

Full Length Research Paper

***Mycobacterium tuberculosis* complex identification by polymerase chain reaction from positive culture in patients from Jamot and Mbalmayo district hospitals**

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Tuberculosis (TB) is a contagious and infectious disease of which the causative agents are mycobacteria. Up to one third of the world's population is infected by these bacteria. Its control and follow up treatment are achieved by the use of antituberculosis drugs after tests and diagnosis. The last two decades have been marked by the development of new techniques for detection and characterization of *Mycobacterium tuberculosis* complex (MTBC). Conventional tests to differentiate MTBC from non tuberculosis mycobacteria (NTM) such as Para nitro benzoic acid (PNB) inhibition tests are often time consuming. This study aim at using PCR amplification of specific markers (*hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB*) for more rapid detection of MTBC in positive cultures. The study was conducted in Jamot Hospital, the largest urban treatment center for tuberculosis in Cameroon and in Mbalmayo District Hospital, a small rural district hospital. Mycobacterial culture was performed on all smear positive sputa. All positive cultures were subjected to drug susceptibility testing (DST) using the indirect proportion method. On the same way, MTBC were differentiated from other mycobacteria using the PNP inhibition test. DNA extracted from positive cultures was subjected to PCR amplification using specific primers (*hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB*). Analysis of PCR products was done by agarose gel electrophoresis. A total of 79 smear positive pulmonary tuberculosis patients were enrolled at the two sites. Drug susceptibility carried out showed that among the samples analyzed, 68 (86.08%) were susceptible to all TB drugs tested, while 11 (13.92%) were resistant to at least one of them. Resistance to streptomycin was the most frequent (8.86%), followed by resistance to isoniazid (5.06%). Identification by PCR using specific markers as *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* revealed that the mycobacterium strains belonged to the MTBC. In short, identification by PCR using these specific markers revealed that mycobacterium species responsible for pulmonary tuberculosis in patients from Jamot and Mbalmayo District Hospital belonged to the MTBC. Also PCR technique is more rapid compared to the PNP inhibition test.

Key words: *Mycobacterium tuberculosis* complex, polymerase chain reaction (PCR), Cameroon.

INTRODUCTION

Tuberculosis (TB) remains a major global health problem caused mainly by *Mycobacterium tuberculosis*. It causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (including 320 000 deaths among HIV-positive people). The probability of developing TB is much higher among people infected with HIV. The number of TB deaths is unacceptably large given that most are preventable. The rate of new TB cases has been falling worldwide for about a decade, achieving the millennium development goal global target. TB incidence rates are also falling in all six WHO regions. The rate of decline (2% per year) remains slow. An estimated 1.1 million (13%) of the 8.6 million people who developed TB in 2012 were HIV-positive. About 75% of these cases were in the African Region (WHO, 2013). The majority of cases worldwide in 2012 were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26 and 12% of total cases, respectively (WHO, 2013).

Cameroon is an independent nation situated in the Central Africa. Tuberculosis (TB) has emerged as a major public health problem in Cameroon and other border countries due to economic decline and the general failure of TB control, and other health services following the economic crisis in the year 1980s. The current incidence of TB in Cameroon has been reported to be about 238 cases per 100 000 population (WHO, 2013). The emergence of multidrug-resistant TB (MDR-TB) has also been documented in Cameroon. According to the National TB Control Programme (NTBCP) in 2012; 25, 576 new sputum smear-positive were reported among all TB cases despite the implementation of the directly observed treatment short course strategy (DOTs) by the NTBCP in 2003 (WHO, 2013).

Tuberculosis is an infectious disease caused by a mycobacterium belonging to *M. tuberculosis* complex (MTBC) which includes: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti* (Singh et al., 2006). Identification of *M. tuberculosis* complex is performed routinely by conventional techniques taking into account cultural (growth rate, colonies morphology and pigmentation) drug susceptibility testing (DST) and biochemical characters. Conventional methods for differentiation of MTBC are time consuming thus, it is imperative to develop and implement a rapid technique that is able to

identify the MTBC.

Advances in molecular biology and knowledge on the genome of *M. tuberculosis* complex have enabled the development of faster identification techniques. Molecular methods like PCR are increasingly used for rapid laboratory diagnosis of TB; different target sequences have been evaluated for detection of MTBC/differentiation between MTBC and NTM (van Embden et al., 2000). In the present study, a combination of 5 targets sequences (hupB, IS6110, IS1081, oxyR, rpoB) was used to detect MTBC in positive cultures by PCR and agarose gel electrophoresis. The five targets were used to see the results are identical to those of the conventional methods and conclude that each of the markers can be used to differentiate the MTBC to other mycobacteria.

