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Full Length Research Paper

Application of the Alamar blue assay to determine the susceptibility to anti-tuberculosis pharmaceuticals

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The aim of this study was to apply the Alamar Blue assay to determine the susceptibility to anti-tuberculosis pharmaceuticals, in *Mycobacterium tuberculosis* isolated strains from patients with tuberculosis (TB). In this study, a TB diagnostic was done by bacilluscope as an essential diagnostic tool for pulmonary TB using the Ziehl-Neelsen (ZN) stain. Furthermore, a culture was prepared in Lowenstein-Jensen (L-J) solid media, Middlebrook 7H9, and Middlebrook 7H10. For all the clinical samples investigated, five reference strains of *M. tuberculosis* were used to standardize the sensibility to streptomycin (SM), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) of the ATCC. Additionally, 97 *Mycobacterium* strains were analyzed from clinical isolations. The microplate Alamar Blue assay (MABA) was standardized and applied. The resistance levels of this disease were obtained in La Comarca Region (Lagunera). The results indicated that the MABA test is fast and easy to apply, however the most important aspect is the reliability of the method to determine the drug sensibility to pharmaceuticals. The MABA colorimetric test can be used in different regional as well as state TB diagnostic laboratories.

Key words: Tuberculosis, Alamar blue, drug resistance.

INTRODUCTION

Tuberculosis (TB) is a reemerging disease that in the actuality constitutes one of the most important public health problems in the world. According to NOM-006-SSA2-1993, TB is a generally chronic infectious disease caused by species of the *Mycobacterium* gender, principally by *Mycobacterium tuberculosis* in humans and *Mycobacterium bovis* in animals. It is transmitted from an infected individual to a healthy one by inhalation of infected material or by ingestion of contaminated cow milk, respectively (Rodríguez, 1995). In the last decades, this disease has been enhanced by reemerging multidrug-resistant strains (MDR) of *M. tuberculosis* and by a co-infection with the human immunodeficiency virus (HIV) (Kibiki et al., 2007). The presence of HIV increases

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the risk of the infection reactivating from latent TB (LTBI) and has increased dramatically the number of deaths related to TB in recent years. TB is a lethal infectious disease that affects millions of people around the world. The WHO has declared that there are eight million new cases each year and as a consequence, is the cause of three million deaths annually (Ritacco et al., 2008). The species in the *Mycobacterium* gender derive from the Mycobacteriaceae family, a potential pathogen for humans. *M. tuberculosis* belongs to the *M. tuberculosis* group, which includes *M. tuberculosis, M. bovis, Mycobacterium africanum,* and *Mycobacterium microti.* Of these, only *M. tuberculosis* and *M. bovis* are considered important pathogens for humans. The rest of the members of the group are little well known, however, all the members of the group have an almost identical sequence in the RNA gene ribosomal 16S (16S rRNA) which is used for specific studies among different micro-bacterial species (Wirth et al., 2008). The most advanced anti-TB therapy consists in INH, pyrazinamide (PZA), and RIF (Gumbo et al., 2007). Of these, RIF and INH are reported in all the regions of the world and the prognosis for patients MD-TB is poor, due to the fact that a patient commonly carries the infection until death (Traore et al., 2007). Extensively, MDR-TB is defined as a disease caused by isolation of *M. tuberculosis* resistant to at least INH and RIF in addition to a fluoroquinolone and second-line antibiotics such as capreomicine, kanamicine (K), or amikacine (AK) (Bonilla et al., 2008). In Mexico, the number of new cases has increased nearly 12% also increasing its resistance and MDR to the anti-TB therapy, in the Comarca Region, but those figures are unknown. Resistance to RIF and INH is associated to the mutation in specific genes like *katG* and *rpoB* in *M. tuberculosis*, which leads to MDR. *M. tuberculosis* has two distinctive characteristics: its ability to resist acid staining and its ability to provoke long-term LTBI in humans. It is a slow-growing bacterium. Four to six weeks are needed to obtain a colony of *M. tuberculosis* that allows us to study its pharmaceutical sensibility (Bhatt et al., 2009). The medium used for its cultivation contains malachite green and other antibiotics to inhibit the growth of other bacteria. It is aerobe obligated and an optional intracellular parasite. To obtain control of TB, it is necessary to implement economical, appropriate, precise, and reliable methods of diagnosis that allow us to give fast results and more importantly, that serve as a means to safeguard the limitations of the conventional bacteriological diagnosis. It is important to consider that the diagnosis of TB needs a follow up treatment and this substantially depends on diagnostic techniques, in the process of clinical samples. Because of this, a need for an application of modern, sensitive, and specific methods arises from this study which will lead us to determine the drug susceptibility of micro-bacteria in strains obtained from patients with TB in the Laguna Region. Due to this fact, it is essential to run susceptibility tests with anti-TB pharmaceuticals in strains from patients with TB using the modern MABA. The aim of this investigation was to apply the MABA method to determine the susceptibility to anti-TB pharmaceuticals of *M. tuberculosis* in isolated strains from patients with TB in the Laguna Region.

