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The Use Of Polymerase Chain Reaction In Real Time To Diagnose The Tuberculosis (TB) for patient from Erbil

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Abstract
were examined sample 30 patients (male 19 and female 11) suspected clinically infected with tb and using RT-PCR the sample from sputum and ICT Assay from blood plasma were ratios 36%, 13% respectively. and test values were Chi square from diagnose tb from sputum by RT-PCR for female $3.798^{n.s}$, P-Value 0.0571 and male were Chi square $3.185^{n.s}$, P-Value0.00714 non significant difference among females and male but the value of male closer to significant. test values were chi square from diagnose tb from blood plasma by ICT for female $3.157^{n.s}$, P-Value 0.1782 and male were chi square 1. $831^{n.s}$, P-Value 0.381 non significant difference among females and male but the value of female closer to significant. diagnose tb from urine by RT-PCR dose not appear any result. Results showed despite the lack of significant differences between the ways studied but the RT-PCR offers a way sensitive, efficient and accurate for the diagnosis and determine tb infection especially from sputum and followed ICT test less efficient and indexation DNA of tb from urin by RT-PCR is non efficient .

Introduction
Tuberculosis (TB) remains an important public health problem world wide. It is responsible for more than 3 million deaths and 8 million new cases annually (1) Tuberculosis continues to dominate infectious diseases globally due to its ability to infect, remain latent in the host for an indefinite period and then re-appear later as overt disease. ***Mycobacterium tuberculosis*** is the most common cause of TB (2). as a result of slow growth of ***M. tuberculosis*** on selective media, the microscopic detection of directly stained organisms on the smear sample processed for clinical specimens could provide quick results, but its specificity and sensitivity is not enough (3). diagnostic challenge since the clinical presentation of pulmonary TB is not always as typical (4). Furthermore, as extra pulmonary TB is most often paucibacillary, acid fast bacilli (AFB) stains are even less sensitive on fluid aspirates and tissue biopsies than they are on sputum samples (5).In this context, improved microbiological diagnosis would be of great importance since the appearance of a caseating granuloma in affected tissues or the crowding of lymphocytes in an aspirated fluid may be the only evidence for extra pulmonary TB (6). The acid – fast smear of sputum sample, is essentially a variant of procedure developed by Robert Koch in his initial experiments (7).The gold standard for TB diagnosis is the demonstration of mycobacteria in various body fluids. This is often not possible, due to the paucibacillary nature of the illness in some cases (8). (RT- PCR) has allowed great progress to be made in the rapid and accurate diagnosis ofinfections due to organisms that are not cultivable by in vitro means, that require complex media or cell cultures and prolonged incubation times, or for which culture is too insensitive**.** We aimed to evaluate the effectiveness of available rapid diagnostic tests to identify TB infection. The main goal of this study was to use the RT-PCR technique for the rapid detection of tuberculosis using sputum and Urine sampling compares with serological test. Quik (Check) ICT for blood samples .

Materials and Methods
Subject
30 samples (sputum, urine and blood) were tacked from outpatients attended to pharma laboratory in Erbil city during the period from January to July 2013. All patients had clinical manifestations such as fever, weight loss, cough, anorexia, and some of them with bloody sputum. The mean of their age ranged from 1–70 years, from both sexes and different residency. Carefully of each patient was taken as the name of the patient, sex, age, residency. The patients were selected according to the following basis:

SAMPLING
to tacked Patients sputum were advised to wash their mouths with antiseptic mouth wash and then three times with water. To obtain a sputum sample, the patient was given a labeled sputum container and was asked to Take a deep breath, Open the container ,bring it close to the mouth and bring the sputum out into it and Not to put saliva or nasal excretion into the container, Not to have sputum in the mouth but immediately spit into the containerand Close the container**.** The respiratory samples were decontaminated and digested by treatment with an equal volume of sputolysin/sodium hydroxide (4%) for 30 minutes at room temperature with rocking. After neutralization with 10 ml of PBS (pH 7.4), the mixture was centrifuged at 3000 rpm for 30 minutes. After discarding the supernatant, the sediment was obtained **(9)**. For urine sampling. Tacked 5ml centrifuged at 12000 rpm for 10 minutes then the sediments were collected. The portion of the sediment from (sputum or urine) was used in DNA extraction process. The remainder of the sediment for was transferred to an Eppendorf tube and stored at- 20°C if not immediately processed (10) The sample of blood was putted in another test tube containing EDAT, and centrifuged by T-30 centrifuge for 20 minutes. Three layers were formed, the upper one

was the plasma which was aspirated by Pasteur pipettes and transferred to test tube and stored at -20˚C until used (11) .

