"ALEXANDRU IOAN CUZA" UNIVERSITY

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PHD STUDENT

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RESEARCH ON RAPID IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* BY MODERN METHODS

PHD THESIS SUMMARY

Phd Coordonator,

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Research objectives

The Research objectives focused on the diagnosis and treatment of Tuberculosis. It is aimed at the development and implementation of modern diagnostic methods currently unavailable in Romania:
1. Developing new processing techniques for sputum which optimizing the culture yield and eliminate the centrifugation step while preserving the quality of decontamination ;
2. Implementing advanced techniques of liquid culture that allows to achieve faster results and increased sensitivity for mycobacteria isolation ;
3. Phenotypic and molecular characterization of Mycobacterium tuberculosis strains with multidrug – resistance isolated in Iasi county.

Introduction

Pulmonary tuberculosis remains one of the most important public health problems in the XXI century. It is estimated that about a third of the world's population is infected with *Mycobacterium tuberculosis*, Therefore, early diagnosis is particularly important as it allows limiting transmission.
Classically the diagnosis of pulmonary tuberculosis is based on culture results on solid medium, but this technique has a long turnover time. The results of molecular biology techniques are available in less than 24 hours. The aim of this study was to test, identify and implement a series of diagnostic techniques aimed shortening of delivery has general application and lowering costs but mainly aimed multidrug-resistant pulmonary tuberculosis.

Chapter 1. Current problems in the diagnosis of tuberculosis

TB diagnosis is a process that can be difficult. and require a positive diagnosis of tuberculosis after bacteriological examination and the physician is responsible for conducting these tests before treatment. In 2013, 44% of new TB cases in Europe came from patients in the age range 25-44 years.
In Romania about 1,300 cases of multidrug-resistant tuberculosis occur annually. Romania ranks high on the list of 18 countries with cases of MDR -TB in the WHO European Region.

Chapter 2. Identifying the species M. tuberculosis by smear microscopy

Mycobacteria, due to biochemical structure, are able to fix fuchsin, crystal violet or auramine phenol in water and resists discoloration with an acid-ethanol and this is the basis for the Ziehl-Neelsen staining
Microscopic examination reveals acid-fast bacilli in various biological products. Is a rapid , inexpensive, but that does not identify the species.

Chapter 3. Methods of cultivation of mycobacteria

Bacteriological diagnosis of tuberculosis identify mycobacteria species, and to perform susceptibility testing of the infecting strain, thus guiding therapeutic decisions .
The solid culture medium used for the growth of *Mycobacterium tuberculosis* is currently Lowenstein-Jensen (LJ).

3.3. MODS culture

MODS is a modified liquid cultivation technique using Middlebrook 7H9 medium (the same used by the MB/BacT system), in microtiter plates. MODS stands for Microscopic Observation Drug Susceptibility because it was used for susceptibility testing to isoniazid (HIN) and rifampicin (RMP), but can be used successfully to isolate also the mycobacteria. The method was first described in 2000 by Caviedes and is faster and allows sensitivity testing.

Chapter 4 Molecular. diagnostics in tuberculosis

Molecular biology techniques can identify M. tuberculosis complex species non-tuberculosis specie, can detect the presence of genetic variants associated with susceptibility/resistance to drugs such (rifampicin, isoniazid) and permit the tracing of infection transmission

Personal Part

Aim of the thesis

The work was thesis aimed at developing diagnostic methods not used in Romania, adapting them to local practical conditions, evaluating their operational characteristics and the development and evaluation of advanced diagnostic molecular methods for studying with multidrug - resistant strains isolated in Iasi County in recent years.
The research conducted during the thesis was directed in several related directions:
-sputum processing methods prior to inoculation in culture. In practice clinical specimens which need to be inoculated is require a decontamination step and concentration to increase culture yield . This involves the use of centrifuges that would not generate aerosols that have a high cost. We developed a processing technique for sputum without a centrifugation step that can be widely used.
-Implementation of a faster culture method in liquid media using microscopic observation for detecting culture positivity
-Implementation of a phenotipic method for detecting *Mycobacterium tuberculosis* (MTB) drug resistance and evaluating Minimal inhibitory concentrations (MIC) to first and second line drugs
-Identification of mycobacteria using molecular biology techniques. We compared the operational characteristics of two molecular biology methods one based on an end-point PCR and another based on real-time PCR

