

## Diagnosis and Drug Susceptibility of *Mycobacterium tuberculosis* from Pulmonary Specimens at Pasteur Institute of Algeria: Comparative Study between Classic Lowenstein-Jensen Culture and *BACTEC MGIT 960* System

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### Abstract

**Background:** Tuberculosis is an old infectious disease and the causative agent is *Mycobacterium tuberculosis* complex. The direct diagnosis stills long and fastidious since microscopic examination, with Ziehl-Neelsen (Z-N) staining, even fast, lacks sensitivity. The culture on Lowenstein-Jensen (L-J), a reference method, sometimes takes up to ten weeks to obtain the result. In order to compensate the slow growth of cultures on solid media, new automated methods have been developed, including *BACTEC MGIT 960*, *VERSA TREK*, *MBREDOX*, *BACTEC 460*, allowing early diagnosis and drugs susceptibility testing, in addition to their good sensitivity and specificity.

**Materials and Methods:** The aim of this study is to verify the contribution of *BACTEC MGIT 960* in the diagnosis of pulmonary tuberculosis, compared to microscopic examination and culture on L-J medium, at the tuberculosis and mycobacteria unite in Pasteur Institute of Algeria. Nine hundred and fourteen specimens were collected between January 2016 and April 2017. One hundred and seventy nine reported positive L-J culture and/or *BACTEC MGIT 960*.

**Results and Discussion:** Among the 179 cases, 155 were detected by the *BACTEC MGIT 960* and confirmed by Ziehl control, L-J sub-culture and MPT64 immuno-chromatographic assay. On classic culture and Z-N staining, nevertheless, only 123 and 95 specimens respectively were positive. These results confirm the high susceptibility of *BACTEC MGIT 960* in improving the diagnosis of tuberculosis in bacilli-poor specimens, compared to classic culture ( $p = 0.037$ ) and direct examination ( $p = 0.014$ ). Contamination rate was higher in L-J culture: 81/914 (8.86%), including 7 microscopic examination positive cases, whereas, in *BACTEC MGIT 960*, only 29/914 (3.17%) specimens were contaminated, with no positive microscopic examination cases.

**Conclusions:** The main advantage of *BACTEC MGIT 960* is its ability to shorten the time of growth to an average of 7 days, compared to the solid medium. Nevertheless, there is an incompressible risk of contamination. Bacilloscopy and L-J culture remain complementary to this automat for a reliable diagnosis.

**Keywords:** Tuberculosis; *BACTEC MGIT 960*; Microscopic Examination of Ziehl-Neelsen (Z-N) Staining Lowenstein-Jensen Culture

### Abbreviations

AFB: Acid Fast Bacilli; B+: Positive Bacilloscopic; EPT: Extra-Pulmonary Tuberculosis; IPA: Pasteur Institute of Algiers; L-J: Lowenstein-Jensen; MDR: Multi Drug Resistant; MGIT: Mycobacteria Growth Indicator Tube ; PT: Pulmonary Tuberculosis; TB: Tuberculosis; WHO: World Health Organization; XDR: Extensively Drug Resistant; Z-N: Ziehl-Neelsen

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## Introduction

Tuberculosis is a very old infectious disease. The etiological agent of this pathology, *Mycobacterium tuberculosis*, was discovered in the 19<sup>th</sup> century by Robert Koch. The tubercle bacilli infected the first hominids and co-evolved with them [1,2]. This infection usually begins with inhalation of contaminated droplets emitted by ill individuals, with a very low minimum infective dose, ranging from 1 to 10 bacilli [3]. According to the World Health Organization (WHO), nearly one-third of the world's population is infected with tubercle bacilli. In 2015, 87% of new cases occurred in 30 countries with high TB burden. Six countries accounted for 60% of new cases: India, Indonesia, China, Nigeria, Pakistan and South Africa. The incidence of this disease has declined by an average of 1.5% per year since 2000 and the evolution of its diagnosis and treatment saved 49 million lives between 2000 and 2015 [4]. Control of the disease begins with the identification of *M. tuberculosis* and the development of detection tools, including X-rays and tuberculin test.

