

Identification and Discrimination of *Mycobacterium tuberculosis* Complex with Traditional and Real-Time PCR in Different Specimens in Iraq

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Abstract:

Background: Tuberculosis (TB) is a major public health issue and a main cause of global morbidity and mortality. TB is the world's ninth leading cause of death despite the numerous treatment strategies for managing the disease.

Objective: To assess the traditional method (direct smear examination and culture) against real-time PCR as pulmonary and extrapulmonary tuberculosis laboratory diagnostic techniques.

Cases and methods: Samples were collected from (612) TB cases, (409) of whom were pulmonary tuberculosis (PTB) and (203) were extrapulmonary tuberculosis (EPTB). The cases were seeking care at the Specialized Chest and Respiratory Disease Center/ National Reference Laboratory for Tuberculosis (NRL) in Baghdad, during the period 1st of May -1st of October 2019. Direct smear examination, Lowenstein-Jensen culture and Real Time PCR were used to diagnose TB.

Results: Out of 612 samples received, 82(13.4%) were positive by smear microscopy, while 90(14.7%) were able to grow on Lowenstein-Jensen (LJ) media. Ninety DNA extracts from the samples which were positive on LJ media and 25 control specimens, were diagnosed with molecular analysis by using real time PCR to determine the species of *Mycobacterium tuberculosis* complex. The results revealed that the 71 samples (78.9%) were *M. tuberculosis*, three specimens (3.3%) were combined *M. bovis* and *M. tuberculosis*, and one *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG, while 15 (16.7%) were negative and subsequently excluded from study.

Conclusion: The comparison between molecular diagnostic methods by using Real time PCR with conventional diagnostic methods, provides a new promising technique and is potentially a practical and rapid alternative to the slower traditional pulmonary and EPTB diagnostic culture. The results show *M. bovis* overall contribution on human TB in comparison to *M. tuberculosis* is minor among PTB and EPTB cases in the sample studied.

Keyword: Pulmonary tuberculosis, extrapulmonary tuberculosis, Real time PCR.

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Introduction:

Tuberculosis (TB) is a major public health issue and a main cause of morbidity and mortality in the world. TB is the world's ninth leading cause of death despite the numerous treatment strategies for managing the disease (1). TB is caused by *Mycobacterium tuberculosis* (MTB) (2). Generally, this bacterium targets the lungs, but the spine, kidney and brain are other exposed organs (3). TB constitutes a major public health issue in Iraq. Iraq is

a middle disease-burden country with TB, ranked 108th worldwide and 7th in the Eastern Mediterranean Region among TB burden-size countries (4). There were an estimated 20,000 TB patients in Iraq in 2014 and nearly 4000 died from the disease (1, 5). Improvement in TB diagnosis and standardized anti-TB therapies is a key to the "end-TB strategy" of the WHO (6, 7). Effective TB treatments primarily depend on accurate TB diagnosis (8). In TB diagnosis, molecular methods are being increasingly used (9). The use of real time-PCR in the diagnosis of TB has increased, as it has been shown to be highly specific and sensitive to detect *Mycobacterium tuberculosis* complex (MTBC) from culture isolates or directly in clinical samples (10, 11, and 12).

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Cases and Methods:

Sample Collection and DNA Extraction: Between 1st of May and 1st of October 2019, 612 samples were received from patients attending the Specialized Chest and Respiratory Disease Center/ National Reference Laboratory for Tuberculosis (NRL) in Baghdad. Specimens were separated into two groups: 409 (63.4%) pulmonary TB (PTB) specimens and 203 (36.6%) extrapulmonary TB (EPTB) specimens. PTB specimens included (285) sputum specimens and (124) Broncho-alveolar lavage specimens. The other specimens that were collected from EPTB cases included pleural fluid (48), urine (42), pericardial fluid (30), ascetic fluid (28), gastric fluid (27), biopsy (10), abscess (7), lymph node aspirate (5), synovial fluid (3), skin swab (2), and Cerebrospinal fluid (CSF) (1). Specimen collection and processing (Decontamination) were carried out according to the laboratory manual guidelines of work (13). Ziehl-Neelsen Stain as well as culture on Lowenstein-Jensen media were investigated for the presence of acid fast bacilli. Only L.J medium culture positive of PTB and EPTB samples and specimens from healthy persons were taken to extract DNA with the sonicator method according to National Tuberculosis Control Program (14).