MATERIALS AND METHODS

Study setting and design

This was a cross-sectional study involving all pulmonary smear positive patients, age ≥ 15 years attending Jamot and Mbalmayo District hospitals from April to June 2010. All patients underwent physical examination and their histories were recorded. The chosen study sites was Jamot and Mbalmayo District hospitals. All the sputa collected from the enrolled patients were evaluated for acid fast bacilli (AFB). Sample processing, confirmatory microscopy, culture, drug susceptibility testing and quality control were performed at the Mycobacteriology Laboratory of the Centre Pasteur of Yaoundé (Cameroon).

A study questionnaire was designed for patient data collection at each study site including sex, age, marital status, educational level, residence (urban vs. rural) and clinical data (previous TB treatment). The study was funded by Central Africa Network for Tuberculosis, AIDS/HIV and Malaria (CANTAM) a network sponsored by the European Developing Countries Clinical Trials Partnership (EDCTP).

Ethical clearance and administrative authorization

Ethical clearance N° 126/CNE/SE/09 and an administrative authorization N° 631. 7-10 were respectively obtained from the Cameroon National Ethic Committee and the Cameroonian Ministry of Public Health. Signed informed written consent was obtained from each enrolled patient.

Sample processing

Ziehl-Neelsen and/or auramine smear examinations were performed at the recruitment sites (Soini and Musser, 2001). Only samples with the highest smear grade were transported in a cold box to the Centre Pasteur du Cameroun (CPC, Yaoundé) for confirmatory microscopy, culture and drug susceptibility testing (DST). Each specimen was submitted to a decontamination step

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Table 1. Characteristics of different primers.

Target gene	Primers	Primer sequence	Size (bp)	Reference
<i>hupB</i>	<i>hupBF</i>	5'GGAGGGTTGGGATGAACAAAGCAG-3'	645	Prabhakar et al. (2004)
	<i>hupBR</i>	5'GTATCCGTGTGTCTTGACCTATTTG-3'		
<i>IS6110</i>	<i>IS6110F</i>	. <i>IS6110F</i> 5'-CCTGCGAGCGTAGGCGTCGG-3	123	Eisenach et al. (1990)
	<i>IS6110R</i>	<i>IS6110R</i> 5'-CTCGTCCAGCGCCGCTTCGG-3 '		
<i>IS61081</i>	<i>IS61081F</i>	5'CGACACCGAGCAGCTTCTGGCTG-3'	306	Niyaz et al. (1998)
	<i>IS61081R</i>	5'GTCGGCACCACGCTGGCTAGTG-3'		
<i>oxyR</i>	<i>OxyRF</i>	5'-GCGTCTGGTCGCGTAGGCAGTG-3'	473	Mokaddas et al. (2007)
	<i>OxyRR</i>	5'GTGAAGTAGTCGCCGGGCTGCT-3'		
<i>rpoB</i>	<i>rpoBF</i>	5'TACGGTCGGCGAGCTGATCCAAA-3'	235	Mokaddas et al. (2007)
	<i>rpoBR</i>	5'ACAGTCGGCGCTTGTGGGTCAAC-3'		

using lauryl sulfate sodium. Quality control procedures (internal and external) were conducted at the mycobacteriology laboratory of the CPC.

Sputum culture and identification

After centrifugation, the sediment of the decontaminated product was used to inoculate two Löwenstein Jensen culture media, with an additional one supplemented with a 0.65% solution of sodium pyruvate. Cultures were incubated at 37°C, read weekly for growth and considered negative when no colony was obtained after 8 weeks of incubation. The PNB inhibition test was used to distinguish non tuberculous mycobacteria from *M. tuberculosis* complex.

Drug resistance assays

For all positive cultures, drug susceptibility testing was performed using the indirect proportion method on Löwenstein Jensen media at the following drug concentrations: isoniazid (H1: 1 mg/ml and H2: 2 mg/ml), streptomycin (S: 4 mg/ml), Rifampicin (R: 40 mg/ml), ethambutol (E: 2 mg/ml), ofloxacin (2 mg/ml), kanamycin (K1: 30 µg/ml and K2: 20µg/ml) (Canetti et al., 1963). Drug resistance was defined as growth on a drug containing medium greater than or equal to 1% for INH and RIF, and 10% for SM and EMB (Canetti et al., 1963).

