EUROPEAN ACADEMIC RESEARCH
Vol. IV, Issue 9/ December 2016

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Molecular identification of *Mycobacterium tuberculosis* causing Pulmonary Tuberculosis in Sudan

FATIMA ABBAS KHALID[1]
Tuberculosis Research Center, University of Kassala
Kassala, Sudan
NAGLA GASMELSEED
Department of Molecular Biology, National Cancer Institute, Sudan
Department of Biology, Faculty of Science University of Hafr Al Batin
Saudi Arabia
ELENA HAILU
Armauer Hansen Research Institute, Addis Ababa, Ethiopia
MUATAZ MOHAMED ELDIRDERY
Tropical Medicine Research Institute, National Center for Research
Khartoum, Sudan
MARKOS ABEBE
Armauer Hansen Research Institute, Addis Ababa, Ethiopia
STEFAN BERG
Animal and Plant Health Agency, New Haw, Surrey, UK
ABRAHAM ASEFFA
Armauer Hansen Research Institute, Addis Ababa, Ethiopia

Abstract:

Background: *Tuberculosis (TB) is a major public health problem in Sudan. However, the strains responsible for the epidemic of TB have been poorly characterized. The aim of this study was to characterize the causative agent of TB isolates circulating in Kassala and Gezira States during 2011- 2012.*

[1] Corresponding author: Fatima Abbas Khalid (fatmaabbas2014@gmail.com)

Method: *Ninety two Mycobacterium tuberculosis complex (MTBC) strains were isolated from sputum samples of pulmonary TB patients, attending the Teaching Hospitals in Kassala and Gezira states. Molecular identification was performed using region of difference 9 (RD9) deletion and spoligotyping methods. Spoligotype patterns were compared with those listed in the international SITVIT2 spoligotyping database.*

Results: *The results determined the circulation of Euro-American, Central Asian, and Indo- Oceanic Lineages. They were clustered in the following clades: Manu2 (64.8%), CAS(22.7%), EAI (4.5), LAM2 (2.3%), H3(1.1%), T (1.1%), T3-ETH (1.1%), T5-RUS1 (1.1%). Comparison with the international multi-marker database SITVIT2, 76.1% of isolates matched the database, while 23.9% of the isolates were not represented in SITVIT2 (orphans). SIT54 (Manu2) was the most common strain circulating in the two states.*

Conclusion*: The study showed that a large variety of Mycobacterium tuberculosis (MTB) genotypes were circulating in the two states. Further studies on identification and molecular characterization of mycobacterial are needed to understand the biodiversity, rate of transmission and the associated risk factors of MTB in Sudan.*

Key words: Mycobacterium, Spoligotype, Sudan

BACKGROUND

Tuberculosis continues to be one of the major public health problems worldwide [1]. The problem has become worse after the emergence of drug resistant tuberculosis and the HIV epidemic. In 2014, 9.6 million cases were estimated to have TB with mortality of about 1.5 million per year [2]. Control efforts are threatened by emergence of drug resistance [1,3] . Sudan

accounts for 15% of the TB burden in the WHO Eastern Mediterranean Region with incidence of 94/100,000 [4]. Gezira and Kassala States are classified among the top of states with high TB prevalence in Sudan [5]e The disease is caused by species belonging to *Mycobacterium tuberculosis* complex (MTBC), which can be characterized by typing of polymorphic genetic loci, including region of difference (RD) typing and spoligotyping that are used as epidemiological tools to discriminate MTB isolates to species and subspecies level. These tools can also be used to understand transmission dynamics that can facilitate control of the disease [6] . Although TB has been a longstanding public health problem, few studies have investigated the genetic diversity of MTBC in Sudan [7]. Molecular typing of MTB would contribute to understanding the population structure of the strains circulating in the country, transmission dynamics, and pathogen biology. Moreover, it could help in control of the epidemics. This study aims at characterizing MTB isolates from pulmonary TB patients from Kassala and Gezira States in Sudan.