Identification and Discrimination of *M. tuberculosis* complex via Real time PCR

All positive LJ culture of PTB and EPTB and 25 control specimens (people who visited NRL and have negative direct smear examination and culture) have been confirmed with real time PCR by MTB Diff Real-TM kit (Sacace, Italy), and performed according to the kit's protocol.

Statistical analysis

The significance of difference of different percentages (quantitative data) was tested using Pearson Chi-square test (χ^2 -test) with application of Yate's correction or Fisher Exact test whenever applicable. Statistical significance was considered whenever the P value was equal or less than 0.05.

Results:

Out of (612) specimen received, 82(13.4%) (55 samples from PTB and 27 from EPTB) were positive by smear microscopy (direct examination), whereas 90(14.7%) specimens (57 from PTB and 33 from EPTB) were able to cultivate on LJ media. It was noticed that eight negative specimens by direct examination were detected by culture and only (27) EP specimens were diagnosed by ZN stain, while 33 specimens were positive by culture as seen in (table 1).

Table 1: Specimens Distribution According to the Type of TB and the Type of Lab Test

	No of specimen	Direct		Cultivation		Positive culture%
		+ve	-ve	+ve	-ve	
Pulmonary (n=409)						
Sputum	285	51	234	52	233	57.8
Broncho alveolar lavage	124	4	120	5	119	5.6
Extra pulmonary (n=203)						
Pleural fluid	48	8	40	11	37	12.2
Urine	42	3	39	3	39	3.3
Pericardial fluid	30	1	29	1	29	1.1
Ascetic fluid	28	6	22	7	21	7.8
Gastric fluid	27	5	22	6	21	6.7
Biopsy	10	-	10	-	10	-
Abscess	7	2	5	3	4	3.3
Lymph node	5	2	3	2	3	2.2
Synovial fluid	3	-	3	-	3	-
Skin Swab	2	-	2	-	2	-
CSF	1	-	1	-	1	-
Total	612	82 (13.4%)	530 (86.6%)	90 (14.7%)	522 (85.3%)	100

The diagnosis by real time PCR revealed that the greater number of DNA extracted belongs to *Mycobacterium* genus. As shown in (Figure. 1). The results showed that out of the 90 DNA extracted from (57) PTB and (33) EPTB samples, 71 samples (78.9%) were *M. tuberculosis* (Figure. 2), three (3.3%) were combined *M. bovis* and *M. tuberculosis* (Figure. 3), and 1 for *M. bovis*, *M. tuberculosis*, and *M. bovis* BCG (Figure. 4). Fifteen extracts (16.7%) (7 samples from PTB and 8 samples from EPTB) were

negative and subsequently excluded from study. The results show that *M. tuberculosis* 47 (47/90) represented (82.5%) of the PTB samples, and 24 (24/90) (72.7%) of the EPTB samples. The two samples of mixed of *M. tuberculosis* and *M. bovis* represented (3.5%) of the PTB, and one (3.0%) of the EPTB, and only one (1/90) (1.8%) the mixed of *M. bovis*, *M. tuberculosis* and *M. bovis* BCG from the PTB as show in (Table. 2).



Figure 1: Distribution of 90 DNA Extracted from PTB and EPTB Specimen Detection by Real Time PCR.

Figure 2 shows that for the 13 samples and three of controls (Negative Amplification Control, Positive Amplification Control, and Negative Extraction Control), specific color for each specimen to recognize it from the other specimens. Twelve of these specimens and positive control showed above the threshold line as colored amplification curves (positive result), one specimen and negative control was showed in the colored line under threshold line.