DNA extraction

Colonies were scraped from *Lowestein-Jensen* slopes, collected into Eppendorf tubes containing Tris-EDTA (10 mM, 1 mM, pH 8) and heated for 30 min at 90°C. After centrifugation at 13,000x g, the supernatant was collected into a new tube and kept at -20°C until further use.

PCR amplification of target sequences and detection of PCR products by gel electrophoresis

The specific primers of markers *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* were used (Table 1). A total of 13 µL PCR mix per sample

was prepared with 6.25 µL of ready mix RedTaq, 5.625 µL of PCR water, forward and reverse primers of 0.125 µL in equal quantities and 1 µL of lysate. Distilled water was used as negative control and *M. tuberculosis* H37 Rv strain was used as positive control. The amplification according to each target sequence was done as follow:

For *hupB* and *rpoB* targets, initial denaturation was done at 95°C for 10 min, followed by 35 cycles at 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (extension), and a final extension at 72°C for 7 min.

For *IS6110* target, initial denaturation was done at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 63°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min.

For *IS1081* target, initial denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min were done.

For *oxyR* target, initial denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min were done.

After amplification, 5 µL of the amplicon were mixed with 15 µL of loading buffer for electrophoresis on 2% agarose gel prepared with ethidium bromide. Visualization was done under the ultraviolet transilluminator (BIO-RAD).

RESULTS

From April to June 2010, 79 smear positive pulmonary tuberculosis cases were enrolled at both study sites. Males represented 53.16% (42/79) of the case versus, 46.84% (37/79) for females. Patient's age ranged from 15 to 60 years.

Culture and phenotypic characteristics

All 79 smear positive samples yielded positive cultures as shown in Figure 6 after two weeks for some samples and one month for others. Then, PNB identification and Valid DST results were available for all the 79 strains after one month. All the above samples were sensitive to PNB. However, 68 strains (86.08%) were sensitive to the six



Figure 1. PCR amplicon profiles with *hupB* primers (100 bp, DNA ladder; Column 1, positive Control; Column, 2-15: Amplicons).

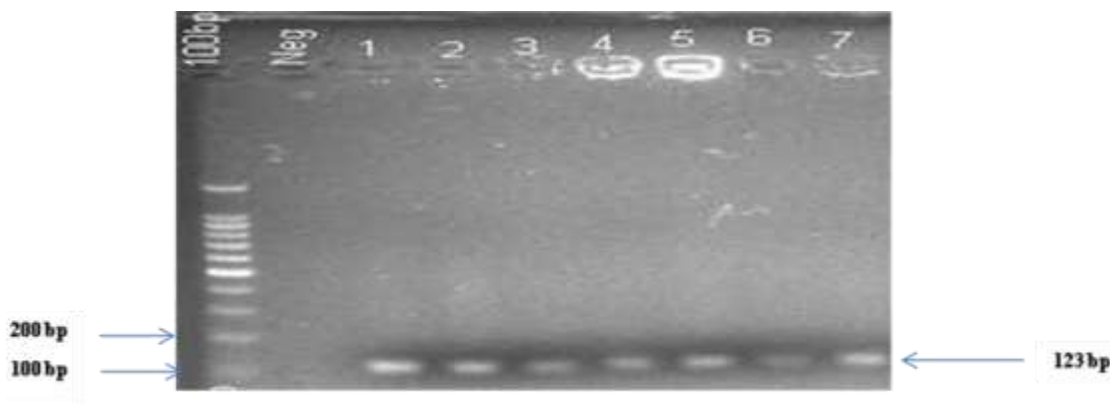


Figure 2. PCR amplicon profiles using *IS6110* primers (100bp: DNA ladder; Neg, négative Control; Column 1, positive Control; Column 2-7, Amplicons).

drugs tested against 11 strains (13.92 %) resistant to at least one drug. Resistance rates were highly observed respectively to streptomycin (8.86%) and isoniazid (5.06%). No resistance to rifampicin, ethambutol, ofloxacin and kanamycin was noted.

PCR amplification of target sequences *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB*

The results of the detection of PCR products by agarose gel electrophoresis have allowed visualization, for each primer, a band of different size and intensity. Thus, application of amplification's technique on *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* markers by PCR, followed by electrophoresis on 2% agarose gel allowed the identification of all samples tested. The target bands, specific for each primer of the *M. tuberculosis* complex were expressed in base pairs (bp) (Figures 1 to 5).