METHODS

Sample collection and processing

Sputum samples were collected from newly diagnosed pulmonary TB patients at chest departments in Kassala and Wad Medani Teaching Hospitals during 2011- 2012. All patients were diagnosed based on clinical symptoms, chest radiography and bacteriological examination. The collected samples were decontaminated and homogenized with sodium hydroxide and N-acetyl L-cysteine. The samples were concentrated by centrifugation at 4000 rpm for 10 min. The supernatants were poured off. Distilled water was added to sediments, mixed and then centrifuged again. Supernatant was removed, and the pellet were re-suspended in 100µL distilled water and inoculated on slanted Lowenstein-Jensen media. The

slants were incubated at 37° C and examined for colony growth weekly for 8 weeks. Any positive growth was analyzed by Ziehl-Neelsen staining to confirm Acid-Fast Bacilli (AFB). AFB isolates were subjected to DNA extraction and molecular analysis.

DNA extraction

Extraction of mycobacterial DNA was performed by boiling method as described by Khosravi [8] . Briefly, a loop full of AFB colonies was taken from Lowenstein-Jensen slants and transferred to 1.5-ml tubes containing 200 µl of distilled water. The re-suspended bacteria were heat-inactivated in a water bath at 85°C for 20 minutes and centrifuged at 10,000x *g* for 10 minutes. The supernatant was processed for target DNA isolation. Extracted DNA was kept at -20 ^{o}C until further analysis by molecular techniques.

Molecular typing

Multiplex PCR was performed to identify MTB lineages. Four primer sets (RD9, RD750, RD239, and TbD1) were employed [9,10]. PCR was done using a HotStarTaq Master Mix Kit (Qiagen; Prod No 203445, Germany) and performed in a total volume of 20 µL, containing 7.1µl of H_2O Qiagen, 10µlof Master mix and 0.3 µL(100mM) for each primer (0.3µl ofRDx Flank Rev, 0.3µl of RDx Flank FW and 0.3µl of RDx Internal). Laboratory strain of *M. tuberculosis*H37Rvand *Mycobacterium bovis* were used as positive controls. The PCR samples were analyzed by agarose gel electrophoresis.

Spoligotyping was performed following the method described by Kamerbeek [11].

Molecular data analysis

Spoligotype patterns were entered in an Excel spreadsheet, converted into octal codes and were then entered in the SITVIT2 data base of the Institute Pasteur de la Guadeloupe

(http://www.pasteur-guadeloupe.fr:8081/SITVITONLINE/) to assign the shared type (SIT) and used the web- based algorithm SPOTCLUST (http://tbinsight.cs.rpi.edu/run_spotclust.html) to assign the lineages of each isolates.

RESULTS

Molecular characterization of isolates determined the circulation of three lineages in Kassala and Gezira states: Euro-American 63 (71.6%), followed by Central Asia (CAS) 17 (19.3%) and Indo-oceanic (IO) 8 (9.1%). 7/8 of IO were isolated from Kassala State. Spoligotype patterns of eighty eight isolates were clustered into 11 families represented as: manu2 57 (64.8%), CAS1-Delhi 19 (21.6%), EAI4 (4.5%), LAM2 2 (2.3%), ill-defined T family 3 (3.3%), and CAS1-Kili 1 (1.1%) (Table1). Absence of hybridization to the spacer was demonstrated in four isolates.

Regarding the frequencies of major shared types, a total of 34 patterns were observed among 88 isolates; 16 patterns corresponding to 19 isolates were described as new patterns not yet reported to the SITVIT2 database (Table2). The remaining 18 patterns (n= 69) matched pre-existing shared types in the SITVIT2 database. As shown in Table1, strains with types SIT54 (50%), SIT26 (3.4%), SIT1088 (3.4%), and SIT1343 (2.3%) were all isolated from both states Kassala and Gezira. However, strains with types SIT21 (1.1%), SIT25 (2.3%), SIT289 (2.3%), SIT952 (1.1%), SIT1401 (1.1%), SIT53 (1.1%), SIT254 (1.1%), SIT1634 (1.1%) and SIT1588 2(2.3%) were isolated from Kassala State. On the other hand, SIT50 (1.1%), SIT149 (1.1%), SIT583 (1.1%) and SIT1690 (1.1%) were isolated from Gezira State.

The isolates which were described as new patterns belonged to three clades; manu2 (7/19), CAS1 Delhi (8/19) and EAI (4/19). Three spoligotype patterns of these isolates were

reported twice (773777740003571, 777777740003771 and 557767777743771) (Table 2).