MATERIALS AND METHODS

This work was approved by the Research Ethical Committee of the Faculty of Medicine of the Juarez University of the State of Durango (17/2009). The selection of participants was done considering two inclusion and exclusion criteria. For the handling of samples, a strict monitoring and immunization protocol was established for the investigator (PPD tuberculin, *M. bovis* bacillus Calmette-Güerin (BCG) vaccine, a thorax X-ray, and bio-security cabin). 97 expectoration samples were processed from patients channeled from the General Hospital in Torreon, the Health Ministry, Sanitary Unit no.6, Health Clinic, Sanitary Unit no. 2, ISSSTE Clinical Hospital in Gomez Palacio and a number of private clinics that had diagnosed pulmonary TB and five controls were processed. The collection of samples was done over eight month period. Direct bacilluscopy was performed and from the concentrate we evidenced the resistance to acid – alcohol (BAAR) using the ZN staining process, in accordance with Technical Note no. 26/Rev. OPS/OMS. To prepare the culture samples, a decontamination process was developed for the samples using the Petroff method (NaOH solution at 4%), in accordance with Technical Note no. 27 OPS/OMS. The reference strains used were ATCC, Rock-Ville, Md. Once the samples were decontaminated using the Petroff method, they were concentrated and planted in two Middlebrook agar 7H11(Beckton-Dickinson) culture media and the conventional L-J culture medium. In the same way, we proceeded to cultivate duplicate reference strains. Middlebrook Stock 7H9 (Beckton-Dickinson) was used for the logarithmic phase. Once a pure culture was obtained from stock 7H9 directly or by a subculture coming from solid measures, a test was performed to determine the sensibility to four pharmaceuticals using the MABA (Luna-Herrera et al., 2007), is a redox colorant used as an indicator of a viable cellular growth whose oxidized form is blue and does not flourish whereas the reduction is pink and flourishes, allowing a detection and quantification of said property in relative units of fluorescence. It was processed with isolated chemicals recovered from stock 7H9 with a two week incubation period (logarithmic phase) and adjusting the turbidity of tube number 1 of the McFarland Nefelometer (equal to 3 × 108 UFC/ml), which were then diluted 1:10 in stock 7H9 supplementing with a 10% OADC (oleic acid, albumin, dextrose, and catalase) enrichment. This suspension was prepared immediately before the microplate inoculation. To run the studies, sterile microplates (Costar®) were used with 96 dregs. In each plate, two strains different to drugs SM, INH, RIF, and EMB (SIRE) were analyzed. In all the peripheral dregs, 200 µl of sterilized water was added (to prevent desiccation). Isolation 1, with its respective problems and free drug controls was distributed in columns 2 to 6, while isolation 2 was placed in columns 7 to 11 (Figure 1). In dregs from columns B to G and columns 2 to 11, 100 µl of stock 7H9 was added without tween 80: later 100 µl of the pharmaceutical was added. After that, with the help of a multichannel micropipette a series of six dilutions were performed. The remaining 100 µl from the last dilution was discarded. Later, 100 µl was added to the bacterial suspension (dilution 1:10 beginning with McFarland tube no. 1) to all the drug containing dregs obtaining a final volume of 200 µl per dreg with the following final concentrations: SM: 8, 4, 2, 1, 0.5, and 0.25 µg/ml; INH: 1, 0.5, 0.25, 0.125, 0.0625, and 0.031 µg/ml; RIF: 2, 1, 0.5, 0.25, 0.125, and 0.062 µg/ml; EMB: 32, 16, 8, 4, 2 and 1 µg/ml.