Algeria, erstwhile considered as a country with high prevalence of tuberculosis, joined since early 1980s, the group of countries with moderate prevalence. The annual incidence of tuberculosis in all forms is about 20 to 99 cases per 100 000 inhabitants (PNLCT, 2011). In 2015, the number of TB cases reported in the country, reached 23,379, among them, 8197 (35.1%) of pulmonary tuberculosis (PT) and 15,174 (64.9%) extra-pulmonary (EPT) [4]. This change is due to the old age of tuberculosis in the country, which is observed in other countries, where tuberculosis is aging, rate of EPT is increasing compared to PT. This is known as the awakening of tuberculosis in EPT, in addition to poor management and misdiagnosis of EPT. A national survey is underway to address this high EPT problem.

Anti-tuberculous therapy must be bactericidal, which requires the combination of several molecules that can radically reduce the TB mortality rate (Meyssonier, 2012). However, the cell wall of *Mycobacteria* is the main cause of natural resistance to several drugs. Mainly, its weak permeability, efflux systems, inactivating enzymes and lack of affinity for the target (Jarlier and Nikado, 1994). In addition to its natural resistance, *M. tuberculosis* can acquire chromosomal point mutations, which can affect either the target of anti-tuberculous drug or the enzymes involved in drug activation. The appearance of spontaneous resistant strains is due to non-respect and inadequate treatment. After selection of resistant bacilli, patients can contaminate their surroundings and new subjects can be infected by the resistant bacilli (Gillespie, 2002).

The diagnosis of tuberculosis has always been hampered by the long and tedious processes involved in the demonstration of the pathogen. Direct microscopic examination, although easy to perform, lacks sensitivity. The culture, which remains of reference and of good sensitivity, sometimes takes up to ten weeks to obtain the result. To compensate the slowness of culture on solid media and the lack of sensitivity of direct examination, new methods have been developed, based on the use of a liquid medium, allowing an earlier growth and more adapted for antibiotic susceptibility [5,6]. The development of new automated techniques based on the use of liquid media, which confers a remarkable time saving, in addition to their well-defined sensitivities and specificities, among them: *BACTEC MGIT 960*, *Versa TREK*, *BACTEC 460* ... etc.

As direct microscopy, culture can confirm the diagnosis of tuberculosis. Egg-based Löwenstein-Jensen solid medium is the most widely used because of its high sensitivity, and the typical appearance of *M. tuberculosis* colonies. In the first culture, *M. tuberculosis* colonies grow on average between 21 and 28 days [7]. Nevertheless, given this delay, other means were developed. These new methods are applied to the use of several non-radioactive automated systems in liquid media, including Mycobacterial growth tube method (*BACTEC MGIT 960*). This method consists of tubes containing 7 ml of modified Middlebrook 7H9 broth. It is based on the presence of a ruthenium (fluorescent substance) at the bottom of the tube. The intensity of violet fluorescence increases as the oxygen concentration decreases, induced by bacterial multiplication (Piersimoni, *et al.* 2006). The *BACTEC MGIT 960* system uses a standard commercial decontamination method including 2% NaOH N-acetyl-L-cysteine and antibiotics (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim and Azlocillin) to reduce the risk of contamination (Eduardo, *et al.* 2016). With this automatic method, the average growth time is reduced to 12 days. The BacT/Alert method is another automated technique based on the acidification of the medium, caused by the bacterial metabolism which causes the color turn of the pellet contained in the bottom of the bottle (Gravet, *et al.* 2011). Versa TREK method is also an advanced method that detects bacterial growth through pressure sensors, in the upper part of a closed vial, monitoring changes in the production or consumption of gas due to microbial growth (Gravet, *et al.* 2011; Jabri, *et al.* 2016). MBRedox is a commercial technique in the form

of a 5ml tube of Kirchner's medium containing a colorless tetrazolium salt in an oxygen medium. It turns into formazan, red in a reduced atmosphere. The presence of a culture of *M. tuberculosis* results in the appearance of red-purple grains. Growth time gain relative to Löwenstein-Jensen medium appears to be less important with other liquid media.