Table 1: Description of spoligotype patterns of MTB isolates from Kassala and Gezira states

State	Spoligotype Patterns	Frequency		Clade	SIT
		No	%		
Kassala	11111111111111111111111111111111100111111111	26	63.42	Manu2	54
	11111111111110111111111111111111100111111111	1	2.4	Manu2	1088
	11111111111111111111111111111110100111111111	1	2.44	Manu2	1634
	11100001111111111111100000000000111011111	2	4.88	CAS1-Delhi	289
	11100001111111111111100000000000110011111	2	4.88	CAS1-Delhi	25
	11100001111111111111100000000000111111111	1	2.44	CAS1-Delhi	26
	11100001111101111111100000000000111111111	1	2.44	CAS1-Delhi	1343
	11000001111111111111100000000000111111111	1	2.44	CAS1-Delhi	952
	11100001101111111100000000000000011111111	1	2.44	CAS1-Kili	21
	11100001111111111111100000000000111101111	1	2.44	CAS1-Delhi	1401
	00001111111001111111000011111111000011111111	2	4.88	LAM2	1588
	11111111111111111111111111111111000011111111	1	2.44	T	53
	11111111111110000000000111111111000011111111	1	2.44	T5-RUS1	254
	Total	41	100		
Gezira	11111111111111111111111111111111100111111111	18	66.7	Manu2	54
	11111111111111011111111111111111100111111111	2	7.4	Manu2	1088
	11111111111101111111111111111111100111111111	1	3.7	Manu2	583
	11100001111111111111100000000000111111111	2	7.4	CAS1-Delhi	26
	11100001111101111111100000000000111111111	1	3.7	CAS1-Delhi	1343
	11111111100000000001111111111110000111111	1	3.7	T3-ETH	149
	11111111111111111111111111111101000011111	1	3.7	H3	50
	11111111111111111100111111111110000111111	1	3.7	H37Rv	451
	Total	27	100		

The spoligotype pattern is shown as a series of 1s and 0s with 1 representing hybridization to the spacer and 0 representing absence of hybridization. International names for these spoligotype patterns were assigned by SITVIT2.

Table 2: Description of 16 spoligotype patterns from Kassala and Gezira States not previously described.

State	Spoligotype patterns	Frequency	Clade
Kassala	11111101111111111111100000000000111011111	2	CAS1-Delhi
	11101001111111111111100000000000111011111	1	CAS1-Delhi
	11111001110111111111100000000000111011111	1	CAS1-Delhi
	00101001111111111111100000000000110111111	1	CAS1-Delhi
	11111111111111111111100000000000111111111	2	EAI
	11111111111101111111100000000000111111111	1	EAI
	11111101110111111111100000000000111011111	1	EAI
	10110111111111011111111111111111000111111111	2	Manu2
	11100101111111111111110000111110001111111111	1	Manu2
	11110001111111111111111110011110001111111111	1	Manu2

Gezira	111111111011011111101111111111100111111111	1	Manu2
	111111111111111111111111111111000111111111	1	Manu2
	111101011111111111111111111111000111111111	1	Manu2
	111100011111110111111100000000000111111111	1	CAS1-Delhi
	101001111111101111100000000000000111111111	1	CAS1-Delhi
	111000001111101111111100000000000011111111	1	CAS1-Delhi
	Total	19	

Sixteen spoligotype patterns were not yet reported in the SITVIT2 database

DISCUSSION

TB remains a great public health concern in Sudan, and identification of genotypes of the predominant strains that circulates in the country is critical to study disease transmission patterns and epidemiological features. This study describes the population diversity of *M. tuberculosis* clinical isolates from the two states Kassala and Gezira based on deletion typing and spoligotyping. Four out of 92 isolates showed absence of spoligotype signature. Absence of spoligotyping signature was previously reported [12,13].This finding might relate to infection by non-tuberculous mycobacteria as shown in the result of large sequence polymorphism (RD9).