The expected sizes of PCR products were 645 bp for *hupB* after 122 min of amplification, 123 bp for *IS6110* after 102 min, 306 bp for *IS1081* after 102 min, 473pb for *oxyR* after 102 min and 235 bp for *rpoB*. PCR products from positive control and all target sequences obtained with all 79 samples had the sizes expected after an hour of electrophoresis. Nothing was obtained from negative controls. Thus, the analysis of 79 amplified samples by gel electrophoresis showed that all bands had the same base pair (bp) numbers according to each primer as shown in the Figures 1 to 5.

DISCUSSION

In countries with a high prevalence of tuberculosis, the majority of cases of pulmonary tuberculosis in adults are sputum smear-positive (Ait-Khaled and Enarson, 2003). From this study with 79 clinical evidences of pulmonary

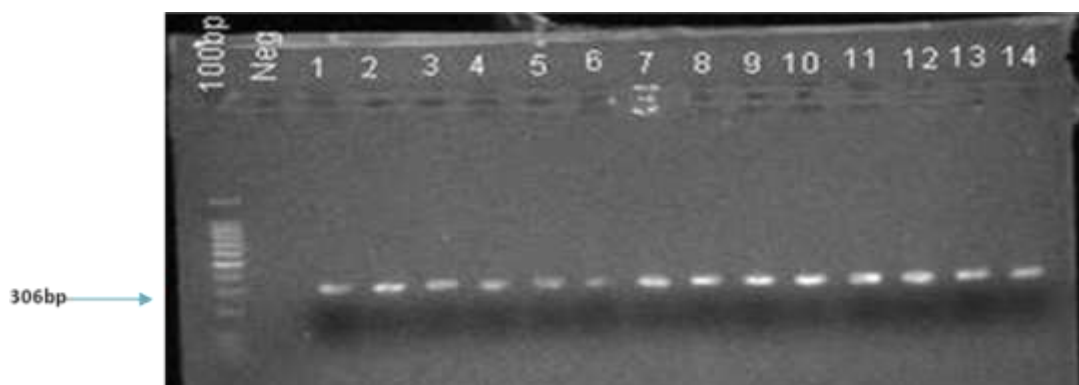


Figure 3. PCR amplicon profiles using *IS1081* primers (Neg, negative control; 1, positive control).



Figure 4. PCR amplicon profiles using *oxyR* primers (Neg, negative control; A, positive control; B-N, amplicons)

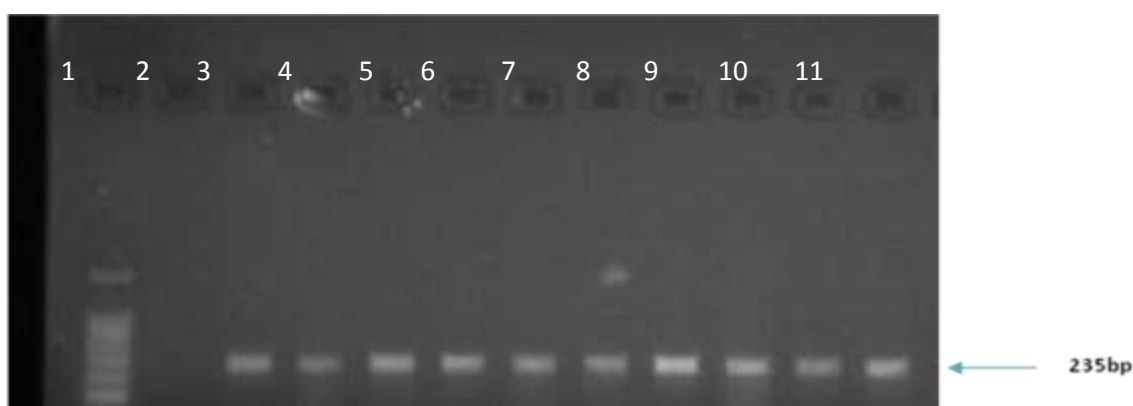


Figure 5. PCR amplicons profile using *rpoB* primers (100 bp, DNA ladder, Column 1, negative control; Column 2, positive control; 3-11, Amplicons)

tuberculosis confirmed, men were more infected 42/79 (53.16%) than women 37/79 (46.84%). The experience of the World Health Organization (WHO) since 1997 in

assisting high TB burden countries to set up standardised TB surveillance systems shows that globally, men account for a higher proportion of notified TB cases (The