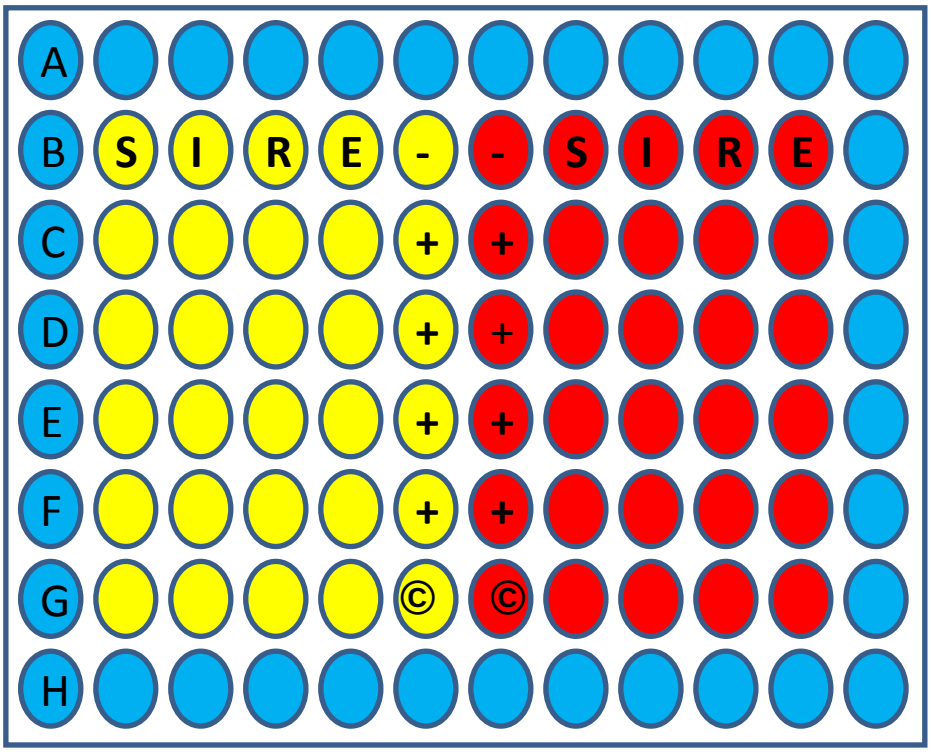

Figure 1. Schematic representation of the distribution of dregs for the Alamar Blue assay and for MTT. Two stains were applied on the plate with four drugs in six different concentrations. SM, INH, RIF, and EMB (SIRE). In columns 6 and 7 the controls are represented in this way: positive (+), negative (-), and dilution 1:100 © (Luna-Herrera et al., 2007).

Next, 100 µl by way of 7H9 were added to dregs B6 and B7 without Tween 80 (control of means) and 100 µl of the bacterial suspension with pharmaceutical (bacterial growth control) were added to dregs C, D, E, and F, and to columns 6 and 7. Finally, 100 µl of 1:100 diluted bacterial suspensions were added to dregs G6 and G7 starting from the bacterial work suspension (control with 1% inoculum). The plates were marked and sealed with adhesive tape. They were then placed in a plastic bag which was then sealed to be then incubated at 37°C for five days. After the incubation period had passed, a revealing resarzurin colorant was applied according the following indications: 20 µl of Alamar Blue to dregs C6 and C7, then another incubation period of 24 h and a reading was taken using the control of 1:100 as a point of reference to obtain the MIC. If no change in color existed in the first control, then another two to three day incubation period was given and in the end a colorant was applied. If a change in color existed, then a colorant was applied to the whole plate.