The study identified co-circulation of three MTB lineages :Euro- American, Central Asian (CAS) and Indo-Oceanic in the study areas. These lineages have also been reported from TB patients in Ethiopia [14,15], indicating an epidemiological relationship between the neighboring countries. Sudan and Ethiopia have strong historical relations, and cross boundary movement between tribes of the two countries facilitates epidemiological links. Sudan hosted large numbers of refugees from Ethiopia and Eritrea in Kassala State during the recent war between the two countries. This and previous similar population movements would be expected to contribute to the circulation of shared lineages in these areas.

Cluster analysis of isolates showed the dominance of the manu2 clade (64.8%) followed by CAS1 Delhi (21.9%) as reported previously in Sudan [7,16]. Manu2 and EAI are

characterized by deletion of RD239 and intact TbD1[10]. Moreover, they are closely related and were probably derived from a last common ancestor [10]. In South Africa, isolation of manu2 was related to mixed infection between Beijing and the Euro-American lineage [17]. Beijing was not detected among isolates in our study. However, the detection of manu2 in Ethiopia was related to co-infection with HIV [18]. Manu is considered ancestral family of principal genetic group1 [19,20]. It is divided into three groups; manu1 (lack spacer 34), manu2 (lack spacer 33-34), and manu3 (lack spacer34-36) [21]. Manu2 has been isolated from different countries including neighboring ones [18, 22,23,24,25,26,27]. High clustering of manu2 might indicate a high rate of transmission in central and Eastern Sudan and this can potentially accelerate the emergence of drug resistance. The result might be mixed circulation of Manu and East African Indian. Both super families have the same ancestral origins [28]. Dual infections of single patients by distinct strains of *M. tuberculosis* is increasingly reported in high tuberculosis incidence areas, raising the possibility of false composite spoligotype patterns if performed upon mixed strain samples [29].

Manu2 was determined by different shared types among which SIT54, SIT1634, SIT1088 and SIT1690 were previously isolated from Sudan (Sharafeldein *et al* 2011, Eldirdery et al 2015). However, SIT583 has not previously been reported in Sudan, but has been reported in France. Shared types of the CAS Delhi clade were represented as SIT25, SIT26, SIT289 which were previously isolated from Sudan [7,16]. These types are also dominant in Ethiopia (Mengistu et al 2015). CAS1 Kili /SIT21, H3/SIT50, TI/SIT53 patterns were identical to that isolated previously in Sudan [7,16]. It is interesting to note that no isolates were characterized as Lineage 7, the newly identified *M. tuberculosis* lineage that is very much defined to the northern highlands of Ethiopia [14]. With significant movement of people across the border to Eastern Sudan, it

would not have been a surprise to have found this strain also in Kassala.

The families which were isolated in this study have also been isolated and genotyped by spoligotyping in several other countries. According to the SITVIT Database, MANU2 family/SIT1588 was prevalent in Bulgaria and Germany and SIT1401 was reported in Bangladesh, Pakistan and the United States. SIT952 has been reported from Bangladesh, India, Sweden and the United States. SIT451 has been reported from different African countries, Europe and the Americas. SIT149 has been isolated in Ethiopia and South Africa. SIT54, which is the dominant isolate in Sudan, circulates worldwide. This may be due to open boundaries of Sudan and movement across states.

Isolates with new spoligotypes were related to CAS1 Delhi, manu2 and EAI families. Some of them had identical octal numbers indicating that they are new shared strains (Table 2). Moreover, one of them was already typed in Iraq [30].

CONCLUSION

The study concludes that a large variation of *M. tuberculosis* genotypes were circulating in the two states. Further studies on identification and molecular characterization of mycobacteria are needed to better understand the biodiversity, rate of transmission and the associated risk factors of MTB in Sudan.

Abbreviations

AFB: Acid fast bacilli, MTB: Mycobacterium Tuberculosis, RD: Region of difference

Acknowledgements

The authors would like to thank the staff of chest departments from teaching hospitals in Kassala and Gezira States, who help in collection of samples, and the staff of Armaur Hansen

Research Institute, Ethiopia, who facilitate the molecular typing of MTB.

Funding
No fund was received for this study.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
The study was approved by the Research Ethics Committee at University of Kassala.

Authors' contribution
FA and NG conceptualized the research idea and study design. Data collection (eg sampling, MTB culturing , molecular typing) was completed by FA, NG ,EH, and ME. Data analyses were performed by FA, EH and revised by AA and SB. AA and SB closely involved in the design, and review of the manuscript. All authors have read and approved the final version of the manuscript and agreed for publication.