### **Aim of the Study**

The aim of this study is to verify the contribution of *BACTEC MGIT 960* in the diagnosis of pulmonary tuberculosis, compared to classic culture on L-J medium, at the Tuberculosis and Mycobacteria unit in Pasteur Institute of Algeria.

### **Material and Methods**

#### **Setting and ethical considerations**

The laboratory of tuberculosis and mycobacteria at Pasteur Institute of Algiers (IPA) occupies a key place in the fight against tuberculosis in Algeria, in addition to being the national reference laboratory for the diagnosis and anti-tuberculosis drug testing, it is implicated in supervision, monitoring and reporting results across the entire network of national laboratories involved in the diagnosis of tuberculosis. It is also a supranational laboratory cooperating with WHO for the Africa region.

Oral consents were obtained from all patients prior to specimens collection, and ethical considerations were taken into account during all steps of the study. The patient's data and results were maintained in secure database.

#### **Materials and procedures**

Three types of pulmonary samples were included in the study: expectoration, gastric tubing and bronchial aspiration. The collection of these samples was done in clean spittoons. Each sample sent to the laboratory was accompanied with information sheet of the patient. The samples were stored at + 4°C and Z-N staining were directly performed. Poly-microbial samples subjected to prior decontamination before they were cultured, and Petroff's decontamination technique was used, without neutralization. For each sample, 2 tubes of Lowenstein-Jensen were inoculated. Before inoculating the MGIT tubes, samples were processed using the *BBLMycoPrep* kit containing a mixture of N-acetyl-L-cysteine and 2% sodium hydroxide (NALC-NaOH), following the Kubica's Protocol. Positive tubes in *BACTEC MGIT 960* were systematically inoculated on Lowenstein-Jensen media and Ziehl-Neelsen staining was performed for each tube. Finally, a rapid identification with immuno-chromatographic TBC ID was applied.

#### **Antibiogram on solid medium, proportions method**

The colonies (about 1 mg) are placed in a 50 ml flask containing glass beads, stirred for 10 min, in order to dissociate the colonies. From the initial suspension and dilution  $10^{-2}$ , two L-J tubes containing, each, one of the first line anti-tuberculosis drugs: Isoniazid (0.2 mg/ml), streptomycin (4 mg/ml), rifampicin (40 mg/ml) and ethambutol (2 mg/ml), were inoculated. For each sample, two single L-J tubes (without antibiotics) were inoculated as controls. L-J containing specific media tubes (TCH, PNB and PAS), for confirmation of identification, were inoculated with the initial suspension. First verification of tubes, after incubation at 37°C, was done the 28<sup>th</sup> day, a second and final lecture at the 42<sup>nd</sup> day. After counting of colonies number on both types of tubes, a proportion ratio between the number of colonies with antibiotics and the number of colonies on the control tubes is expressed as a percentage. Below 1% "critical proportion", the strain is sensitive, above or equal to 1%, it was considered as resistant. MDRs isolates were submitted to a second antibiogram, against Ofloxacin and Kanamycin, to verify the existence of XDR isolates.

#### **Antibiogram on liquid medium, BACTEC MGIT 960**

The first day of the positivity of a MGIT culture tube is considered as day "D-0". Inocula for this antibiogram should be prepared between "D-1" and "D-5". Five tubes were labeled for each isolate to be tested, a growth control tube and 4 tubes containing the antibiotics; streptomycin (1 µg/ml), Isoniazid (0.1 µg/ml), rifampicin (1 µg/ml), ethambutol (5 µg/ml). Reading is done automatically every 60 minutes, the sensitivity test is recorded between 5 to 12 days, and the result is obtained in the form of a printed report.