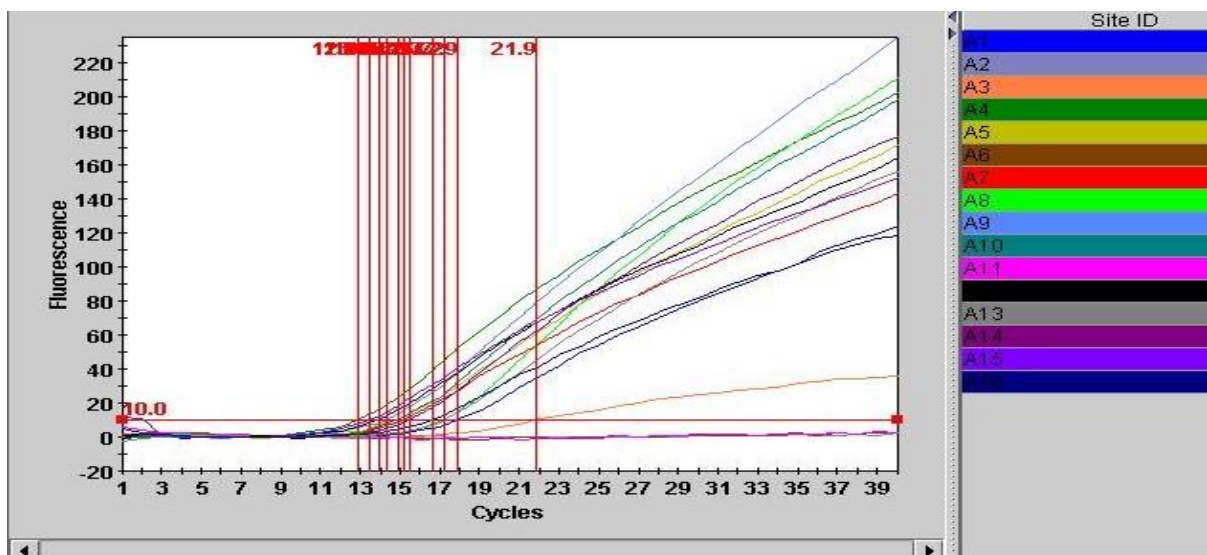


Figure 2: Real Time PCR FAM channel runs to detect *M. tuberculosis*

Figure 3 revealed that for the nine specimens as well as three of controls (Negative Amplification Control, Positive Amplification Control, and Negative Extraction Control), only three colored curves were showed the progress of the amplification for the

positive specimens, one of them represent positive control and the other were positive result for *M. bovis*, and all the specimens appear as a line under the threshold line (negative results) with the negative controls.