REFERENCES:

1.Global tuberculosis report 2014. World Health Organisation. www.who.int/tb/data.
2.Global tuberculosis report 2015. World Health Organisation. www.who.int/tb/data
3. Jagielski T, Augustynowicz-kope E, Pawlik K, Dziadek J, Zwolska Z, Bielecki JA. Two-Step Strategy for Molecular Typing of Multidrug-Resistant *Mycobacterium tuberculosis* Clinical Isolates from Poland. Polish Journal of Microbiology. 2011; 60 (3): 233–241.

4.Global tuberculosis report 2016. World Health Organisation. www.who.int/tb/data.

5. Federal Ministry of Health, General directory of Primary health care, National TB control program (Sudan) – report 2012.

6. Kontsevaya IS, Nikolayevsky VV, Balabanova YM. Molecular epidemiology of tuberculosis: objectives, methods, and prospects," *Molecular Genetics, Microbiology and Virology*. 2011; 26 (1): 1–9.

7. Sharaf Eldin GS, Fadl-Elmula I, Ali MS, Ali AB, Salih AL, Mallard K, Bottomley C, McNerney R. Tuberculosis in Sudan: a study of *Mycobacterium tuberculosis* strain genotype and susceptibility to anti-tuberculosis drugs. *BMC infectious Diseases*. 2011; 11: 219.

8. Khosravi A.D, Dezfulian A andAlavi S.M (2006). Detection of Isoniazid and Rifampin resistant *Mycobacterium tuberculosis* isolated from tuberculosis patients using conventional method and PCR. *Pak. J. Med. Sci*. 2006; 22 (1) 47–50.

9. Berg S, Firdessa R, Habtamu M, Gadisa E, Mengistu A, Yamuah L, et al. The burden of mycobacterial disease in ethiopian cattle: implications for public health. PLoS One. 2009;4(4):e5068. doi: 10.1371/journal.pone.0005068.

10. Gagneux S, DeRiemer K, Van T, Kato-Maeda M and de Jong BC.Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *ProcNatlAcadSci U S A*. 2006; 103: 2869–2873.

11. Kamerbeek J, Schouls L, Kolk A, Van Agterveld M, Van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M and Van Embden J. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.1997; 3: 907–914.*

12. Streicher EM, Victor TC, van der Spuy G, Sola C, Rastogi N, van Heldenp D, Warren RM (2007). Spoligotype Signatures in the *Mycobacterium tuberculosis* Complex. *Journal of Clinical Microbiology*. 2007; 45 (1): 237–240.

13. Zink AR, Sola C, Reischl U, Grabner W, Rastogi N, Wolf H, Nerlich AG. Molecular identification and characterization of *Mycobacterium tuberculosis* complex in ancient Egyptian mummies. International Journal of Osteoarchaeology. 2004; (14). DOI: 10.1002/oa.724

14. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G,Gadisa E, Kiros T, Habtamu M, Hussein J, Zinsstag J, Robertson B.D, Ameni G,Lohan M.J, Loftus B, Comas I, Gagneux S, Tschopp R, Yamuah L, Hewinson G, Gordon S.V, Young D.B, and Aseffa A. Mycobacterial Lineages Causing Pulmonary and Extra-pulmonary Tuberculosis, Ethiopia. Emerging Infectious Diseases. 2013; 193).DOI:10.3201/eid1903.120256

15. Mengistu A, Enquselassie F, Hailu E, Aseffa A, and Beyene D (2015). Identification and Characterization of *Mycobacterium Tuberculosis* Isolates from Cattle Owners in North Western and North Eastern Parts of Rural Ethiopia. *International Journal of Innovation and Applied Studies.* 2015; 10 (1): 85-94

16. Eldirdery MM, Alrayah IE, AwadElkareim MO, Khalid FA, Elegail A MAS, Ibrahim NY, Nour EOM, Ali R. H, Hailu E, Abebe M, Aseffa A, Osman N.M, Mukhtar MM, Elhaj NMA and Elagib AA. Genotyping of Pulmonary *Mycobacterium tuberculosis* Isolates from Sudan Using Spoligotyping. *American Journal of Microbiological Research. 2015; 3(4): 125-128*

17. Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, Gey van Pittius NC, van Helden PD. Patients with active tuberculosis often have different strains in the same sputum specimen. *Am J Respir Crit Care Med*. 2004; 169(5):610-614.