### Statistical analysis

Statistical analysis was performed to calculate frequencies and significant differences, using one-way ANOVA test or Kruskal-Wallis, when appropriate, or by *chi*-square and Fisher's exact test respectively. Association between variable under consideration were evaluated using contingency tables, and all reported *p*-values were two-sided. *p* < 0.05 was considered significant.

### Results and Discussion

During the last decades there has been a significant upsurge in the incidence of TB infections. It is obvious that the increase of the favorable factors to this disease as well as the emergence of multi-drug resistant strains, are major health problems for the international community, whether, in developed or developing countries [8]. This alarming situation has led to research and development of a more rapid and efficient means of detection and treatment, with the aim of reducing mortality rates. The race against this disease is justified, among other things, by the fact that it is the most deadly, in front of AIDS in 2014 [4]. Some studies have shown that the combination of the liquid and solid medium is an essential principle for the diagnosis of tuberculosis [9,10]. Nevertheless, in most African laboratories, this combination is rarely available because of the high costs of liquid media. As a result, only the solid medium (L-J) is used for culture and susceptibility testing [11].

Among the automated mycobacteria culture systems, *BACTEC MGIT 960* has demonstrated good sensitivity and excellent ability to shorten growth detection time [12]. To date, a number of studies have been conducted worldwide on the efficacy of TB diagnosis by this system on clinical specimens [13,14]. The objectives of our study are to verify the efficacy of this system in detecting *M. tuberculosis* from pulmonary specimens and its susceptibility and reliability in anti-tuberculosis drugs testing.

During a 16-month period, 914 samples collected by the Pasteur Institute of Algeria were cultured, and 179 (19.58%) were positive. Of these 179 strains, 68.71% were detected on L-J medium and 86.59% on MGIT, a difference of 17.88%. This result is confirmed statistically (*p* = 0.037). With *BACTEC MGIT 960*, only 29/914 (3.17%) specimens were contaminated, compared to L-J medium (*p* < 0.0001). The synchronous use of MGIT and LJ increases the sensitivity of the culture method, as described in the studies by Lee and Hassan and their collaborators, who achieved rates of 10.65% and 23.8% respectively [15]. Unlike the solid medium, the liquid medium has the advantage of recovering the bacteria present in the sample even at a reduced number, but also the bacteria stressed by the treatment preceding the culture [11]. Nevertheless, some other studies found no significant difference between the two methods [8,10,16,17].

The most important step in the diagnosis of tuberculosis is the differentiation between *M. tuberculosis* and other species. The first cultures obtained on MGIT are identifiable by a Zielh control, immuno-chromatographic test MPT-64, and a possible transplanting on L-J medium. For this purpose, the Center for Disease Control (CDC) recommends the use of a combination of liquid and solid medium for better performance [18-20].

Pfyffer and his collaborators have shown that the combination of two liquid media does not improve the culture yield significantly, compared to the liquid-solid medium combination [21].

From a diagnostic point of view, 10.39% (95/914) of the isolates are bacilloscopic positive (B+). Of the 179 isolates detected positive on culture, 49.72% (89/179) were to B+ and 50.27% (90/179) to B-. The discrepancies between the positive direct examination and the negative culture are due, on the one hand, to the fact that the observed AFB cannot be cultivated, on the other hand, to the problems of delay of routing as well as the conservation of the samples which normally should be maintained at + 4°C [7]. For negative direct examination results that are positive in cultures, they are due to the fact that there must be 5,000 to 10,000 bacilli per milliliter of sample to allow the detection of bacilli, so much so that culture can detect only 10 bacilli per milliliter [22]. MGIT showed good sensitivity by isolating 22.91% (41/179) of the strains from B- samples, which thus escaped diagnosis by sputum smear and culture on L-J, while L-J detected 8.38% (15/179).

Although diagnosis, chemotherapy, vaccination are available, TB is far from to be eradicated, due to several factors, such as antibiotic resistance and ineffective management of TB by the public health facilities [23-25]. Antibiotics are probably the most important factors in minimizing the spread of contagion. The phenotypic study of the resistance of *M. tuberculosis* to these anti-tuberculous drugs remains

the method of choice, the sensitivity tests in liquid media is currently the most suitable method at the laboratory level, the advantage of accelerating the reports of the results [26].