RESULTS AND DISCUSSION

In the present project, we were able to show the microscopic BAAR in the 97 expectoration samples and the five controls, after analyzing the processed slides that corresponded to each sample, using the ZN staining process, where an abundance of BAAR was observed, which were then quantified as positive and given a cross according to the established protocol. This suggests a higher success probability in finding resistant populations. This procedure turned out to be the easiest, the most economical and fastest way to offer the investigator a diagnostic orientation of TB. Of the total number of samples processed, 91.9% corresponded to the positive bacilluscopy readings. 53 men and 43 women participated in this study representing 55 and 45%, respectively. In other studies, while diagnosing TB, the same conclusion has been reached, where ZN by BAAR was detected and a culture of *M. tuberculosis* in the appropriate media. Although the bacilluscopy study is fast, the culture is the “true test” for the bacteriological diagnosis; however it takes an average of six to eight weeks to run (Vera-Cabrera et al., 1999). In media 7H11, a development of small colonies of typical mycobacteria was observed. As its colonial morphology was analyzed through an optic microscope, it was possible to identify the morphology in each case from the images conserved for this purpose (Mejia et al., 1999). A faster growth rate was obtained (three to five days) using the thin layer method in media 7H11. A typical growth rate was obtained in media L-J, however more incubation days were necessary (15 to 30 days). These results concur with a study done by Mejia et al. (1999). They calculated the sensibility and the positive predictive value with agar

7H11 comparing it to the conventional L-J method which was 83 and 93%, respectively. In this experiment, through a comparison with microcolonies, a result of a presumptive TB identification was obtained and at the same time the purity of the culture was established, making it possible to identify one type of colony, a characteristic of mycobacteria, where even the thin layer culture was considered positive when the development of the micro-colony was observed until it reached a size that was manipulatable. This occurred in a period of 10 to 12 days. To verify this result even further, the micro-bacterial nature was corroborated using a ZN stain. In the same way, Mejia et al. (1999) proved that the thin layer method from agar 7H11 for mycobacteria allows for a rapid detection of growth in more than 80% of the positive isolations during the first two weeks of the culture, while only 10% of the cases were positive using L-J, this representing a clear advantage for early diagnosis using the thin layer method with agar 7H11 for mycobacterial growth, therefore concluding that this method allows for an early identification of presumptive *M. tuberculosis*. In this study, after planting in media L-J, very good results were obtained as far as the colonial morphology of the mycobacteria; they were typical colonies with a confluent growth that corroborates with the bacterial population observed in the corresponding bacilluscopy test. The color of the colonies goes from white to a cream color, with a rough aspect, that changed, after a greater period of culture time, into the shape of a cauliflower, a characteristic specific to *Mycobacterium.* To corroborate on the result that was in front of the Koch bacillus, a sample was taken in each case, which was then stained using the ZN technique, which were confirmed in the BAAR cultures. From the total number of samples processed, the positive cultures corresponded to 82.7%. In Brazil, Bonini et al. (2001) carried out a similar study to isolate the *Mycobacterium* spp. in samples taken from milk from a dairy cow that was suspected of having or that had been positively tested for TB, from a total of 780 samples of milk from 52 animals. The samples were cultivated in L-J media with reduced glycerol (0.5%) and in Stonebrink media. They were kept at a temperature of 37°C for a period of 90 days. The gender of each colony observed in that investigation was determined using the ZN staining methods and auramine. The isolation was confirmed in 78 samples taken from 19 animals. According to the chromatography in the fine layer, under the appropriate growth conditions the following results were obtained: *M. avium* complex (5.26%), *M. fortuitum* (10.52%), *M. bovis* (5.26%), and *Mycobacterium* spp. (78.95%) (Bonini et al., 2001), those results showed a relatively high (78.95%) incidence with subclinical cases with positive isolation of the mycobacteria present in over one fourth of the affected population. Those results reinforce the participation of milk in the transmission of the mycobacteria in diverse animal species. In Brazil there is evidence that approximately 47% of the milk sold in the urban and rural areas is raw milk (Bonini et al., 2001). The direct detection of the sensitivity to the medications is relevant in the prediction of the answer to therapy, and in this way, offers a specific treatment in each case. The use of molecular methods has contributed a greater efficiency in diagnosing pulmonary TB (TBP) and extra-pulmonary TB up to an 80% positiveness with respect to the diagnosis of the expectoration bacilluscopy. In the actuality, the molecular techniques are necessary to evaluate the presence of genomic mutations that confer resistance. In our investigation, when standardizing and developing the MABA test, good results were obtained when plates were placed in a polyurethane bag and by adding in the last dregs, both vertically as well as horizontally, 200 µl of sterile water to avoid desiccation during the incubation period. However, the micro-plates can also be sealed with parafilm giving acceptable results (Franzblau et al., 1998). Redox activity was observed from the MABA reagent, which changed its physical – chemical properties while being subject to a high potential reduction level, such as the level in which the drug resistant mycobacteria grows unrestrictedly in the culture media. At the end of the 24 h incubation period, the plate was observed to determine if there was a change in coloring from blue to pink. This observation was possible because MABA is a resarzurin-based oxidation-reduction indicator which measures colorimetric drug MICs for *M. tuberculosis*. The results obtained in relation to the method reproducibility in determining sensibility of the anti-TB pharmaceuticals: INH, RIF, SM, and EMB, in isolated strains of *M. tuberculosis* in patients with TB in the Laguna Region, by applying the MABA method, coincides with a study run by Franzblau et al. (1998) where a MABA color index was used to identify the MICs of the INH, RIF, SM, and the EMB of 34 isolations of *M. tuberculosis*. In this study, tetrazolium, a redox indicator was used to revealing agent to obtain a measure of the susceptibility of the bacteria. Also during this study, a comparison was done using the BACTEC 460 method - another radioactive method used to determine the susceptibility of the medications – where it was discovered that out of every 19 isolations, 16 turned out to be sensitive to 0.1 µg/ml of IMH in the BACTEC 460 method and the MIC in the MABA method was ≤0.25 µg/ml. The sensibility result obtained by the MABA is shown in Table 1. In Table 2, the critical concentrations obtained for the antibiotics are shown. The results of this study also coincide with an evaluation applied to determine the precision of the MABA color index, where they quickly detected the resistance to RIF and the INH in clinical isolations of *M. tuberculosis* in Peru (Chauca et al., 2007), in which it is stated that MABA is an excellent alternative as a way of testing the susceptibility to anti-TB medications, considering the advantages of the susceptibility to the phenotypical medications, such as micro-dilution tests, cost/benefit, velocity, and quantitative