Methods

Detection of DNA in sputum and urine samples using the Real Time PCR Technique

The Real Time PCR of the modern techniques used to detect DNA by cells multiplied using specialized primers Specific Primers and enzymes Taq Polymerase chain specializes in building DNA (12). This technique is used for the diagnosis tb in the sputum and urine of patients with multiple extraction and amplification for DNA samples examined, the working methods and diagnosis as follows: The extraction process was by using several Promegal Reliaprep sputum and urine g DNA Miniprep System U.S. based on the work of this kit is the Binding Column link in the centrifuge tubes precise high-speed Microcentrifuge tubes, 200 micro liter were drawn from a sufficient sample purification processes (13), either way amplified DNA was using several tb Real-TM Quant which several specialized to amplify the tb DNA and produced by the company Sacace Biotech-nologies-Italian. It has been prepared kit Home Master Kit according to the manufacturer's instructions, was to add a leaky 12.5 microliter of a DNA sample to 12.5 microliter of the main solution kit and put in the pipeline Smart cycle RT instrument. Then a pipe was transferred to a RT PCR instrument, which can detect the DNA of 200 copies / mL and upwards, As less than this level is undetectable for the device, and was reading the mix according to the software installed in the instrument, after fully reading instrument gave two results for reading are FAM and Cyanine 3 (CY3), Where the value was divided CY3 the FAM and beat the result in the coefficient of the device to give the final result of the viral load found in the sample and be units (IU / mL).

Serological Assay for detecting anti–TB antibodies in blasma using direct examination: diagnostic kit (ACON Laboratories Inc., USA).

It is a rapid chromatographic immunoassay for the qualitative detection of anti –TB antibodies (Isotypes IgG, IgM and IgA) in whole blood, serum or plasma samples. pouch was testing temperature prior flat surface. Theplaced on clean labeled with an ID. Pipette dropper filled the samples. It device was held vertically 2-3 drops dispensed into the sample well without air bubble. Timer was set up. After 10 minutes of adding the sample, test results were read.

Statistical analysis

All results were performed by Chi square test at the level of significant when P-value < 0.01. (14).

Result and Discussion

The results showed that have been obtained from testing RT-PCR with tb the sample from sputum were ratios 36%, Its high ratios gave test in this study (Fig-1), (Fig-2) and (Fig-3) the number female infected 7/11Chi square values $3.798^{n.s}$, P-Value 0.0571and the number male infected 4/19 Chi square values $3.185^{n.s}$, P-Value0.00714 non significant difference among females and male but the value of male closer to significant table (1).

Table (1) shows the numbers of patients(male and female) with TB in sputum detected by RT-PCR

Female x` = 3.798 $^{n.s}$ P-Value=0.0571 male x` = 3.185 $^{n.s}$ P-Value=0.00714

TB detected by RT-PCR Sputum		Male	TB detected by RT-PCR Sputum		Female	Total	Age
Non detected	detected		Non detected	detected			
1	0	1	0	0	0	1	1-10
1	0	1	1	2	3	4	10-20
5	2	7	1	1	2	9	20-30
2	0	2	1	1	2	4	30-40
3	0	3	1	0	1	4	40-50
3	2	5	0	2	2	7	50-60
0	0	0	0	1	1	1	60-70
	4	19		7	11	30	total