Of the 83 interpretable results, a concordance was observed on 61 strains, of which 58 were sensitive and 3 resistant, at least to one antibiotic, by the two methods (73.49%). The absence of a statistically significant difference shows that both media have the same overall value. Even if the concordance rate is lower than those reported by Cambeau and his collaborators (1996) who obtained 81% concordance, Gerome and his collaborators [27] 89%, while Bergmann and his collaborators (1997) recorded a concordance rate of 93.2% between the MGIT and L-J medium. According to the studies conducted by Palaci [28], Reisner [29], Walters [30] and their collaborators, a 100% concordance in testing rifampicin, was obtained, contrary to the results of our study, a rate of 92, 77% concordance with 73 susceptible strains, and 4 resistant ones. A concordance rate of 85.54% is obtained for isoniazid in both media. Despite this, a statistically significant difference was observed for the results obtained by the two methods ( $P = 0.046$ ). Zapata [31] and Birinci [32] and their collaborators reported a good correlation for streptomycin, rifampicin and ethambutol, but a lower correlation for isoniazid (Table 1).

Anti-tuberculosis drugs	MGIT liquid medium				L-J solid medium			
	Streptomycin	Isoniazid	Rifampicin	Ethambutol	Streptomycin	Isoniazid	Rifampicin	Ethambutol
58	S	S	S	S	S	S	S	S
1	R	R	R	S	R	R	R	S
2	S	R	S	S	S	R	S	S
5	S	R	S	S	S	S	S	S
1	S	S	R	S	S	S	S	S
1	S	S	S	S	S	R	S	S
2	S	R	R	S	S	S	S	S
1	S	S	S	S	R	S	S	S
2	S	S	S	S	S	R	R	S
1	R	R	R	S	S	S	S	S
1	R	R	S	R	R	R	S	S
1	R	R	S	R	S	S	S	S
4	R	S	S	S	S	S	S	S
1	R	R	R	S	R	R	R	R
2	R	R	R	R	S	R	R	S

**Table 1:** Comparative study of the susceptibility testing of *M. tuberculosis* to the four first-line anti-tuberculosis drugs, tested by two L-J solid medium and MGIT system, at Pasteur Institute of Algiers.

According to Walters and Hanna [29], the MGIT has the accuracy of the BACTEC 460 TB ratio and speed method, studies currently under way with streptomycin, isoniazid and pyrazinamide indicate that MGIT can be an alternative appropriate to these methods. Rusch-Gerdes, *et al.* [33] reported rates of 1.9% for ethambutol and 0.9% for streptomycin, while Bergmann and Associates (1997) reported rates of 9.5% and 6.8% In our study rates of 6.02% and 10.84% were respectively obtained. According to Rusch-Gerdes and his collaborators (1999), two elements can explain these results, on the one hand the heterogeneity of resistance to ethambutol, which groups together a high level resistance (MIC > 20 µg/ml) associated with mutations of the gene *embB*, and low level (MIC < 10 µg/ml). On the other hand, the critical concentrations chosen for the method of proportions or methods derived from them are different according to the techniques [34]. The reason for the discrepancies between the results of the MGIT and the proportion method was unclear, however the lack of calibration of the inoculum and the representation of *M. tuberculosis* populations in the MGIT was the possible main reason according to

Fang-Lan and his collaborators [35]. Birinci, *et al.* [31] suggest using *BACTEC MGIT 960* routinely instead of the Solid Proportion Method, given the shorter average time to obtain results [36].

## Conclusion

The main advantage of *BACTEC MGIT 960* is its ability to shorten the time of mycobacterial growth to an average of 7 days, compared to the solid medium. Nevertheless, the bacilloscopy and culture on L-J remains complementary to this automat, for a reliable diagnosis. Despite the good laboratory practices, there is an incompressible risk of contamination.

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## Conflicts of Interest

All authors declare no conflicts of interest.

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