-Early detection of mycobacterial cultures positivity by combining MODS culture with gene amplification techniques in order to achieve faster results of culture positivity . Combining these methods would allow an improvement of about one week of the diagnosis of mycobacterial infections .

- Sanger sequencing of rpoB RRDR for identifying rifampin related mutations;
- Implement high throughput sequencing techniques (Whole Genome Sequencing) for mycobacterial characterization.

The documents from which we adapted the techniques are the tuberculosis WHO manual and the articles in which the differents methods methods were initially described

Chapter 5. Material and Research Methods

5.1. Material investigated

Investigated material consisted of strains isolated from pathological products addressed for diagnosis at the bacteriology laboratory of the Pneumology Hospital.
We used a total of 7446 of pathological products between January 2009 and March 2009.
From the samples currently being processed, a total number of 614 samples were used for the implementation and adaptation of the MODS culture in liquid medium. The samples were retrieved between March 2011 and February 2012.
For molecular characterization were used all strains of multidrug - resistant isolated in the laboratory between January 2009 - February 2014.

6.1. Optimizing the processing technique of sputum

Culture of spontaneous expectorated sputum generally includes a decontamination step before media inoculation and then concentration usually done by centrifugation. The aim of this study was to test a simple decontamination method lacking a centrifugation step to be used in conjunction with the Löwenstein- Jensen culture media. The most widely used decontamination method is with sodium hydroxide 4%. This step is time consuming and requires an aerosol safe centrifuge which is a relative expensive piece of equipment and therefore may represent a strain for low resources settings.
Of the 7446 samples collected between 12 March 2009 and 12 June 2009 from 3229 patients, a total of 819 samples (from 378 patients) were either only positive direct microscopic examination a total of 84, representing 10.3% , from 42 patients, are associated with at least one positive culture of mycobacteria and non-contaminated (735, 89.7% of 336 patients) (Fig. 42). 84 samples were positive microscopic but cultures were negative. Regarding the positive culture results - out of a total of 735 positive cultures (from 336 patients), 492 were positive at microscopy (66.9%) and 243 were microscopically negative (33.1 %). These data are similar to those of the

regional register of tuberculosis (Fig. 43). The average time to positivity was 21 days (interquartile range 21 to 30 days) (Fig. 44)

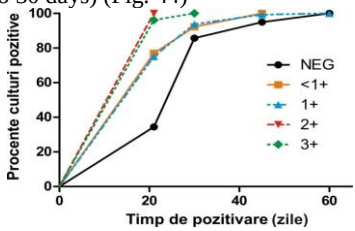

Fig 44. Time to positivity

Discussions

The data of the present study show that the culture positivity is significantly higher in scanty positive samples (countable and 1+ versus 2+, 3+, at microscopic examination) suggesting minimal loss of sensitivity with this protocol. The contamination rate was less than 2% being within the limits recommended by the WHO. This modified Petroff method does not requires expensive equipment, is faster compared to conventional methods and requires only inexpensive reagents. These elements make the method applicable under conditions of limited resources thus increasing laboratory capacity to perform culture even if the tubes are then sent to a central laboratory.

Conclusions

These data suggest that this approach may be viable as a temporary measure in low resource laboratory setting at the putative price of a tolerable sensitivity loss.