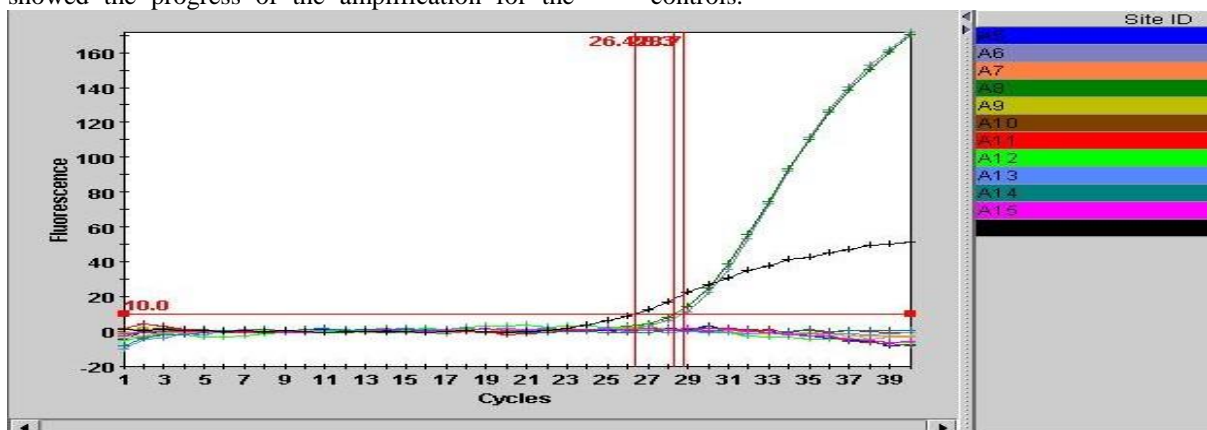


Figure 3: Real Time PCR Cy3 channel runs for detection of *M. bovis*

Figure 4 shows that for the six specimens for detection of *M. bovis* BCG in addition to three of controls (Negative Amplification Control, Positive Amplification Control, and Extraction Negative Control), five specimens and negative controls were

appeared as a line under the threshold line (negative result), the positive control (one curve) and one specimen positive was appeared above the threshold line.

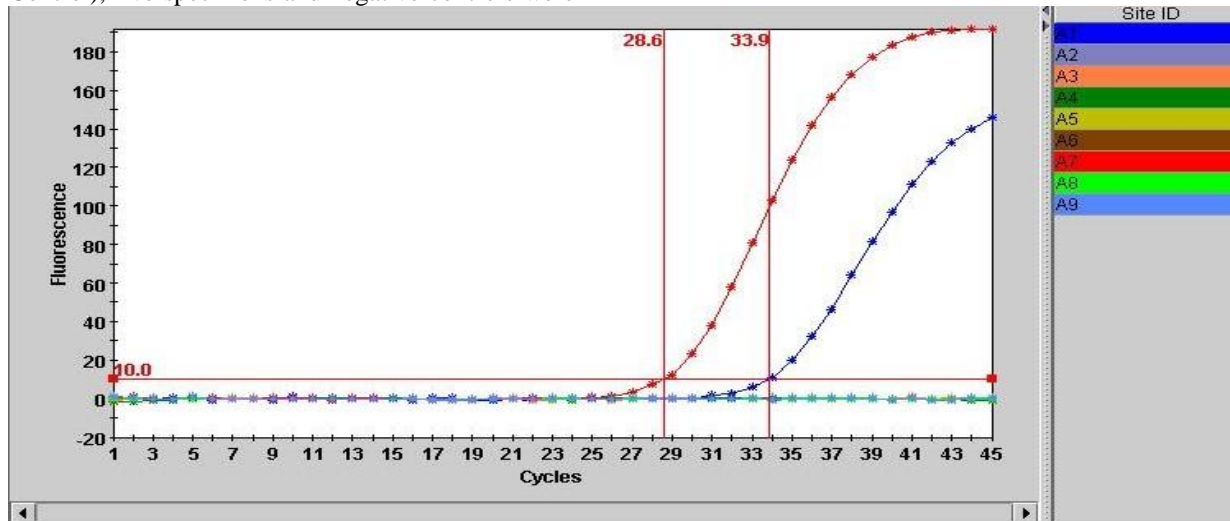


Figure. 4: Real Time PCR TexasRed channel runs for detection of *M. bovis* BCG

Table. 2: Detection of *Mycobacterium* Species by Real Time PCR.

Tuberculosis type	MT		MT & MB		MT & MB & MB- BCG		Negative	
	No	%	No	%	No	%	No	%
Pulmonary (57)	47	82.5	2	3.5	1	1.8	7	12.3
Extra pulmonary (33)	24	72.7	1	3.0	-	-	8	24.2
Total (90)	71	78.9	3	3.3	1	1.1	15	16.7
P value compared to negative	0.155		0.527		0.302		-	
P value comparing all	0.451							

Table 3 shows the Sensitivity and Specificity of Direct Microscopic Examination (DME) compared to culture Sensitivity 91%, and Specificity 100%, and table 4 shows the Sensitivity and Specificity of PCR compared to culture.