Table 1. Results of the sensibility tests using the Alamar Blue method.

Sensibility	Isoniazid (%)	Rifampicin (%)	Streptomycin (%)	Ethambutol (%)
Sensitive	71.9	86.4	85.4	82.5
Resistant	28.1	13.6	14.6	17.5

Table 2. Initial and critical concentrations (ug/ml) for antibiotics.

A	32	4	8	128
	STR	INH	RIF	EMB
B	8	1	2	32
C	4	0.5	1	16
D	2	0.25	0.5	8
E	1	0.125	0.25	4
F	0.5	0.060	0.125	2
G	0.25	0.03	0.060	1

Sensitivity regions, Critical regions, Resistance regions.

results of the minimum inhibitory concentration (MIC).

In this investigative work, we were able to observe that MABA is a convenient method to determine the sensibility to the anti-mycobacterian isolations – it is fast and its application requires very little technology. These observations coincide with the study carried out by Franzblau et al. (1998). With respect to this, Collins and Franzblau (1997) besides the observations already mentioned, concluded that the MABA method is, above all, useful in scanning anti-TB medications. In this study, a comparative study was also run with BACTEC 460, where three mycobacterial strains were used: H37Rv and H37Ra of *M. tuberculosis* and *M. avium*, where they concluded that the difference between the MIC of H37Rv and H37Ra of *M. tuberculosis* were quite significant with nine of the tested medications and no significant difference was observed between the MIC determined with BACTEC 460 and the MABA. The method standardization took place in five ATCC strains, with presented monoresistance to the anti-TB drugs (SM, INH, RIF, EMB). The MIC for each sample and for each drug (MIC of the necessary antibiotic to prohibit 99% or less of bacterial growth) was determined, according the MABA indicator, using as a reference the color tone obtained in the dreg containing 1% of the bacteria used, in such a way that the dregs with this tone (or less blue), were identified as inhibitors, while the dregs with a more pinkish tone, were considered resistant. Also, to verify the sensibility and specificity of the MABA test, a study by Kumar et al. (2005) was published where they carried out a comparative study with the color index method on a fast microplate based on the reductase nitrate with the MABA method to determine *M. tuberculosis* in clinical isolations, the reproducible results were obtained in seven days using both techniques. The levels of primary and secondary resistance were higher than those reported by Sifuentes (1995), where he reported the levels of MDR found in the National Institute of Nutrition “Salvador Zubiran” in Mexico with results that indicated levels of INH primary resistance of 9%, RIF at 6%, and MDR at 6%, and also elevated levels of secondary resistance to INH at 44%, RIF at 35%, and MDR at 35%. A survey run in Latin America by the WHO and the OPS between 1986 and 1990 indicated that Mexico presented a preoccupying frequency of primary resistance of 19.1%. However, between 1989 and 1993, The National Institute for Diagnosis and Epidemiology Resistance (INDRE) evaluated 1,811 isolations from diverse states of the republic finding a primary resistance of 8.3%. In 1997, in a survey carried out in three states of the Mexican Republic, with the WHO guidelines, levels of resistance were found in 12.9% of the new cases, and cases with TB with prior treatment at 50%, to one or more first hand pharmaceuticals like INH, RIF, and PZA; and levels of MDR of 2.4 and 22.4% for new cases and cases with prior treatment, respectively (Granich et al., 2000). Peter (1998) found levels of MDR at 17% in 427 isolations carried out in Baja California (Peter and Moser, 1998). In the year 2000, the National Institute for Respiratory Diseases reported an ascending tendency in secondary resistance between the periods 1994-1997 and 1997-2000 of 13.0 and 15.8%, respectively (Olvera, 2001). The Laboratory of Public Health in Baja California reported that from January 1, 1998 to April 31, 1999, a resistance to at least one anti-tuberculosis pharmaceutical was found in 41% of the patients and 17% of those were MDR, reason for which Laniado-Laborin has planted the necessity to regionalize the diagnostic laboratories for TB with the purpose of facilitating the realization of regional studies and optimizing the diagnostic time and