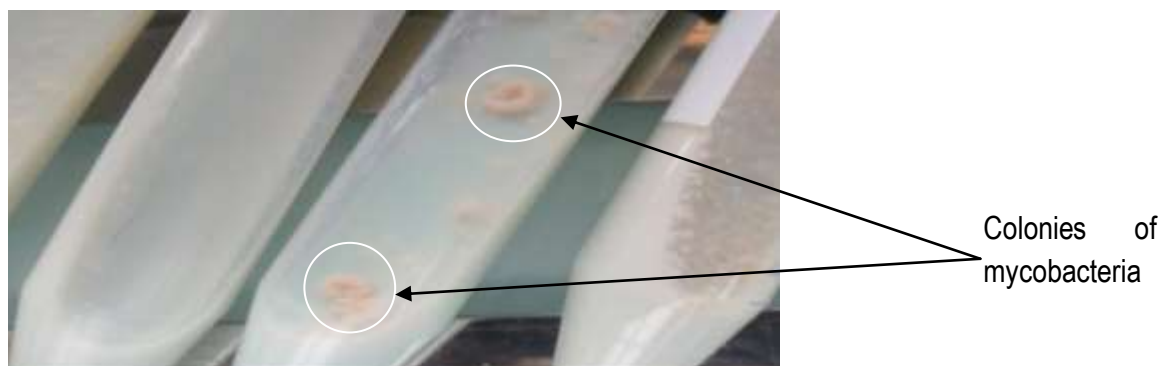


Figure 6. Aspects of some mycobacterial cultures growing.

Union, 2008). These results are similar to another recorded in South Africa which showed a high predominance of pulmonary TB in male (Dhedea et al., 2009). This is due to specific factors related to socio-cultural phenomena (Godfrey-Faussett et al., 2000) that contribute to the acceleration of the TB epidemic and in addition, the general factors such as poor health infrastructure, a poor organization and management, poverty, weak health systems and poor management of human resources.

For culture, growth of *M. tuberculosis* complex species was inhibited by p-nitrobenzoic acid (PNB), thus confirming their presence. However, non-tuberculous mycobacteria are resistant (Giampaglia et al., 2007).

The use of PCR amplification technique for the routine diagnosis of TB has been improved in recent years. The results and interpretation of PCR may be shortly summarized. The results obtained here are similar with respect to amplicon sizes to those of Prabhakar et al. (2004) for *hupB* (645 bp); of Eisenach et al. (1990) and Sekar et al. (2008) for *IS6110* sequence (123 bp); of Van Soolingen et al. (1992) and Bahador et al. (2005) for *IS1081* (306 bp); finally of Mokaddas et al. (2007) for *oxyR* (473 bp) and *rpoB* (235 bp).

Since *rpoB* has been published to have correlation between its mutations and rifampicin resistance (Halse et al., 2010) and used in differentiation of MTBC from non-tuberculosis mycobacteria, it is an essential gene that is uniformly present in all mycobacterial species. The chance occurrence of nearly identical sequences in the *rpoB* gene corresponding to MTBC-specific primers may result to misidentification of some non tuberculous mycobacteria as MTBC (Mokaddas and Ahmad, 2007).

For primer *oxyR*, the expected target characterizing the presence of *M. tuberculosis* complex species in all sample tested confirms the results reported by Mokaddas et al. (2007) who used the same primer to differentiate *M. tuberculosis* complex species of non-tuberculous mycobacteria and got the same band at 473 bp.

The insertion sequences for instance *IS1081* and *IS6110* were reported to be specific for *M.*

tuberculosis complex and hence are extensively exploited for laboratory detection of the agent of tuberculosis and for epidemiological investigations based on polymerase chain reaction.

Amplification of the fragment of the *IS6110*, which is (Das et al., 1995) specific for the *M. tuberculosis* complex, belongs to IS_3 family and is found in almost all members of the *M. tuberculosis* complex. Most strains of *M. tuberculosis* carry 10 to 15 copies of *IS6110*, this characteristic helps to increase sensitivity of PCR over that obtained in amplification of a single DNA sequence (Gill et al., 2012).