Table 3: Distribution of studied samples according to results of both culture and DME

		Culture	
		Positive	Negative
DME	Positive	82	0
	Negative	8	25

The of Sensitivity and Specificity DME compared to culture

Sensitivity : 91%
Specificity : 100%

Table4: Distribution of studied samples according to results of both culture and PCR

		Culture	
		Positive	Negative
PCR	Positive	75	0
	Negative	15	25

The of Sensitivity and Specificity PCR compared to culture

Sensitivity : 83%
Specificity : 100%

Discussion:

The present study revealed that similar to many developing countries, the direct smear microscopy method is widely used to diagnose TB in Iraq. The LJ culture medium has been considered the gold standard for TB diagnosis in developing countries (15). Although the microscopic examination and culture remain the cornerstone of the TB diagnosis, there are several drawbacks to these conventional bacteriological procedures, due to the long time need for the culture results, and the low sensitivity of the ZN smearing particularly with small numbers of the organisms in clinical specimens (16). Positivity of cultures in this study was high compared to microscopy of AFB smears. Approximately 5000-10000 AFB/ml of specimens is required in order to produce positive results with AFB smear microscopy, while the LJ medium culture advantage is that it is very specific with a sensitivity of 80-85% and only 10 bacteria per milliliter of specimens can be detected (17, 18). Our results confirmed the limited sensitivity of the AFB smear although it is a specific test, as well

as the culture on LJ medium is being the gold standard with higher sensitivity. The detection rate is always 30-50% higher compared to microscopy (19). There are different indications that ZN stain sensitivity for sputum specimens does not exceed 60-70% compared to sputum culture specimens (20), and ZN stain sensitivity for EP samples ranged from 0% to 75% (21). In this study these results were clear, only 27 EP specimens were diagnosed with ZN stain, while 33 specimens were diagnosed with solid culture. Diagnosis of EPTB, in particular, it is difficult because of the pauci-bacillary nature of the disease, the distribution of the sample for different diagnostic tests resulting in a non-uniform distribution of microorganisms, the difficulty in collecting sufficient specimens, the variable clinical presentation, and the need for invasive procedures to secure an appropriate sample (22), lack of laboratory facilities in the resource-limited settings and the lack of appropriate specimens processing methodology generally applicable to all forms of extra-pulmonary samples (23). All these limitations lead to the poor establishment of an EPTB diagnosis through bacteriological techniques (24). Our results were in discordance with those reported by Abbadi *et al* in Egypt (25) who stated that among the (45) *M. tuberculosis* complex isolates from sputum specimens, (44) were identified as *M. tuberculosis* and only one as *M. bovis*. The results of our study also disagree with those of Ali (26) in Iraq which revealed the absence of *M. bovis*, and with the study conducted by Ahmed in 2018 that showed *M. tuberculosis* as the only species from *M. tuberculosis* complex circulating in Iraqi patients (27). The present finding is in disagreement with study by Al-Hajoj *et al* (28) who found only *M. tuberculosis* isolates with no other species in EPTB specimen, and with results by Siala *et al* (29) in south Tunisia who reported the occurrence of the following species: (20) *M. tuberculosis*, (75) *M. bovis*, (2) *M. bovis* BCG from total of 97 EPTB specimens. Our results on species identification by Real-time PCR in different specimen of pulmonary and EPTB are in disagreement with a study in Ethiopia which reported that out of 964 isolates from pulmonary and EPTB, most of those isolates were classified as *M. tuberculosis*, with only four (0.4%) *M. bovis* isolates obtained from cases of PTB (30). They were also incompatible with Torres-Gonzalez *et al* (31) study that found from EP disease 41.6% of cases was *M. bovis*, and 30.1% *M. tuberculosis*, while 36% of *M. bovis* was observed in pulmonary disease, compared to 52.6% of *M. tuberculosis*. In the present study, 15 (16.7%) samples were found to be positive by cultivation method but was negative by PCR, this may be attributed to non-tuberculous mycobacterial strains in the samples, or the presence of PCR inhibitors in various clinical specimens (32), low bacterial load as previous studies have confirmed (33), and unequal distribution of AFB in these samples (34). Negative PCR results may be attributable to the nil or very low number of

Mycobacterium copies in the sample (35). The study findings are similar to the study results by Ahmed (27), who found that 6(3.8%) isolates were negative results and identified as non-tuberculous mycobacteria (NTM).