6.2. Improvement of liquid culture techniques through detection of culture positivity by microscopy observation (MODS)

Culture is currently the standard for confirming tuberculosis; Culture is cheap, but the results are obtained after at least fourteen days and up to three months;
Caviedes et al have developed a new method of liquid culture, the culture positivity being evaluates by direct microscopic observation which has been referred as MODS. The aim of this study was to verify whether this technique could be applicable under conditions of limited resources, to evaluate its performance for the isolation of mycobacteria from clinical specimens (sensitivity, rate of contamination) and to assess the costs associated with this method.
A total of 488 samples were cultured of which 306 (62.7%) were sputum , 116 (23.8 %) bronchial aspirates , 13 (2.7%) gastric aspirates , 22 (4.5%) collected biopsy , 30 (6.1%) and pleural fluid specimen and one urine (0.2%).
Of these samples, 401 were addressed to diagnose tuberculosis (initial diagnosis samples) while 87 were done for monitoring response to treatment (follow -up samples).

Of the total number of samples, 113 were positive for mycobacteria on solid media LJ, 125 per MB/BacT and 162 with the MODS (Fig. 48). A number of additional positive samples were contaminated with other types of bacteria: 23 LJ tubes (4.7 %) and 7 samples each for the MB/BacT and MODS (1.4%).

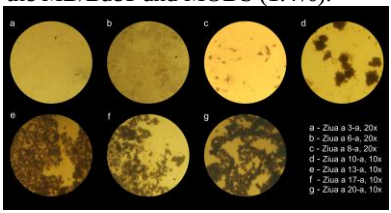

Fig. 48. Mycobacterium tuberculosis in MODS culture (original photo**)**

Mycobacteria were isolated from 166 samples with at least one of the three methods used. Thus, taking into account the presence of a mycobacterial isolate in any of the three culture methods as the reference standard , respective sensitivities (confidence interval 95%) were of 68.07 % (60.4-75.08) for the solid medium LJ, 75.3% (68.02-81.65) for MB/BacT and 97.59% (93.94-99.32) for MODS.

Sensitivity was slightly lower for the group of patients undergoing treatment than for those being investigated for diagnostic purposes namely 92.6% vs. 99.2% (Fig. 52).

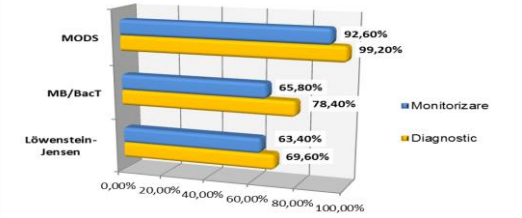

Fig. 52. Sensitivity of different culture methods according to the purpose of culture.

Globally the MODS method of were found to be statistically significant better than the other two methods ($p < 0.001$)

The average time to positivity was 26.8+/ 9.1 days on solid media , 13.8+/-6.5 days on MB/BacT 8.2+/-4.6 days and new method.

Regarding the cost analysis, the fixed costs related to processing samples were estimated at 3.3€/sample. The additional costs directly related to tehnical manoeuvres was estimated at 1.1€ per sample for MODS culture, 1.07 € for Löwenstein -Jensen culture and culture €10 for MB/BacT.

Discussions

Data obtained local support the implementation of MODS culture as possible within a limited resource environment. The MODS technique detected M. tuberculosis in samples with different origins, with greater sensitivity and in less time than the Löwenstein-Jensen culture and automated mycobacterial culture through the MB/BacT system. The average gain of time (±SD) was two weeks compared with solid medium and more than 11 days, compared to MB/BacT.

Another aspect that emerges from this approach is that MODS is also more sensitive than culture on solid media or MB/BacT, which is a new element; it is interesting to elucidate this improvement in sensitivity, although it used the same type of culture medium as in MB/BacT.
MODS culture can be used not only to obtain quick results rifampicin susceptibility testing, but also to increase the yield of isolation of mycobacteria.