Despite the results with others primers in this study, PCR targeting *IS6110* has shown higher positivity than PCR for other targets. Also the methodology of PCR for *IS6110* has been widely carried out in different technical set up and has been proved to be simple and reproducible, compared to methodologies for PCR (Negi et al., 2007) targeting other gene sequences.

The similarity at points of migration on the gel with identical bands, 645 bp for *hupB* leads us to conclude that the species belong to *M. tuberculosis* complex. Knowing that the *IS6110* sequence identifies the *M. tuberculosis* complex and the *HupB* establishes the differences between *M. tuberculosis* and *M. bovis* (Prabhakar et al., 2004; Mishra et al., 2005), it is likely that *M. tuberculosis* is responsible for tuberculosis in patients involved in this study since MTB is the most common species and most frequently encountered in the Centre region of Cameroon (Assam et al., 2011). However to be sure, a study of genomic characterization using techniques such as line-probe assays should be required.

This study demonstrates the wide distribution of the *M. tuberculosis* complex species in the areas of Jamot and Mbalmayo District Hospitals. These results confirm the specificity of the PCR and the identity of all strains tested as species belonging to the *M. tuberculosis* complex. For this study, PCR technique has proven useful for the detection of *M. tuberculosis* complex with a sensitivity of

100% both for all strains. This may indicate that a single agent would be responsible for TB in all patients, therefore homogeneity of the mycobacterial population. The high activity of the PCR would be obtained due to multiple copies of the target sequence frequently present in the genome of mycobacteria. The routine hospital's technique requires use of conventional species-specific identification, for instance PNB in the growing media of mycobacteria to identify the species present since most mycobacterial infections are still caused by MTBC. Then, conventional species-specific identification and proper patient management are delayed due the slow growing nature of mycobacteria (Mokaddas and Ahmad, 2007). The nucleic acid amplification using specific primers provides a good opportunity to identify the *M. tuberculosis* complex from cultures of MTB. The amplification of the target sequence is a quick and relatively simple and the results are available within a day instead of several weeks as the identification tests with PNB. As PCR can detect both living and dead mycobacteria (Bauman et al., 2003), some patients may remain PCR-positive for mycobacterial DNA for several months after treatment. This study shows the importance of PCR in the diagnosis and identification of mycobacterial species, and thus can allow establishment of an appropriate anti-tuberculosis chemotherapy early. The introduction of the PCR technique in health services may increase the number of tuberculosis cases treated and can also reduce the time required for diagnosis of tuberculosis.

Conclusion

In short, identification by PCR using specific makers as *hupB*, *IS6110*, *IS1081*, *oxyR* and *rpoB* revealed that the mycobacterium species responsible for pulmonary tuberculosis in patients from Jamot and Mbalmayo District Hospital belonged to the MTBC. This technique is more rapid compared to the identification test with Para nitro benzoic acid (PNB).