Conclusion:

The comparison between molecular diagnostic methods by using Real time PCR with conventional diagnostic methods provides a new promising technique and is potentially a practical and rapid alternative to the slower traditional pulmonary and EPTB diagnostic culture. *Mycobacterium tuberculosis* plays a major role in producing pulmonary and EPTB in Baghdad compared to *Mycobacterium bovis*.

Author's contributions:

Shaymaa M. Ali: PhD student
 Mohammad A. Al-Faham: first supervisor
 Ahmed A. Mankhi: second supervisor

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تشخيص وتفريق بكتريا التدرن من مختلف النماذج باستخدام الطرق التقليدية وتفاعل البوليميراز المتسلسل اللحظي في العراق

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الخلاصة:-

خلفية البحث: يعتبر مرض التدرن من المشاكل الصحية العالمية ومن المسببات الاساسية للمراضة والوفاة في معظم انحاء العالم. فهو المسبب التاسع للوفيات في العالم بالرغم من وجود عدة استراتيجيات للعلاج.
هدف الدراسة: تقييم الطرق التقليدية (المسحة المباشرة والزرع) مقارنة بفحص تفاعل البوليميراز المتسلسل اللحظي لتشخيص التدرن الرئوي وخارج الرئوي.

المرضى وطرق العمل: تم جمع 612 عينة توزعت الى 409 عينة تدرن رئوي و 203 عينة خارج رئوي من المرضى المراجعين للمختبر المرجعي للتدرن التابع لمركز الامراض الصدرية والتنفسية التخصصي في بغداد خلال الفترة من الاول من ايار وحتى الاول من تشرين الاول 2019. استخدم الفحص المباشر للمسحة والزرع على وسط لويشتاين-جنسن وكذلك فحص تفاعل البوليميراز المتسلسل اللحظي لتشخيص التدرن.
النتائج: من مجموع (612) عينة اظهرت نتائج الفحص المجهرى المباشر للمسحات المصبوغة بصبغة زيل-نيلسون ثم الزرع بعدها على وسط لويشتاين-جنسن ان 82 (13.4%) عينة كانت ايجابية للفحص المباشر في حين 90 (14.7%) عينة كانت ايجابية للزرع على وسط لويشتاين-جنسن. تم استخلاص الجينوم (DNA) من 90 عينة رئوية وخارج رئوية الايجابية للزرع على الوسط الصلب للدراسة الجزيئية باستخدام تفاعل البوليميراز المتسلسل اللحظي لتشخيص عائلية البكتريا الى جنس Mycobacterium tuberculosis complex. اظهرت النتائج وجود 71 مريضا (78.9%) مصاب ب M. tuberculosis و ثلاث حالات (3.3%) مصابة ب M. bovis مع M. tuberculosis وحالة واحدة (1.1%) لمريض مصاب بخليط M. tuberculosis, and M. bovis BCG بينما اظهرت النتائج 15 عينة (16.7%) بنتيجة سالبة لهذا الفحص وتم استبعادها من هذه الدراسة.

الاستنتاجات: وجد ان فحص تفاعل البوليميراز المتسلسل اللحظي هو اسرع مقارنة مع الطرق التقليدية لتشخيص التدرن الرئوي وخارج الرئوي. تشير هذه النتائج الى أن M. tuberculosis في بغداد لها دور اساسي مقارنة ب M. bovis في كونه المتسبب لمرض السل الرئوي.
مفتاح الكلمات: التدرن الرئوي، التدرن الخارج رئوي، تفاعل البوليميراز المتسلسل اللحظي