterium* pathogens.

Our work was supported by the Ministry of Health of the Czech Republic (NS 10367-3).

PP-124

GAMMA-INTEFERON ASSAY FOR TUBERCULOSIS DIAGNOSIS IN CATTLE AND GOATS: STUDY OF FACTORS AFFECTING RESULTS OF THE ASSAY

Bezos J[1,2], Aranaz A[1,2], Romero B[1,2], Álvarez J[1], Casal C[1], Moya N[1], Lozano F[1], Mateos A[1,2], Domínguez L[1,2]

[1]VISAVET Health Surveillance Centre, Complutense University of Madrid, Avenida Puerta de Hierro, s/n, 28040 Madrid, Spain
[2]Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Avenida Puerta de Hierro, s/n. 28040 Madrid, Spain
Contact: jbezosga@visavet.ucm.es

The gamma-interferon assay is as a useful tool (together with the tuberculin test) for the diagnosis of tuberculosis in cattle and goats. In these experiments, we have evaluated the effect of factors such as phenol concentration and dialysis of PPDs, delay in processing samples and use of single or duplicate results on the performance of the assay. Moreover, we have evaluated the influence of tuberculin test on gamma-interferon production in infected animals. Finally, presence of lesions and microbiological results have been correlated with the production of gamma-interferon. To assay the effect of phenol concentration, bovine and avian PPDs with three different phenol concentrations (0.1, 0.3 and 0.5%) were prepared and results were compared with those obtained using dialyzed 0.5% phenol PPDs. For the study of the effect of a delay in blood culture, samples were stimulated within first 8 hours and 16 and 24 hours after collection and results were compared. Gamma-interferon assay was performed in duplicate to study differences between repeated samples. Finally, a group of goats were necropsied and lesion score was calculated and correlated with the gamma-interferon release. Results using two cut-off points (0.05 and 0.1) were analyzed using SPSS and WINPEPI software. Proportion of positive samples were lower in samples stimulated with dialyzed PPDs: using the 0.05 cut-off point, significant differences were observed between dialyzed PPD and non-dialyzed 0.5% phenol PPD ($p<0.1$). Using a threshold of 0.1, significant differences ($p<0.05$) were also obtained. Analysis of variance revealed significant differences between results with blood stimulated within the first 8 hours compared with those stimulated after 16 and 24 hours post-collection. When the test was performed in duplicate, the number of positives was very similar in both runs of the test, and concordance between outcomes was very high (kappa>0.85). Moreover, differences in the correlation between lesion score and gamma-interferon release based on the antigen assayed for stimulation were observed.

These findings can improve the application of gamma-interferon tests for the diagnosis of tuberculosis in cattle and goats.

PP-125

A MULTI-ANTIGEN ELISA-TEST FOR SEROLOGY DETECTION OF *MYCOBACTERIUM BOVIS* INFECTION IN CATTLES

Casto B[1], Donati C[1], Zoppi S[2], Pacciarini M[1], Boniotti MB[1]

[1]Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, via Bianchi 9, 25124 Brescia, Italy
[2]Istituto Zooprofilattico Sperimentale Piemonte Liguria e Valle d'Aosta, via Bologna 148, 10154 Torino, Italy
Contact: mariabeatrice.boniotti@izsler.it

Bovine TB can be detected in live animals by the use of tests of cellular immunity: the intradermal (IDT) and γ-interferon (γ-INF) tests. Assays of humoral immunity may detect animals in which the cell mediated response is poor and thus complement IDT and γ-INF for the detection of infected animals.

In this study the humoral responses of individual animals were tested by means of a multi-antigen indirect ELISA assay developed in our laboratory. PPDB, PPDA and 4 recombinant proteins expressed in *E. coli* (MPB70, MPB83, ESAT-6 and CFP-10) were used. A total of 570 cattle blood sera were analysed. A group of 385 animals from 6 different officially TB-free herds were used to assess the specificity of the test. To determine sensitivity of the assay a further 93 culture isolation positive animals were tested. The remainder of the samples tested, coming from 21 TB positive herds (localized in a low incidence area), were negative by culture isolation but classed positive by other diagnostic tests or classed negative by all the performed tests.

The multi-antigen ELISA test showed moderate sensitivity, comparable to other studies, and high specificity. Among the antigens, MPB70 detected most of the ELISA positive samples, followed by PPDB, MPB83, ESAT-6 and CFP-10. Compared to IDT or γ-INF, the multi-antigen ELISA test showed lower sensitivity, nevertheless this test was able to detect humoral response in animals were the other tests failed.

These results support the use of the ELISA test to complement other techniques based on cellular response to identify mycobacterial infection in cattle and to improve the overall probability of removing all infected animals in herds where eradication is ongoing.