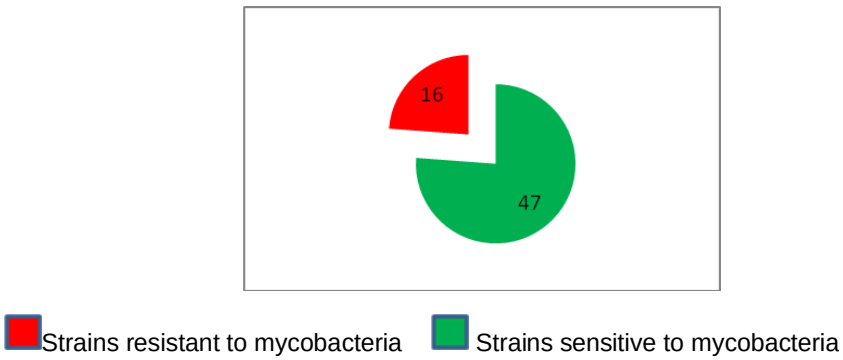

Figure 2. Levels of primary resistance in 64 new cases without prior treatment.

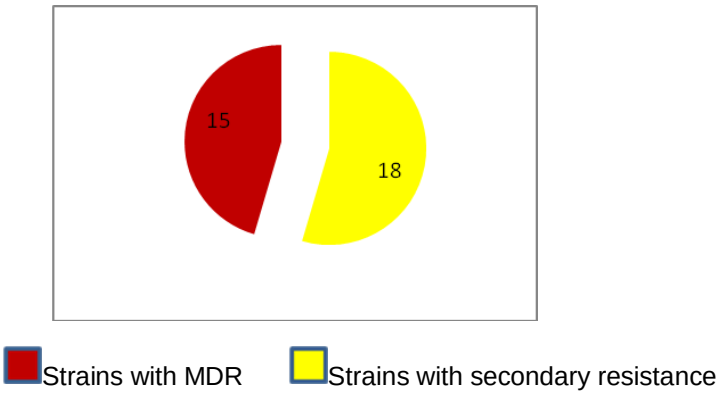

Figure 3. Levels of secondary resistance in 33 cases with prior treatment where a therapeutic flaw was present.