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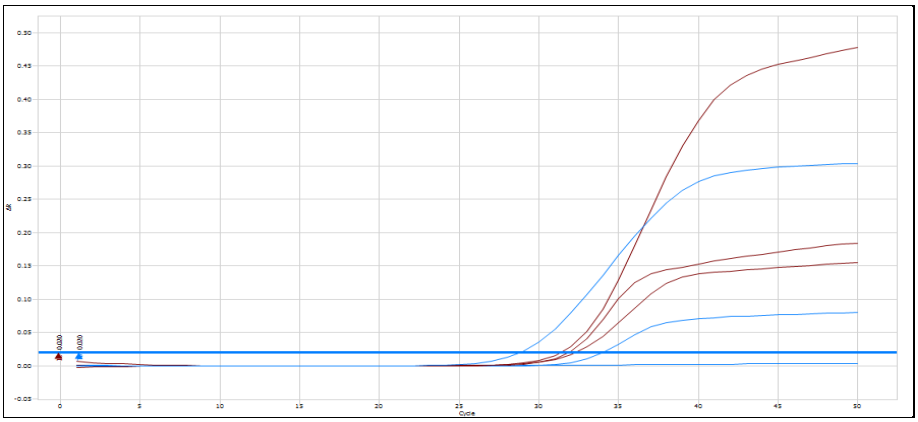
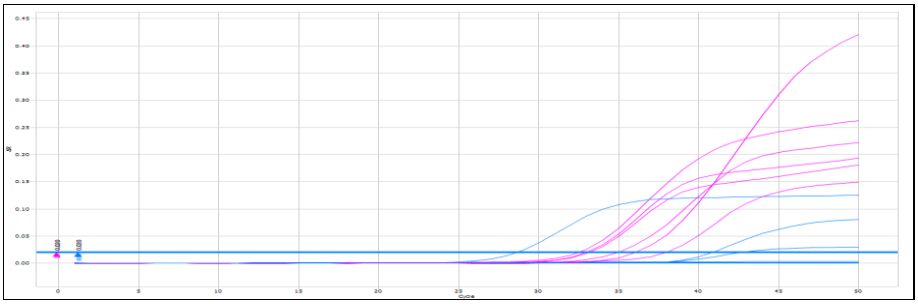
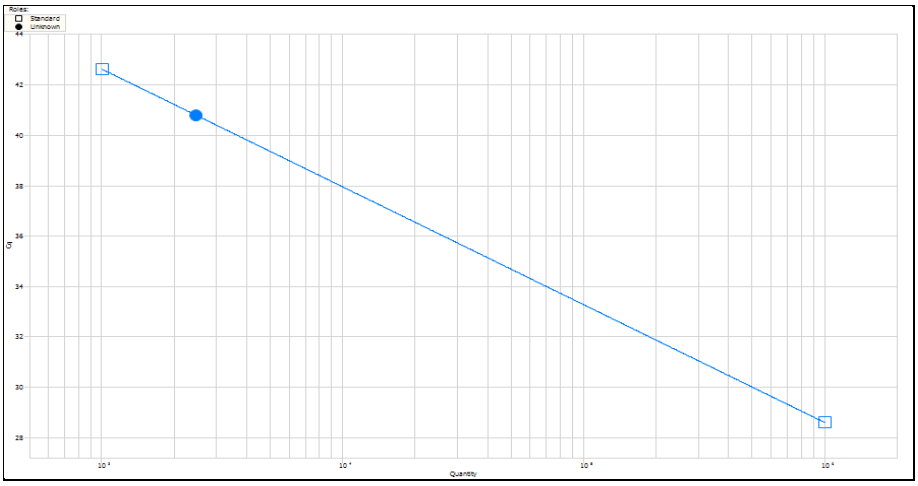


(Fig-1)Sample for Positive Result

In the left fluorescence intensity, in the below number of cycles PCR , in brown color standard containing a known concentration of DNA and in blue an known concentration DNA of the TB sample, Both DNA of TB samples and standard have CT value(The threshold cycle) is the cycle number at which the fluorescent signal of the reaction crosses the threshold.



(Fig-2)Sample For Positive with TB DNA quantification, Determine the amount of DNA been through RT-PCR interaction.



(Fig-3) standard curve, In the left CT value of an known concentration of DNA of TB sample , in the below standard that have a known concentration of DNA , by comparison between as determine the concentration of DNA of TB samples .

were ratios from diagnose tb from blood plasma by ICT test 13% the number female infected 2/11values chi square for female 3.157 $^{n.s}$, P-Value 0.1782 and the number male infected 2/19 Chi square 1. 831 $^{n.s}$, P-Value 0.381 non significant difference among females and male but the value of female closer to significant table (2).