18. Belay M, Ameni G, Bjune G, Couvin D, Rastogi N, Abebe F. Strain diversity of *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients in Afar Pastoral Region of Ethiopia. *BioMed Research International*. 2014; 12. doi: 10.1155/2014/238532.238532.

19. Singh UB, Suresh N, Bhanu NV, Arora J and Pant H. Predominant tuberculosis spoligotypes, Delhi, India. *Emerg Infect Dis*. 2004; 10: 1138–1142.
20. Singh UB, Arora J, Suresh N, Pant H and Rana T. Genetic biodiversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in India. *Infect Genet Evol*. 2007; 7: 441–448.
21. Swarnkar P, Katoch VM, Soni H, Singhai AK, Nayak G, Mishra K. Spoligotyping of *Mycobacterium tuberculosis* strains from Ziehl-Neelsen stained sputum slides from DTC, Satna (M.P.). *I JRPBS. 2010; 1 (2): 2229-3701.*
22. Al-Hajoj SA, Zozio T, Al-Rabiah F, Mohammad V, Al-Nasser M, Sola C and Rastogi N. First insight into the population structure of *Mycobacterium tuberculosis* in Saudi Arabia. *J. Clin. Microbiol*. 2007; 45:2467- 2473.
23. Namouchi A, Karboul A, Mhenni B, Khabouchi N, Haltiti R, Ben Hassine R, chir Louzir B, Chabbou A, Mardassi H. Genetic profiling of *Mycobacterium tuberculosis* in Tunisia: predominance and evidence for the establishment of a few genotypes. *J. Med. Microbiol*. 2008; 57: 864–872.
24. Helal ZH, Ashour MS, Eissa SA, Abd-Elatef G, Zozio T, Babapoor S, Rastogi N, Khan MI. Unexpectedly high proportion of ancestral Manu genotype *Mycobacterium tuberculosis* strains cultured from tuberculosis patients in Egypt. *J ClinMicrobiol.2009;* 47(9):2794-2801.
25. Stavrum R, Myneedu VP, Arora VK, Ahmed N, Grewal HM. In-depth molecular characterization of *Mycobacterium tuberculosis* from New Delhi -predominance of drug resistant isolates of the 'modern' (TbD1) type. 2009; PLoS One4: e4540.
26. Viegas SO, Machado A, Groenheit R, Ghebremichael S, Alexandra Pennhag A, Paula S Gudo PS, Cuna Z, Miotto P, Hill V, Marrufo T, Cirillo DM, Rastogi N, Kallenius G, Koivula T . Molecular diversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mozambique.

BMC Microbiology. 2010; 10:195. [195]. DOI: 10.1186/1471-2180-10-195

27. Sekati EM, Molepo J, Nchabeleng M. Molecular characterization and associated drug susceptibility patterns of *Mycobacterium tuberculosis* isolates from South African children. *South Afr J Infect Dis*. 2015; 30(1):11-16.

28. Thomas S K, Iravatham CC, Moni B H, Kumar A, Archana BV, Majid M, Priyadarshini Y, Rani PS, Valluri V, Hasnain SE and Ahmed N. Modern and ancestral genotypes of *Mycobacterium tuberculosis* from Andhra Pradesh, *India.PLoSOne*. 2011 ;6 (11):e27584.).

29. Lazzarini LCO, Rosenfeld J, Huard RC, Hill V e, Silva JRL, DeSalle R, Rastogi N, Ho JL. *Mycobacterium tuberculosis* spoligotypes that may derive from mixed strain infections are revealed by a novel computational approach. Infection, Genetics and Evolution. 2012; 798–806

30. Ali RM, Trovato A, Couvin D, Al-Thwani M N, Borroni E, Dhaer FH, Nalin Rastogi N, and Cirillo DM (2014).Molecular epidemiology and Genotyping of *Mycobacterium tuberculosis* Isolated in Baghdad. *BioMed Research International. 2014* .doi:10.1155/2014/580981.