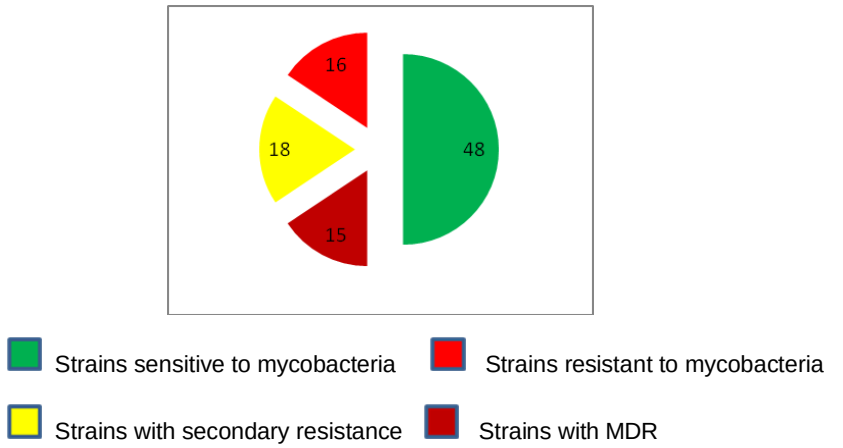

Figure 4. General outline of the drug resistant levels by the Alamar Blue assay.

commencement of treatment (Laniado-Laborin, 2000). The levels of resistance to drugs were obtained by the MABA method as shown in Figures 2, 3, and 4. The direct bacilluscopy allows for a BAAR reading by using the ZN staining process. Moreover, with the bacilluscopy the mycobacterian nature of the colonies obtained in selective culture media for *Mycobacterium* was confirmed. The results obtained in each bacilluscopy allowed us to have a more precise idea of TB in our region, the reason for this being that the majority of the

clinical samples contained a considerable bacilar charge (2+ and 3+). This data suggests a higher probability of finding mutant strains. The combination in the culture samples in the three selective media used to obtain the respective strains of mycobacterias, agar 7H11, stock 7H9, and the conventional L-J culture media allowed for a higher recuperation than that obtained with only one media, with the media the major recuperation in the *Mycobacterium* strain and in a shorter incubation period was that of stock 7H9, followed by the culture in the agar thin layer 7H11. The media for agar thin layer 7H11 allowed us to establish the purity of the culture and to assign a presumptive diagnosis of TB by observations using an optic microscope and the comparison of *Mycobacterium* colonies established in previous studies. Furthermore, a reliable result was obtained in less time than that considered in a conventional culture. The determination of the sensibility to the anti-TB pharmaceuticals: INH, RIF, SM, and EMB, in isolated *M. tuberculosis* strains from patients with TB in the Laguna Region, was successful by applying the MABA, with high sensitivity and specification. That is why this essay, firstly, permutes with the cultures previously carried out, the opportune diagnosis of TB, and secondly, concede to the precise treatment with the determination of the resistance and MDR to the specific medications for this disease. The levels of primary and secondary resistance were determined, as well as the MDR in the strains obtained from clinical samples. The levels of primary and secondary resistance, as well as the MDR were higher than those reported by other authors. This data corroborates the high impact and prevalence of TBP cases. This information provides regional epidemiologic information for TB cases, providing important data to the epidemiologist in the health ministry, with the purpose of improving the attention given to this ailment and in this way prevents the elevated resistance level and MDR as shown in the study. The implementation of the MABA diagnostic technique will give us the opportunity to the Regional Reference Laboratory in our Region, due to the fact that the Federal Government, through CENAVECE InDRE, in Health Project -2004-01-089, contemplate the MABA as a strategy for the control and resistance prevention and MDR carrying out studies at a regional laboratory level in different states of the Mexican Republic.

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REFERENCES

Bhatt A, Fujiwara N, Bhatt K (2009). Deletion of kasB in Mycobacterium tuberculosis causes loss of acid-fastness and subclinical latent tuberculosis in inmunocompetent mice. Harvard School of Public Health, 41: 681-690.

Bonilla CA, Crossa A, Jave HO, Mitnick CD, Jamanca RB, Herrera C, Asencios L, Mendoza A, Bayona J, Zignol M (2008). Management of extensively drug-resistant tuberculosis in Peru: cure is possible. PLoS One, 3: e2957.

Bonini PR, Langoni H, Pereira MLJ, Dahr CK (2001). Isolation of M*ycobacterium* spp. in milk from cows suspected or positive to tuberculosis. Braz. J. Vet. Res. Anim. Sci., 38: 284-287.

Collins L, Franzblau SG (1997). Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob. Agents Chemother., 41: 1004-1009.