6.3. Rapid detection by PCR positivity MODS cultures

We aimed to improve liquid culture techniques using the detection by PCR of culture positivity and not by microscopic observation. Was performed a classical MODS culture of which was drawn at predetermined intervals culture medium for mycobacterial DNA extraction.
We collected two sets of samples: 488 samples from patients with a clinical suspicion of TB between January 2010-May 2010 and 613 samples from 355 subjects between March 2011 and March 2012.
A total of 88 specimens were cultured in triplicate in May 2011 and harvested at 3, 4 and 5 days after incubation for identification of the optimal culture day for performing the molecular tests. None of the 88 samples from day 3 was positive by PCR but at days 4 and 5 the results were consistent with Löwenstein-Jensen cultures as well as with MODS. Consequently culture were harvested at 4 days and samples were stored frozen at -30^{o}C until performing the tests for molecular diagnosis. For specific amplification of the M. tuberculosis complex, we used a nested PCR kit (GeneProof) for amplifying the genomic DNA based on the detection of Mycobacterium tuberculosis insertion sequence IS6110 (fig. 58).

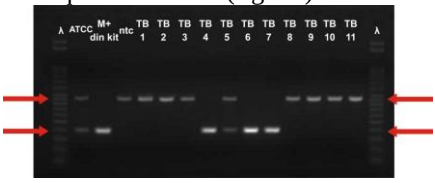

Fig. 58. Example of gel migration of PCR products resulting from TB detection by enpoint PCR

A positive amplification have a 197 bp amplicon resulting from the *Mycobacterium tuberculosis* IS6110 target sequence while the 460 bp amplificon represents the internal standard.
In the period March-May 2013 were also amplified by real-time PCR 257 DNA samples from MODS cultures harvested et the 4th day after inoculation.
Of the 257 samples 19 were true positives by PCR and in culture; 12 samples were positive by PCR although they were negative culture; were also 21 false negative samples and 206 samples were negative in both culture and by PCR.
The rate of false positive results show a high rate of contamination in the culture, DNA extraction step or in the preparation of PCR; DNA extractions from were MODS cultures resumed and samples were also amplified by end point PCR standard kit .

The results obtained after examination by LJ cultures, MB to MODS culture and PCR can be traced in figure 69.

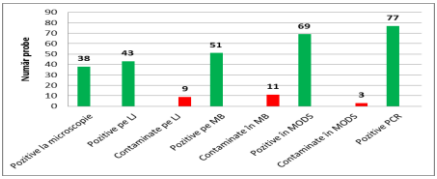

Fig. 69. Results of PCR amplification by endpoint PCR

Conclusion

The combination of the two techniques - MODS culture and amplification by polymerase chain reaction has the potential to generate microbiological results about 20 days faster than the traditional method based on solid culture medium. The sensitivity and specificity are insufficient but with current methodology.

6.4. Developing a method for determining the MIC to rifamycins for mycobacteria using the MODS technique

The also determined the minimum inhibitory concentration (MIC) to rifamycins of the MDR MTB strains from the collection of our bacteriology laboratory isolated between January 2009 February 2014.

Minimum inhibitory concentration was made using MODS technique as previously described. An example is presented in Figure 73.

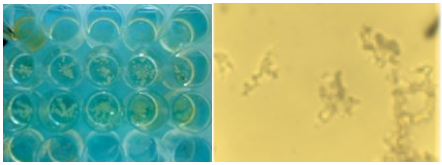

Fig. 73. *Mycobacterium tuberculosis* in MODS liquid culture at inverted light microscope 40x (original photo)

Chosen breakpoint concentrations were: 0,12 µg/ml, 0,25µg/ml, 0,5µg/ml and 1µg/ml for rifabutin and rifapentine and 0,5 µg/mL1 µg/mL and 2 µg/mL rifampicin.

After testing , 70 strains (95.89%) have a higher MICs than 2µg/ml at RMP and one strain (1.36%) a MIC 1 mg/ml and a strain proved sensitive.