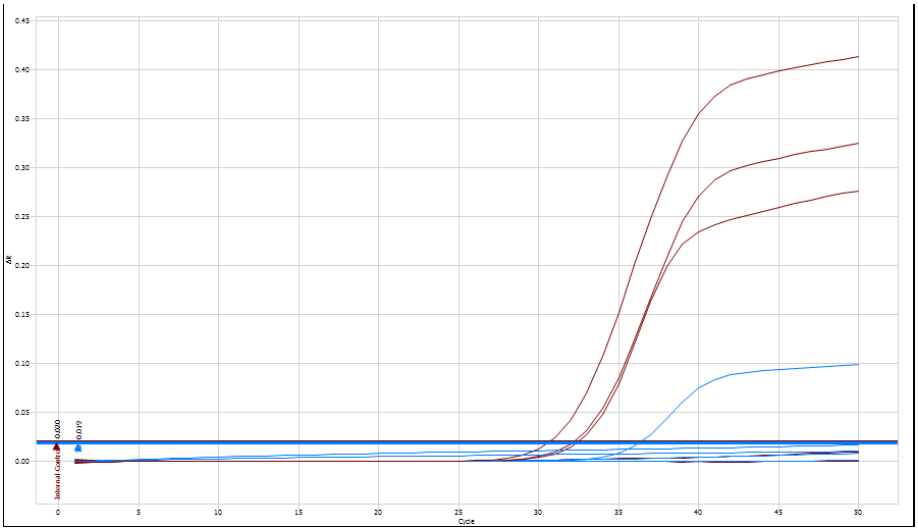
Table (2) shows the numbers of patients(male and female) with TB detected by using direct serological examination in blood plasma. ***Female x` = 3.157 n.s P-Value=0.1782 male x` = 1.831 n.s P-Value=0.381***

TB detected by blood plasma		Male	TB detected by blood plasma		Female	Total	Age
Non detected	detected		Non detected	detected			
1	0	1	0	0	0	1	1-10
1	0	1	2	1	3	4	10-20
5	2	7	2	0	2	9	20-30
2	0	2	2	0	2	4	30-40
3	0	3	1	0	1	4	40-50
5	0	5	1	1	2	7	50-60
0	0	0	1	0	1	1	60-70
	2	19		2	11	30	total

and indexation DNA of tb from urin in pulmonary tb by RT-PCR non give any result table (3) (Fig-4) .

Table (3) shows the numbers of patients(male and female) with TB in Urine detected by RT-PCR. Some expected frequencies zero, completion of computation impossible

TB detected by RT-PCR Urine		Male	TB detected by RT-PCR Urine		Female	Total	Age
Non detected	detected		Non detected	detected			
1	0	1	0	0	0	1	1-10
1	0	1	3	0	3	4	10-20
7	0	7	2	0	2	9	20-30
2	0	2	2	0	2	4	30-40
3	0	3	1	0	1	4	40-50
5	0	5	2	0	2	7	50-60
0	0	0	1	0	1	1	60-70
	0	19		0	11	30	total



(Fig-4)Sample for Not Detected Results
In the left fluorescence intensity, in the below number of cycles PCR, in brown color standard containing a known concentration of DNA and in blue the TB sample but it not have a concentration of DNA because

Just DNA of standard have CT value(The threshold cycle) is the cycle number at which the fluorescent signal of the reaction crosses the threshold.

The results showed that have been obtained from testing RT-PCR method quick diagnose suit with tb symptoms which do not have atypical scenario and it slow-growing(16). while the dependence in the diagnosis on clinical symptoms or diagnose X-rays, cultivate sample the basis of sputum and Serological tests and the basis of which the blood and sputum smears (AFP) that are affected by two factors how to prepare smears and on the experience of the reader diagnosis (17). The results showed that have been