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

- Ait-Khaled N, Enarson D (2003). *TUBERCULOSE*, Manuel pour les étudiants en Médecine. (Organisation Mondiale de la Santé, Union Internationale contre la Tuberculose et les Maladies Respiratoires). pp : 11-16.
- Assam-Assam JP, Penlap BV, Cho-Ngwa F, Tedom JC, Ane-Anyangwe I, Titanji PV (2011). *Mycobacterium tuberculosis* complex drug resistance pattern and identification of species causing tuberculosis in the West and Centre regions of Cameroon. *BMC Infectious Dis.* 11:94.
- Bahador A, Etemadi H, Kazemi B, Ghorbanzadeh R, Nakhjavan FA, Ahmadi NZ (2005). Performance Assessment of IS1081-PCR for Direct Detection of Tuberculous Pleural Effusion: Compared to rpoB-PCR. *Res. J. Agric. Biol. Sci.* 1(2):142-145.
- Canetti G, Rist N et Grosset J (1963). Mesure de la sensibilité du bacille tuberculeux aux drogues antibacillaires par la méthode des proportions. *Revue de Tuberculose et de Pneumologie.* 27: 217-72.
- Das S, Paramasivan CN, Lowrie DB, Prabhakar R and Narayanan PR (1995). IS6110 restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, south India. *Tuberculosis Lung Diseases.* 76:550-554.
- Dheda K, van Zyl-Smit RN, Meldau R, Meldau S, Symons G, Khalfey H, Govender N, Rosu V, Sechi LA, Maredza A, Semple P, Whitelaw A, Wainwright H, Badri M, Dawson R, Bateman ED, Zumla A (2009). Quantitative lung T cell responses aid the rapid diagnosis of pulmonary tuberculosis. *Thorax.* 64:847-853.
- Eisenach KD, Cave MD, Bates JH, Crawford JT (1990). Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J. Infect. Dis.* 161:977-981.
- Giampaglia CM, Martins MC, Chimara E, Oliveira RS, de Oliveira Vieira GB, Marsico AG, Mello FC, de Souza Fonseca L, Kritski A, da Silva Telles MA (2007). Differentiation of *Mycobacterium tuberculosis* from other mycobacteria with p-nitrobenzoic acid using MGIT960. *Int. J. Tuberculosis Lung Dis.* 11 (7):803-807.
- Gill M, Sharma S, Aggarwal A, Kukreja S (2012). Comparative evaluation of conventional methods and nested polymerase chain reaction in the diagnosis of extra-pulmonary tuberculosis. *Nat. J. Basic Med. Sci.* 3(1):1-4.
- Godfrey-Faussett P, Sonnenberg P, Shearer SC, Bruce MC, Mee C, Morris L (2000). Tuberculosis control and molecular epidemiology in a South African gold-mining community. *Lancet.* 356:1066-1071.
- Mishra A, Singhal A, Chauhan DS, Katoch VM, Srivastava K, Thakral SS, Bharadwaj SS, Sreenivas V, Prasad HK (2005). Direct detection and identification of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in bovine samples by a novel nested PCR assay: correlation with conventional techniques. *J. Clin. Microbiol.* 43:5670-5678.
- Mokaddas E, Ahmad S. (2007). Development and Evaluation of a Multiplex PCR for Rapid Detection and Differentiation of *Mycobacterium tuberculosis* Complex Members from Non-Tuberculous Mycobacteria. *Journal of Infectious Diseases.* 60: 140-4.
- Negi SS, Anand R, Pasha ST, Gupta S, Basir SF, Khare S, Lal S (2007). Diagnostic potential of IS6110, 38 kDa, 65 kDa and 85B sequence based polymerase chain reaction in the diagnosis of *Mycobacterium tuberculosis* in clinical samples. *Indian Journal of Medical Microbiology.* 25: 43-9.
- Prabhakar S, Mishra A, Singhal A, Katoch VM, Thakral SS, Tyagi S, Prasad HK (2004). Use of the hupB Gene Encoding a Histone-Like Protein of *Mycobacterium tuberculosis* as a Target for Detection and Differentiation of *M. tuberculosis* and *M. bovis*. *J. Clin. Microbiol.* 42(6):2724-2732.
- Sekar B, Selvaraj L, Alexis A, Ravi S, Arunagiri K, Rathinavel L (2008). The utility of IS6110 sequence based polymerase chain reaction in comparison to conventional methods in the diagnosis of extra-pulmonary tuberculosis. *Indian J. Med. Microbiol.* 26(4):352-5.
- Halse TA, Edwards J, Cunningham PL, Wolfgang WJ, Dumas NB, Escuyer VE, Musser KA (2010). Combined Real-Time PCR and *rpoB* Gene Pyrosequencing for Rapid Identification of *Mycobacterium tuberculosis* and Determination of Rifampin Resistance Directly in

- Clinical Specimens. J. Clin. Microbiol. 48(4):1182-8.
- Singh JPN, Rishendra V, Chaudhuri P (2006). Random amplified polymorphic DNA (RAPD) analysis of *Mycobacterium tuberculosis* strains in India. J. Vet. Sci. 7(2): 181-187.
- The Union (2008). Gender and TB: socio-cultural aspects. Special section. The Int. J. Tuberculosis and Lung Dis. 12(7):825-866.
- van Embden JD, van Gorkom T, Kremer T, Jansen R, van der Zeijst BA, Schoulds LM (2000). *Genetic variation and evolutionary origin of the direct repeat locus of Mycobacterium tuberculosis complex bacteria*. J. Bacteriol. 182:2393-2401 .
- Van Soolingen, Hermans PWM, De Haas PEW, Van Embden JDA (1992). Insertion Element IS1081-Associated Restriction Fragment Length Polymorphisms in *Mycobacterium tuberculosis* Complex Species: a Reliable Tool for Recognizing *Mycobacterium bovis* BCG. J. Clin. 30(7):1772-1777.
- WHO (2013). Global report against Tuberculosis 2013. Genève, Suisse. Retrouvé sur:<http://www.OMS.org>.