For rifapentine one strain (1.36%) had an MIC of 0.5 microgram / ml, two strains (2.73%) had an MIC of 1µg / ml, and 66 strains (90.41%) had higher MICs of 1µg / mL, and two (2.73%) have a MIC less than 0.5 mg/ml.

In the case of two strains rifabutin (2.73%) MIC was 0,5µg/ml, 2 strains (2.73%) had a MIC of 1µg/ml and 55 strains (75.34%) had a MIC concentrations above 1µg / ml; 14 strains (19.17%) were considered sensitive 12 having an MIC of 0.125 mg / ml and 2 strains of 0.5 mg/ml.

Conclusions

MIC determination by MODS culture shows a very good agreement with the results solid Löwenstein -Jensen medium .

MODS technique offers an alternative for antibiotic susceptibility testing and enables rapid identification of strains with resistance multidrog- .

MODS is a simple technique , cheap, fast what could be an excellent method for routine testing of sensitivity especially in developing countries

6.5. Rifamycins resistance prediction through molecular biology techniques

Multidrug resistance represents a significant threat to tuberculosis control. The majority of rifampicin resistant strains have mutations affecting the 81 bp region of the rpoB gene called RRDR. We retrospectively evaluate the relationship between minimum inhibitory concentrations for rifampicin, rifapentine, rifabutin and RRDR (Rifamycin Resistance Determining Region) mutations in MDR Mycobacterium tuberculosis.

A total of 66 isolated strains underwent first and second line sensitivity testing and rifampicin, rifabutin and rifapentine minimal inhibitory concentrations were determined using a modified MODS technique. Sanger sequencing was performed for the RRDR region of rpoB gene.

Twenty-five percent MDR strains showed extended drug resistance. All strains were rifampicin and rifapentine resistant and 18% maintained rifabutin sensitivity. All detected RRDR mutations involved five codons 511, 516, 526, 531 and 533. Two strains did not show any mutation. One case presented two mutations involving codon 516. XDR strains only associated pS531L and pS516V.

Twelve strains had rifabutin MIC below 0.5 µg/ml (table 2) – and therefore may be considered as sensitive. Rifabutin sensitive strains showed a different mutation profile, codon 516 and 511 mutations were particularly frequent. Even so the use of codon 516/511 mutation as a test for inferring rifabutin susceptibility in MDR strains is not clinically usable because of insufficient sensitivity-75%.

Tabel 4. Comparison of RRDR mutations frequencies in rifabutin resistant and rifabutin sensitive multidrug resistant *Mycobacterium tuberculosis* strains isolated in the north east region of Romania

Codon number	Rifabutin resistant	Rifabutin sensitive	Total
511	0	1	1
516	4	8	12
526	7	0	7
526/516	0	1	1
531	41	0	41
533	0	2	2
Not mutated	**2**	**0**	**2**
Total	**54**	**12**	**66**

There was a case for which two mutations were found involving both codon 516 (GAC->GAG; Asp->Glu) and 526 (CAC->AAC: His->Asn); to our best knowledge this association is not currently reported to the public databases.
A more comprehensive sequencing approach could be useful extending the search for genetic variants beyond the rpoB RRDR, potentially including other genes as for example efflux pumps and additional described mechanisms.

Conclusion

These data suggest that molecular analysis of RRDR is useful as a rifampicin resistance detection tool. This approach is not able to reliably predict rifabutin susceptibility in MDR M. tuberculosis strains. Whole genome sequencing is necessary to identify other relevant regions for this purpose.

6.6. Assess the feasibility of the implementation of Mycobacterium tuberculosis hole genome sequencing in laboratory practice

In this chapter we present the results of the feasibility study for mycobacterial hole genome sequencing. We used 68 strains with multidrug-resistant *Mycobacterium tuberculosis* previously tested for the detection of mutations in the region of 81 base pairs.
The genetic material was processed to obtain library (Figure 83).