obtained from testing RT-PCR with tb the sample from sputum were ratios 36%, Its high ratios gave test in this study because of the inhalation or nerosolined droplets originate in the air way, including the mouth, pharynx , larynx (18) .those patients who have eavitary lunge cavities encourage growth because they are awarm, wet, dark, oxygen-rich this cause Sputum its beast to growth tb bacilli's and it's the beast to diagnosed(19). But ratio to diagnose tb it few because it normally takes about two months of exposure to acquire the disease, The immune system sends macrophage they surround and engulf the organisms, but do not kill them ,instead, they wall them off in small hardend capsules called tubercles (18). and maybe some patients infect by tb but it have little bacilli then need more time for incubate in order to increase the bacilli number and shows the disease (20). As well as possible be the reason for the return to low concentrations of copies of the tb DNA in the sputum that are less than the level at which can RT-PCR instrument diagnosis of tb DNA, where the instrument portability used in this research in the diagnosis up to 200 copies\ml (21،22) Tb bacillis accumulate in the alveoli and gain access to lymphatic channels through the cervical nodes and thoracic duct where they are then disseminated through out the blood stream then bone marrow, liver and spleen. (18) this lead to use direct serological test ICT has additional advantages in situations when the patient is unable to produce adequate sputum(23). And when sputum smear results are negative (24) ratio to diagnose tb it few13% and some patients who have been diagnosed tb from sputum by RT-PCR could not the ICT diagnose tb from them because Mycobacterium tuberculosis can survive for alife time inside such granulomas datent tb infection (LTBI) but may escape from the host's immune response, (25). and can multiply inside macrophage Alveolar where they grow slowly and are divided roughly from 25-32 hours, does not have known endotoxins or exotoxins, therefore, there is no immediate host response grow from 2-12 wk until they reach103 to 104 in number ,which is responsipal for late acellular immune response(26).the infection distribution in all body by blood stream the deposit and prolife rate in sites that are beast known as being high in oxygen kidneys vertebral bodies, the epiphyses of long bons(27,28). This is what prompted us to confirm that it is detected small DNA fragment from cell dying throughout the body have been detected in urine Mycobacterium tub erculosis transrenal DNA (Tr-DNA) in pulmonary tuberculosis (tb). After the use of screening RT-PCR. has not been diagnosed DNA of tb in urine in any of the infected individuals despite the positive first test of sputum, followed by the test, followed by direct testing of blood plasma this maitard with (29) who said he can detect of DNA of tb in the urine of 34of 43 patients with pulmonary tb in the study conducted by possible to be for the same reason in the lack of diagnosis of sputum reason return to low concentrations of copy DNA in urine which is less than the level that could have RT- PCR diagnosis DNA(22). Back that this study did not agree with the results of the previous study because of the small number of samples in this study compared to the large number of samples in the aforementioned study as well as different geographic areas served by the research and the type of equipment and materials used in this research.

Conclusion

The study is concluded from the foregoing indexation DNA of tb from sputum by RT-PCR is the easy way. Precise , efficient and good sensitivity in the diagnosis the tb Comparison with ICT test less efficient and indexation DNA of tb from urin by RT-PCR non efficient.

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استخدام التفاعل السلسلي للبوليميراز في الزمن الحقيقي لتشخيص التدرن الرئوي لعينه من المصابين في اربيل

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الملخص

هدفت دراستنا الى تقييم هذه الطريقة من عدة جوانب منها دراسة جدوى استخدامها مقارنة بالطريقة التقليدية الفحص السيرولوجي (ICT) المباشر من بلازما الدم ومعرفة مدى قدرة RT-PCR على تشخيص (TB) من القشع والبول للمصابين بالتدرن الرئوي. وهل هنالك علاقة بين تردد الإصابة ونوع الجنس.

جرى فحص عينه دم 30 مريضا من (الذكور19 والاناث 11) يشتبه سريريا بإصابتهم ب(TB) وباستخدام RT-PCR و ICT المباشر كانت النسب 36%، 13%. وكانت قيم اختبار مربع كاي الخاصة بتشخيص TB من القشع ضمن فحص RT-PCR بالنسبة للاناث 3.798 وقيمة P-Value 0.0571 اما بالنسبة للذكور وكانت قيم اختبار مربع كاي 3.185 وقيمة Value- 0.00714 ولم يظهر اختلافا معنويا ولكن قيمة P-Value للذكور اقتربت للمعنوية. وكانت قيم اختبار مربع كاي الخاصة (ICT) بالنسبة للاناث 3.157 وقيمة P-Value 0.1782 اما بالنسبة للذكور وكانت قيم اختبار مربع كاي 1. 831 وقيمة P-Value 0.381 ولم يظهر اختلافا معنويا ولكن قيمه P-Value للاناث اقتربت للمعنوية. اما بالنسبة لتشخيص TB من البول ضمن فحص RT-PCR فلم تظهر اي نتائج.اظهرت نتائجنا على الرغم من عدم وجود اختلافات معنويه بين الطرق المستخدمة. ان RT-PCR يقدم طريقة حساسة وكفؤة ودقيقة في التشخيص وخصوصا من القشع إلا أن وجود بعض الصعوبات (drawbacks) تجعل منه اقل استعمالاً في تشخيص المرض يليه فحص ICT اما تشخيص TB من البول من خلال RT-PCR فانها الاقل كفاءة.