PP-126

USE OF SEROLOGY TESTING FOR *MYCOBACTERIUM BOVIS* INFECTION IN GOATS: A CASE REPORT

Casto B[1], Donati C[1], Zanardi G[1], Gaffuri A[1], Zanoni M[1], Pacciarini M[1], Boniotti MB[1]

[1]Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, via Bianchi 9, 25124 Brescia, Italy
Contact: mariabeatrice.boniotti@izsler.it

Mycobacterium bovis causes Tuberculosis (TB) also in goats. Clinical diagnosis is not easy as symptoms are not typical, whereas gross lesions are similar to those found in cattle. Tuberculin skin test is as effective as in cattle and remains the *in vivo* diagnostic method of reference. Few data are available about the use of serology tests in goats.

In this study we analysed the infection status of 34 goats coming from a cattle/goats mixed herd where TB was firstly confirmed in cattle. The 34 goats underwent single intradermal comparative cervical test (SICCT), γ-interferon (γ-INF) and a home–made multi-antigen indirect ELISA. This test was standardized using PPDB, PPDA and 4 recombinant *M. bovis* antigens expressed in *E. coli*: MPB70, MPB83, ESAT-6 and CFP-10. Previously, 290 goat blood sera coming from 13 officially TB-free herds were analysed in order to determine the OD cut-off values for each protein. By SICCT 20 goats were found positive, 19 by γ-INF and 12 by the multi-antigen ELISA. Among the antigens, ESAT-6, CFP-10 and PPDB detected most of the ELISA positive samples, followed by MPB70 and MPB83. After stamping-out, necropsy was performed and gross lesions were found in 15 animals. Culture isolation and IS*6110* PCR, carried out directly on tissue homogenate, were attempted in few SICCT-positive subjects and in all the animals with negative results both by *in vivo* tests and by post-mortem examination. *M. bovis* infection was confirmed by culture isolation in 4 goats. The results of SICCT, γ-INF and ELISA were compared: the concordance between SICCT and γ-INF is very high while the multi-antigen indirect ELISA showed a surprising ability to detect 5 new positive animals previously classed negative by all the other tests.

The results of this study show that the multi-antigen indirect ELISA method assessed in this work appears to be a useful tool to detect *M. bovis* infection in goats, in particular when there can be a loss of reactivity to the tuberculin test.

PP-127

LYMPH NODE TUBERCULOSIS

Sanja Grm Zupan, Petra Svetina Šorli

University Clinic of Respiratory and Allergic Diseases Golnik, SI-4204 Golnik, Slovenia
Contact: Tel. +386 4 2569411, Fax. +386 4 2569117, sanja.grm@klinika-golnik.si

Lymphatic tuberculosis is the second most common group of extrapulmonary tuberculosis in Slovenia. In year 2007 tuberculosis of intrathoracal lymph nodes accounted for 7.5% of the cases of extrapulmonary tuberculosis and tuberculosis of extrathoracal tuberculosis 22.5%.

We made a retrospective analize which included patients treated in KOPA Golnik between years 2004 and 2009. In 70% of cases cervical lymph nodes were the most commonly involved (SCLM muscle, submandibular region). The following regions were: supraclavicular (12.7%), axillar (8.5%), abdominal (4.2%), ingvinal (2.1%) and mediastinal lymph nodes (2.1%).

Clinical findings are painless swelling of one or more lymph nodes, often process is bilateral. In the begining nodes are discrete and the skin above it is not affected. Later the nodes flucuate as central necrosis begins and nodes become swollen and painful. Nodes can rupture. In one third of our patients sinus tract formation happened. Sistemic features of tuberculosis are sometimes present: fever, malaise, weight loss, cough, night sweating. Sistemic signs are often when tuberculosis is present in many organs.

M. tuberculosis reaches lymph node by dissemination during initial airborne infection in the lung. Later in life reactivtion occurs when host defance mechanisms weaken. Lymp nodes can be affected as a part of primary complex or during disseminated tuberculosis.
In 47% of cases history of tuberculosis, contacts with active tuberculosis or radiographic features of ex tuberculosis were present.
More common children then adults are affected, more frequently women.
Diagnosis of lymph node tuberculosis can be made in different ways. In 19% swap of discarging sinus tract was made and *M. tuberculosis* was cultivated from these speciman. In 38% aspirats were used and in 17% lymph node biopsy. In 25.4% of cases *M. tuberculosis* was not cultivated from different samples. The diagnosis was only histologically confirmed. In half of those cases At test, which was made later from histological samples, was positive. Our whish is that when tuberculosis is suspected, one sample should be sent to Micobacterial laboratory. Cultivation of *M. tuberculosis* is golden standard in diagnosing tuberculosis.
In our group of patients radiologic examination was made, induced sputum or aspirats was collected. In 21% sputums were negative by microscopy, but *M. tuberculosis* ws cultivated.