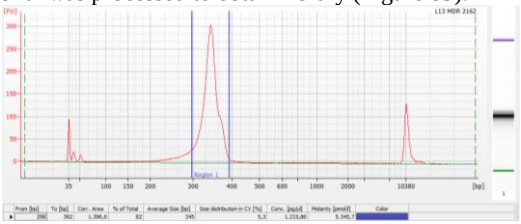

Fig. 83. Library quantification on bioanalyzer

We obtained a satisfactory load for the sequencing chips Ion 316 and Ion 318, values that exceed of the minimum amount described by the manufacturer (50%).

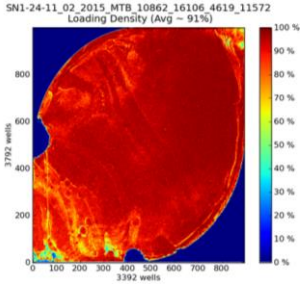

Fig. 84. Loading density on 318 Ion Torrent chip

The complexity and amount of data obtained from sequencing needs a thorough analysis. Data obtained are still under analysis.

General conclusion

•Sputum processing method without centrifugation is applicable in conditions with limited resources thus increasing the capacity to realize culture by poorly equipped laboratories.

•The MODS culture for mycobacteria has been demonstrated as feasible in a limited resource environment. The sensitivity of the method proved to be superior to Löwenstein-Jensen cultures or MB/BacTculture. The gain in culture time was estimated to be about two weeks compared with conventional methods.

•MODS culture technique has proved useful for determining the minimum inhibitory concentrations of tuberculostatic agents, the results are consistent with those obtained through traditional approaches but have the advantage of time savings.

•RRDR region sequencing is a suitable technique for the detection and prediction of the multidrug-resistance status.

•Introduction of high througput sequencing techniques WGS (Whole Genome Sequencing) for mycobacterial strains characterization is possible for laboratories with high level of equipment and ensures high accuracy both in terms of identifying species and groups plotting and predicting resistance to all transmission tuberculostatics in the genotype-phenotype relationship is known.

Elements of originality

•This research has allowed for significant results whose main original elements consist of:

1.Develop a simplified method of growing mycobacteria, to eliminate the concentration step by centrifugation. This type of processing of samples gives the advantage of being faster, and compared with traditional methods rely solely on the availability of inexpensive reagents.

2.We implemented the first time at the national level a new method for rapidly growing mycobacteria in liquid culture initially described by Caviedes et al in 2000, the novelty consists of superior performance in terms of paucibacilare samples.

3.We developed a combination method between MODS technique and a method of identification based on PCR in order to improve the speed of mycobacteria identification. The current proposed method has the potential to get results faster by about seven days.

4.Using the experience gained from the local implementation of MODS we developed the determination of MICs to usual tuberculostatic agents; the method is reliable and reproducible and has the advantage of a reduced price.

5.We implemented under usual clinical the molecular detection of rifampicin resitance based onSanger sequencinf of rpoB RRDR.

6.We performed first time in Romania whole genome sequencing of Mycobacteria

Perspectives

The results obtained in this study allowed the developing and implementation of advanced methods of TB diagnosis and phenotypic and genotypic characterization of multidrug resistant strains isolated in Iasi. These results open interesting perspectives for research that could be further developed as follows:

•Practical implementation of the advanced diagnostic techniques developed.

•Further development of the molecular detection techniques of Mycobacterium tuberculosis in practice.
•The promising results of PCR detection of mycobacterial growth in MODS type liquid cultures enable us to further develop this technique locally. In this regard we should improvethe sampling process from liquid cultures that is a critical step to reduce false positive rate.
•The element that is the most promising is the development of WGS (Whole Genome Sequencing) for mycobacterial characterization. This project will allow improving the processing of samples ("wet lab") and the development of data analysis capacity.