Chauca JA, Palomino JC, Guerra H (2007). Evaluation of the accuracy of the microplate Alamar Blue assay for rapid detection of MDR-TB in Peru. Int. J. Tuberc. Lung Dis., 11: 820-822.

Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM (1998). Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J. Clin. Microbiol., 36: 362-366.

Granich RM, Balandrano S, Santaella AJ (2000). Survey of drug resistance of *Mycobacterium tuberculosis* in 3 Mexican States, 1997. Arch. Intern. Med., 160: 639-644.

Gumbo T, Louie A, Liu W, Ambrose PG, Bhavnani SM, Brown D, Drusano GL (2007). Isoniazid's bactericidal activity ceases because of the emergence of resistance, not depletion of *Mycobacterium tuberculosis* in the log phase of growth. J. Infect. Dis., 195: 194-201.

Kibiki GS, Mulder B, Dolmans WM, de Beer JL, Boeree M, Sam N, van Soolingen D, Sola C, van der Zanden AG (2007). M. tuberculosis genotypic diversity and drug susceptibility pattern in HIV-infected and non-HIV-infected patients in northern Tanzania. BMC Microbiol., 7: 51.

Kumar M, Khan IA, Verma V, Qazi GN (2005). Microplate nitrate reductase assay versus Alamar Blue assay for MIC determination of *Mycobacterium tuberculosis*. Int. J. Tuberc. Lung Dis., 9: 939-941.

Laniado-Laborin R (2000). Urge regionalizar los laboratorio de salud pública de México. Salud Pública de México., 42: 171-177.

Luna-Herrera J, Costa MC, Gonzalez GH, Rodriguez AI, Castilho PC (2007). Synergistic antimycobacterial activities of sesquiterpene lactones from Laurus spp. J. Antimicrob. Chemother., 59: 548-542.

Mejia GI, Castrillon L, Trujillo H, Robledo JA (1999). Microcolony detection in 7H11 thin layer culture is an alternative for rapid diagnosis of *Mycobacterium tuberculosis* infection. Int. J. Tuberc. Lung Dis., 3: 138-142.

Olvera CR (2001). Farmacorresistencia secundaria en tuberculosis. Tendencia en el Instituto Nacional de Enfermedades Respiratorias. Rev. Inst. Nal. Enf. Resp. Mex., 14: 151-159.

Peter CRES, Moser K (1998). Drug-resistant pulmonary tuberculosis in the Baja California-San Diego County border population. West J. Med., 169: 208-213.

Ritacco V, Lopez B, Cafrune PI, Ferrazoli L, Suffys PN, Candia N, Vasquez L, Realpe T, Fernandez J, Lima KV (2008). *Mycobacterium tuberculosis* strains of the Beijing genotype are rarely observed in tuberculosis patients in South America. Memorias do Instituto Oswaldo Cruz., 103: 489-492.

Rodríguez DJ (1995). Norma oficial mexicana, NOM-006-SSA2-1993, Para la prevención y control de la tuberculosis en la atención primaria a la salud. Diario oficial. Primera sección. 20. Dirección general de medicina preventiva. Secretaría de Salud., pp. 1-12.

Sifuentes OJ, Ponce de León A, Camacho MFE (1995). Resistencia de Mycobacterium tuberculosis en pacientes mexicanos: Características clínicas y factores de riesgo. Rev. Invest. Clin., 47: 273-281.

Traore H, Ogwang S, Mallard K, Joloba ML, Mumbowa F, Narayan K, Kayes S, Jones-Lopez EC, Smith PG, Ellner JJ (2007). Low-cost rapid detection of rifampicin resistant tuberculosis using bacteriophage in Kampala, Uganda. Ann. Clin. Microbial. Antimicrob.,

6: 1.
Vera-Cabrera L, Rendon A, Diaz-Rodriguez M, Handzel V, Laszlo A (1999). Dot blot assay for detection of antidiacyltrehalose antibodies in tuberculous patients. Clin. Diagn. Lab. Immunol., 6: 686-689.
Wirth T, Hildebrand F, Allix-Beguec C, Wolbeling F, Kubica T, Kremer K, van Soolingen D, Rusch-Gerdes S, Locht C, Brisse S (2008). Origin, spread and demography of the *Mycobacterium tuberculosis* complex. PLoS Pathog., 4: e1000160.