Treatment of lymph node tuberculosis is based on the same principles and drug regimens as pulmonary tuberculosis. Standard 6-month regimen is used (2HRZ + 4HR, when *M. tuberculosis* is susceptible to drugs of first line of ATL). When discarge from sinus tract is present we use streptomycin locally. During therapy lymph nodes can enlarge or new nodes can appear. The phenomena is immunologically mediated (hypersensitivity to tuberculoprotein. In literature the use of sistemic corticosteroid is used in these cases. Surgical excision or biopsy has no imporatnt role in tuberculosis treatment.

PP-128

MUTATION SPECTRUM OF RIFAMPICIN AND ISONIAZID RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES IN GREECE

Ioannidis P[1], Papaventsis D[1], Mantadakis E[2], Nikolaou S[1], Karabela S[1], Konstantinidou E[1], Marinou I[1], Raftopoulou A[1], Panagi M[1], Samonis G[3], Kanavaki S[1]

[1]National Reference Laboratory for Mycobacteria (NRLM), Sotiria Chest Diseases Hospital, Mesogion 152, 11527 Athens, Greece
[2]Department of Paediatrics, Democritus University of Thrace, 68100 Alexandroupolis, Greece
[3]Department of Internal Medicine, University of Crete, 71110 Heraklion, Greece
Contact: reflabtbsotiria@yahoo.gr

There is limited information on the prevalence of specific mutations within the *rpoB*, *katG* and *inhA* genes in rifampicin and isoniazid resistant *M. tuberculosis* strains isolated in Greece.

953 clinical strains were analyzed by the line probe assay MTBDR*plus* (Hain Life Science, Germany). For several strains the analysis was further extended by DNA sequencing of the *rpoB* hot spot region, the *inhA* promoter and portions of the *katG* and *inhA* ORFs. The molecular results were compared to drug susceptibility testing (DST) performed by the proportion method on Löwenstein-Jensen medium.

RpoB mutations were identified in 53/953 (5.6%) strains. The most prevalent ones were the S531L substitution (29/53; 54.7%) and the silent mutation 1341C>T (7/53; 13.2%). Mutations identified less frequently were: H526Y, H526N, H526R, H526C, H526D, H526L, S531W, S531F, 513dupF, D516V, D516Y and L511P. In 2 strains the double substitutions L511P&D516G and L511P&M515V were identified. For isoniazid resistance mutations were identified in 98/953 (10.3%) strains. The most common were the *katG* S531T1 (AGC>ACC) and the *inhA* [-15C>T] identified in 64/98 (65.3%) and 22/98 (22.4%) strains, respectively. Mutations identified at lower rates were: for *katG* the S315T2 (AGC>ACA) and S315I, and for *inhA* the [-34C>T], [-8T>G], [-8T>C], [-17G>T] and the [-16A>G]. 4/98 strains contained mutations in both *katG* and *inhA* genes whereas heteroresistance was found in 3 isolates. 36/953 (3.8%) strains harbored mutations in genes affecting susceptibility to both drugs (MDR). All wild type *rpoB* strains as well as those carrying the 1341C>T polymorphism, were sensitive to rifampicin. The remaining *rpoB* mutants were resistant with the notable exception of two strains harboring the mutations H526L and H526N (drug concentration 20 μg/ml). All *katG* and/or *inhA* mutants with the exception of 5 strains harboring *inhA* promoter region mutations were found resistant to isoniazid (0.2 μg/ml concentration). 7

strains with no detectable mutation in the genetic regions analyzed by the line assay and by sequencing were found to be resistant to isoniazid by DST.

The data presented herein indicate that, although commercial molecular tests are extremely useful for the rapid detection of rifampicin and isoniazid resistance, the absence of a WT probe signal with no concomitant detection of a known mutation involved in drug resistance should be interpreted with caution and additional methods such as sequencing should be considered.

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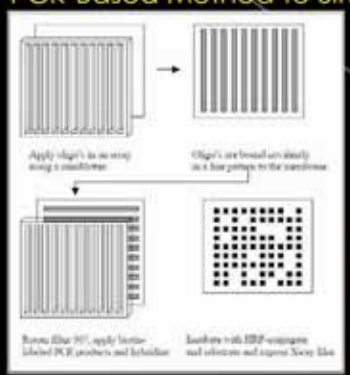

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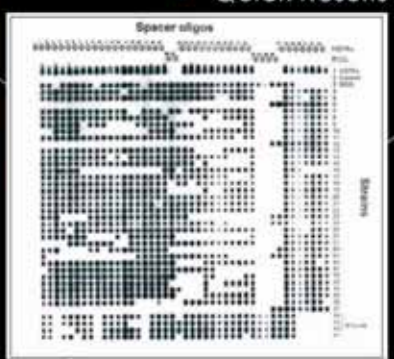

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